Matrix metalloproteinases as predictive markers of coronary in-stent restenosis

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This thesis is dedicated to each of the participants who volunteered to be a part of the enclosed studies. Thank you for your time, your effort, and above all, your trust.
Abstract

Background
Matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs) are involved in remodelling the extracellular matrix of the vascular system. Altered MMP expression has been linked to the development of atherosclerosis, acute coronary syndrome, myocardial dysfunction and complications after vascular procedures including in-stent restenosis (ISR) after percutaneous coronary intervention. Other studies have focussed on the inactive pro-form of matrix metalloproteinases. However, an enzyme-linked immunosorbent assay (ELISA) system that measures the activity of specific matrix metalloproteinases is available. This thesis aimed to investigate whether circulating active MMPs could act as risk markers for ISR, and progression of coronary atherosclerosis and myocardial dysfunction.

Methods
Two groups of patients were studied. Initially, a case-control study was recruited, in which patients with a history of bare-metal stent restenosis were compared to patients who were asymptomatic after coronary stenting. A panel of MMPs was measured in the plasma of patients with restenosis and compared to controls. MMPs and TIMPs were measured using both conventional and activity ELISA. The contribution of MMPs to the risk prediction, beyond that afforded by established clinical and demographic risk models was investigated.

In the second study a prospective cohort of patients undergoing coronary stenting were followed for 12 months. This study provided an opportunity to confirm the results of the first study. Additionally, data on clinical presentation and echocardiographic function allowed correlation of active MMPs and TIMPs with acute coronary syndrome, myocardial infarction and diastolic myocardial dysfunction. Serial blood samples over six months allowed tracking of MMP activity with each condition. The occurrence of both ISR and other coronary endpoints were collected and correlated with MMP activity.

Finally, the seasonal variation and stability during storage of MMPs at -80°C and the relationship between the ratio of pro-MMP-9:TIMP-1 and MMP-9 activity were assessed.

Results
Active MMPs -3 and -9, as well as TIMP-1 were strongly associated with a history of ISR, and were independent of clinical and demographic risk factors. When these markers were included in conventional risk models, they appeared to provide additional predictive information, with improved area under the curve and net reclassification index.

In the prospective study, there was no association between active MMP-9 or TIMP-1 prior to intervention, but the change in active MMP-9 at three months was significantly greater in the group that developed ISR. Conversely, there was no association between active MMP-9 and non-ISR cardiac events. Initial presentation with ST-segment elevation myocardial infarction was also associated with increasing active MMP-9 at three months. Both active MMP-9 and TIMP-1 were associated with the occurrence of diastolic, but not systolic, myocardial dysfunction. The above findings were independent of clinical and demographic variables.

Both active MMP-9 and TIMP-1 appeared to be stable in storage for up to three years, and not affected by variation with the season of sample collection. The relationship between the pro-MMP-9:TIMP-1 ratio and active MMP-9 suggests that the pro-MMP-9:TIMP-1 ratio is not a surrogate measure for MMP-9 activity.

**Conclusions**

Active MMPs-3, -9 and TIMP-1 appear to be associated with ISR, and measuring them may allow improved prediction of ISR. Additionally, increasing MMP-9, but not TIMP-1, after coronary stenting may indicate those patients who are developing ISR. Active MMP-9 also rises in the months after ST-elevation myocardial infarction, and adjustment for this may be necessary if active MMP-9 is to be used as a clinical predictor of ISR. Active MMP-9 and TIMP-1 do not appear to be associated with the development of non-ISR events.

Both active MMP-9 and TIMP-1 appear to be markers of diastolic, but not systolic, dysfunction, and active MMP-9 may be a sensitive early marker for diastolic dysfunction.

Active MMP-9 and TIMP-1 appear to be stable in storage for up to three years, and not subject to seasonal variation, therefore these factors do not appear to be important biases. The pro-MMP-9:TIMP-1 ratio does not indicate the activity of MMP-9, and therefore active MMP-9 should be measured directly.
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Thank you firstly to my supervisors, Associate Professors Gregory “Big Greg” Jones and Michael Williams. You took me on when I was so young, and knew so little. If I am any good now, it is thanks to the opportunity you offered me, and the hours of direction you provided. Thank you. These have been great years, and while they have not always been easy, I do not regret them.

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To everyone in the vascular lab: Gerry, Riordan, Josie, Maria, Matthew, Sarah and both Judys.

To Denice Whiunui, you are so well organised you make me look bad on my best days and your attitude to constant learning is inspirational.

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Abbreviations

ACC/AHA = American College of Cardiology/American Heart Association
ACE-I = angiotensin converting enzyme inhibitor
ACS = acute coronary syndrome
ANCOVA = analysis of covariance
ANOVA = analysis of variance
AP-1 = activator protein-1
APMA = 4-aminophenyl mercuric acetate
ARB = angiotensin receptor blocker
AUC = area under the curve
BIC = Bayesian Information Criteria
BMI = body mass index
BMS = bare-metal stent
CABG = coronary artery bypass grafting
CAD = coronary artery disease
CD = cluster of differentiation
CI = confidence interval
DD = diastolic dysfunction
DES = drug-eluting stent
ECM = extracellular matrix
eCrCl = estimated creatinine clearance, Cockraeft-Gault model
EDTA = ethylenediaminetetraacetic acid
ELISA = enzyme-linked immunosorbent assay
GRACE = Global Registry of Acute Coronary Events
HbA1c = haemoglobin A1c
HDL = high-density lipoprotein
hs-CRP = high sensitivity C-reactive protein
ICAM-1 = inter-cellular adhesion molecule-1
IQR = interquartile range
ISR = in-stent restenosis
ITVR = Ischaemic Target Vessel Revascularisation score
LDL = low-density lipoprotein
MLD = minimal luminal diameter
MMP = matrix metalloproteinase
PDGF = platelet-derived growth factor
NGAL = neutrophil gelatinase-associated lipocalin
NIH = neointimal hyperplasia
NRI = net reclassification index
NSTEMI = non-ST-segment elevation myocardial infarction
NT-proBNP = N terminal pro-B-type natriuretic peptide
OR = odds ratio
PCI = percutaneous coronary intervention
PRESTO = Prevention of REStenosis with Tranilast and its Outcomes score
ROC = receiver operating characteristic curve
SA = stable angina
SD = standard deviation
STEMI = ST-segment elevation myocardial infarction
TDI = tissue Doppler imaging
TGF-β = transforming growth factor beta
TIMP = tissue inhibitor of matrix metalloproteinases
UA = unstable angina
VCAM-1 = vascular cell adhesion molecule-1
VD = vessels diseased
VSMC = vascular smooth muscle cell
WHR = waist:hip ratio
Publications, posters and conference proceedings

The full text of publications I – IV are attached in Appendix 11.7.

1.1.1. Publications

http://dx.doi.org/10.1016/j.atherosclerosis.2009.05.036

http://dx.doi.org/10.1016/j.ijcard.2010.12.093

http://dx.doi.org/10.1016/j.clinbiochem.2011.08.1139

http://dx.doi.org/10.1016/j.clinbiochem.2011.09.019

V. Tarr. GP, Williams. MJA, Wilkins. GT, Chen. VHT, Phillips. LV, van Rij. AM, Jones. GT. Intra-individual changes of active MMP-9 are associated with in-stent restenosis of bare-metal stents. (submitted for publication)

http://dx.doi.org/10.1016/j.ijcard.2012.03.147

1.1.2. Oral presentations

GP Tarr. MJA Williams, GT Wilkins, VHT Chen, LV Phillips, AM van Rij, GT Jones
http://dx.doi.org/10.1016/j.hlc.2011.03.097

*New Zeal Med J*. June 2007, Vol 120 No 1255
http://www.nzma.org.nz/journal/120-1255/2574/

1.1.3. Posters


1. Introduction

1.1. Coronary artery disease

At the beginning of this new century, coronary artery disease (CAD) is the single leading cause of mortality, killing an estimated 4.0 million males and 3.3 million females every year worldwide, out of a total of 30.4 and 26.5 million deaths, respectively. The burden of CAD affects all people, but the incidence is distributed along ethnic, gender and socio-economic lines, disproportionately affecting those with fewer resources. Rates of CAD are higher in those with lower socio-economic status, a trend seen in a number of Western countries. Females are relatively protected, with a relative delay in onset compared to males of as much as seven years.

Age-adjusted death rates for CAD have declined in the West in recent decades from 543/100 000 in 1980 to 267/100 000 in 2000 for males, and from 263 to 134/100 000 for females. However, CAD is predicted to rise again, due to current trends in obesity and diabetes. It has been predicted that CAD will cause a billion deaths during the first half of the 21st century. In developing countries the mortality rates from heart disease are lower, but nevertheless, heart disease is a major killer and again is increasing in rate; 82% of the expected future increase of CAD mortality will occur in developing countries. The burgeoning economies of India and China are associated with increasing rates of CAD, and the combination of increasing populations (1 billion and 1.3 billion peoples respectively) and increasing CAD rates, could mean that the absolute burden of atherosclerosis-related deaths may increase as much as two-fold. While as much as one-half of CAD-related death occurs suddenly, CAD is also a chronic disease, with a large cost in terms of morbidity. The morbidity of CAD causes the third greatest worldwide loss of disability adjusted life years, comprising 37 million of all disability-adjusted life years for males and 25 million for females, or 4.6% and 3.3% of all disability-adjusted life years lost. In economic terms, the impact is enormous; for example, the cost in terms of lost productivity in the United States of America is estimated at $US62 billion, on top of $US103 billion in medical costs, totalling more than for all neoplasms combined. New Zealand is no exception; in 1998 the age standardized coronary heart disease rate was 91/100,000, killing 6,203. Māori populations are particularly at risk, with rates approximately double that of Europeans. The pathology causing this major health problem is atherosclerosis.
1.2. Atherosclerosis

Aristotle’s description (circa 350 BCE) of “bone-in-heart” may be the first description of atherosclerosis,\textsuperscript{13} perhaps describing the characteristic dystrophic calcium deposits of advanced atherosclerosis. Due to prohibition of human dissection, and dogmatic views on the anatomical insights put forth by Galen of Pergamon (129 – 200 CE), no significant insight into the cardiovascular system and its pathology was made until the 16th century, with Andreas Vesalius’ (1514 – 1564) description of the heart.\textsuperscript{13}

With the basic structure of the heart described, interest turned to diseases of the heart. Others built on this anatomical foundation, describing gruel-like deposits, and “petrification.”\textsuperscript{13} Reportedly, there was intense rivalry between the Baron Carl von Rokitansky (1804 – 1878) and Rudolph Virchow (1821 – 1902) as to whether fibrin deposition or inflammatory cell infiltrate represented the primary initiating factor of atherosclerosis, with Rokitansky favouring fibrin and Virchow favouring inflammatory cells.\textsuperscript{14} Soon after Virchow’s death, Felix Marchand (1846 – 1928) coined the term atherosclerosis from the Greek “athero” – gruel and “sclerosis” – hardening,\textsuperscript{15} and Nikolai Anichkov (1885 – 1964) demonstrated that high cholesterol diets in rabbits produced atherosclerosis-like lesions.\textsuperscript{15} However, CAD remained an intractable killer; no one knew when it would strike, what caused it or how to prevent it.\textsuperscript{16}

The seminal ecological studies by Ancel Keys (1904 - 2004) demonstrated large inter-country variation in rates of CAD, attributing this to variation in dietary practices and serum cholesterol.\textsuperscript{17} This, along with the insights from the Framingham study on the role of blood pressure, smoking and lipids, ushered in the era of preventative cardiology.\textsuperscript{18}

Growing advances in molecular biology lead to the discovery of the mediators and receptors associated with multiple physiological processes, and along with improved clinical trials, led to the implementation of a number of effective therapies for the prevention and treatment of coronary artery disease; including β-adrenergic blocking agents, angiotensin-converting enzyme inhibitors, 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins), and the application of the fibrinolytic bacterial enzyme, streptokinase, to those with acute myocardial infarction.\textsuperscript{19} Through the 1980s and 90s, pioneers such as Peter Libby were able to demonstrate the role of multiple cell types and an array of chemical mediators, realising that inflammation was the key mediator of atherosclerosis development.\textsuperscript{20}
1.2.1. An overview of the pathophysiology of atherosclerosis

The pathophysiology of atherosclerosis consists of an inflammatory response to arterial wall injury by neointimal hyperplasia (NIH), the uptake and oxidation of low-density lipoprotein (LDL) and the formation of foam cells as macrophages take up oxidised LDL.\(^{21}\) Inflammation can be defined as ‘coordinated delivery of white blood cells and plasma proteins in order to remove a stimulus’,\(^ {22}\) and is characterised by a large number of mediators with specific roles, complex regulatory networks,\(^ {22}\) and a high degree of redundancy.\(^ {23}\) This process has a clearly beneficial rationale in combating invading bacteria or viruses, where the response is mounted then resolves when the infection resolves. However, it appears that the chronic inflammation associated with non-resolving stimuli that accompany chronic human conditions in the modern world, like obesity and type II diabetes mellitus, is maladaptive, and promotes atherosclerosis.\(^ {22}\)

Noxious stimuli, including by-products of cigarette smoke, hyperglycaemia, high blood pressure and high wall shear stress damage the arterial wall.\(^ {24}\) The normal intima consists of a continuous layer of endothelial cells attached to a basement membrane, however, in humans focal thickenings of vascular smooth muscle (VSMC) and extracellular matrix (ECM) occur (Figure 1.1B).\(^ {25}\) This is probably reflective of areas of local wall stress, indicated by the predilection for these lesions developing at vessel branch points. These stimuli lead endothelial cell activation, resulting in expression of vascular adhesion molecules and increased permeability, as well as recruitment of macrophages and VSMCs to the developing lesion, causing intimal thickening.\(^ {26}\) LDL molecules accumulate in the developing neointima and are oxidised through a number of mechanisms, including via interaction with homocysteine, glycation and reactive oxygen species, and are then taken up by macrophages, forming the characteristic foam cells.\(^ {20}\) Early atherosclerotic lesions are characterized by these macrophages filled with oxidised LDL particles, visible by light microscopy. These lesions then progress to contain more foam cells as well as lipid droplets in VSMCs, and appear, grossly, as “fatty streaks.”\(^ {20}\) These changes begin in utero, with lesions described in fetal aortas.\(^ {27}\) As these fatty streaks acquire more lipid, large cores of extracellular cholesterol form and displace the intimal cells and matrix to form atheroma, pools of lipid within the intimal layer.\(^ {25}\) The foam cells then contribute to the inflammatory reaction which leads to the atherosclerotic plaque developing and remodelling, through the elaboration of signalling molecules including reactive oxygen species\(^ {28}\) and interleukin-1.\(^ {29}\)
Figure 1.1 Histology of normal and atherosclerotic coronary arteries
From a 65 year old male. All histology is stained with Verhoeff’s elastic stain and van Geison’s counter stain (elastic fibers stained blue/black, collagen red, and other tissue elements stained yellow).
A. Wedge of left ventricle and left anterior descending coronary artery (*). Gross specimen. Scale bar 3mm.
B. Histology of left anterior descending coronary artery shown in figure A. Although no atherosclerotic plaque is apparent, over half of the wall thickness is chronic neointimal hyperplasia. Note the intra-myocardial coronary artery.
C. Great cardiac vein, note the irregular shape due to post-mortem collapse of this thin walled vessel.
D. Left anterior descending coronary artery with atherosclerotic disease (†). Scale bar 3mm.
E&F Histology of the lesion shown in D.
E. Note positive outward remodeling of vessel wall to preserve the luminal diameter.
F. Note cholesterol crystals in necrotic core underneath a thick fibrous layer.
Reproduced with permission from Assoc Prof Greg Jones, Vascular Research Group, University of Otago.
This then leads to the recruitment of T lymphocytes and VSMCs.\textsuperscript{20} The latter synthesize collagen to form a fibrous cap on the luminal side of the plaque, over the necrotic, cholesterol-filled core,\textsuperscript{21} protecting the developing plaque from rupturing (Figure 1.1F). While these changes progress, the entire artery remodels outward to keep the lumen patent, a process termed positive remodelling.\textsuperscript{30} Atherosclerosis may be widespread in areas that look normal on coronary angiography due to positive remodelling, with large lipid cores sitting in the arterial wall, but not narrowing the lumen (Figure 1.1E).\textsuperscript{31}

Atherosclerosis progresses to cause clinically recognisable disease by two methods. The growth of the atherosclerotic plaques can exceed the ability of positive remodelling to maintain a patent lumen, leading to a progressive stenosis. This typically limits blood flow during periods of increased demand, such as during exercise, causing stable angina. Alternatively, inflammatory mediators can cause the cells resident in the atherosclerotic plaque to release proteases, including matrix metalloproteinases, which lead to the break down of the fibrous plaque. The thinned fibrous caps appear to spontaneously rupture, exposing the lipid core to the blood flow. The encased necrotic lipid material is highly thrombogenic and can lead to the rapid formation of an occlusive blood clot, stenosing the artery and leading to myocardial infarction.\textsuperscript{26}

\section*{1.3. Coronary stenting}

\subsection*{1.3.1. History}

Until the second half of the 20th century, atherosclerosis and its complications were seen as unpredictable and untreatable.\textsuperscript{16} Morbidity and mortality from both the acute and chronic burden of coronary atherosclerosis have been mitigated by intervention using coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI).\textsuperscript{7,32,33} The two procedures commonly undertaken today both have roots in Ohio’s Cleveland Clinic. It was in this hospital that F. Mason Sones, Jr. developed selective coronary arteriography in 1960,\textsuperscript{34} which subsequently led to the development of percutaneous transluminal coronary angioplasty, or percutaneous coronary intervention, by Andreas Grünzig in 1977.\textsuperscript{35,36}

The first coronary stents were used in humans in 1987, introduced with the aim of reducing acute vessel recoil and restenosis after balloon angioplasty.\textsuperscript{37} The United States’ Food and Drug Administration approved the first coronary stent for the treatment of coronary artery disease in 1994 after key randomized control trials demonstrated their efficacy over
balloon angioplasty.\textsuperscript{38,39} Better outcomes from stenting led to its increasing popularity, and with further advances PCI now accounts for two-thirds of all coronary revascularizations in the US (800,000 PCI vs. 350, 000 CAGB annually), with 1.4 million stents implanted.\textsuperscript{40} Coronary stenting is effective in reducing mortality in high-risk acute coronary syndrome (ACS) patients,\textsuperscript{41} and improving anginal symptoms in stable coronary artery disease.\textsuperscript{42}

\textbf{1.4. In-stent restenosis}

In-stent restenosis (ISR) was the primary complication of coronary stenting during the bare-metal stent era,\textsuperscript{43,44,45} with rates from 10% up to 60% in people with multiple risk factors.\textsuperscript{46} Typically ISR reproduces clinical symptoms within six months of intervention.\textsuperscript{47,48} Restenosis has often been thought of as benign, but more recently it has been understood to be associated with a significant rate of myocardial infarction.\textsuperscript{49,50} Our knowledge of the pathophysiology of ISR has been gained from studies in animals, experimental human models and clinical populations. Unless stated otherwise, the mechanistic insights included in this section are derived from experiments in animals.

Historically, three distinct processes were thought to cause restenosis after balloon angioplasty. These were:

1. Elastic vessel recoil.
2. Negative arterial remodelling.

The development of coronary stenting largely eliminated the effect of elastic recoil and negative remodelling, and today neointimal hyperplasia appears to be the main cause of ISR.\textsuperscript{51} The first drug eluting stents (DES) were widely accepted into clinical practice from 2003 onwards.\textsuperscript{52} DES are effective at reducing restenosis, lowering rates by 60% in comparison to bare-metal stents (BMS), to an absolute rate of around 5%.\textsuperscript{53-57} As such DES has been touted as “solving the restenosis problem”.\textsuperscript{53,58} DES are used in between 30%\textsuperscript{59} – 60%\textsuperscript{60} of PCI. However, the rate of restenosis in those who have no apparent risk factors is still around 5%, translating to hundreds of thousands of cases of ISR worldwide each year.\textsuperscript{61} More recently, a small, but significant, rate of late stent thrombosis with DES was seen and safety concerns arose.\textsuperscript{62} Regardless of the long-term decisions and changes in clinical practice due to safety concerns and cost, rates of restenosis remain significant, especially on a population level.
Coronary stent implantation is a traumatic process, and the phenomenon of ISR is related to vascular healing. A decrease in the size of the arterial lumen from the native diameter is termed stenosis, whereas “loss of gain” after intervention refers to the reduction of lumen diameter from the improvement with the original procedure. This is termed restenosis, and defined either clinically, as a return of anginal symptoms, or angiographically, as a decrease in luminal diameter of >50%.

1.4.1. Pathophysiology of in-stent restenosis

Neointimal hyperplasia is the characteristic response to arterial injury. In some ways, the development of neointimal hyperplasia after coronary stenting is analogous to the intimal thickening posited to be the first step of atherosclerosis. Evidence for this includes the necessity of vascular adhesion molecules for the development of restenosis, and recent reports of atherosclerotic transformation of the neointima within coronary stents. The resulting lesions of fibrous connective tissue and vascular smooth muscle resemble the fibrous cap over atherosclerotic lesions. This has led to the suggestion that the induction of intimal hyperplasia by stenting may have been able to prevent plaque rupture, if unstable plaques could be identified beforehand.

1.4.1.1. Injury to vessel wall: endothelial destruction, intimal/medial tearing

As the artery is mechanically expanded it undergoes de-endothelialization, rupture of the intimal elastic lamina (IEL) and tearing of the intimal and medial layers. The implantation of a coronary stent adds a further variable, namely the presence of the foreign body of the stent. The compliance mismatch between the metal struts and the stented artery places stress on the arterial wall, and the foreign nature of the stent leads to fibrin deposition and accumulation of inflammatory cells. These effects are the stimulus for a cascade of reactions comprising an initial thrombus formation followed by simultaneous re-endothelialization and neointimal hyperplasia.

At the stented site a thrombus composed of red blood cells, fibrin, platelets and neutrophil granulocytes forms and completely covers the struts by up to four weeks after stent placement. The initial injury also appears to cause apoptosis of VSMCs within the media, occurring in the first few hours after injury. Platelets and fibrin adhere to the stented site, with activated platelets expressing adhesion factors such as P-selectin. Circulating leukocytes attach to platelet receptors, and then tightly adhere to the injured area through
macrophage-1 antigen (Mac-1).\textsuperscript{76} Mac-1 binds to multiple receptors, including the glycoprotein receptors Ib\textalpha\textsuperscript{77} and IIb/IIIa,\textsuperscript{78} which attach to platelets and fibrinogen/platelet complexes, respectively. Leukocytes probably then migrate into the lesion, driven by gradients of chemical mediators. Leukocytes are scattered throughout the stented area with a high proportion directly next to the stent struts.\textsuperscript{79} The distribution of the cells suggests an acute inflammatory response, invoking non-specific inflammatory mechanisms consistent with a response to injury. It was postulated that the formation of thrombus was important in this response,\textsuperscript{80} as early inhibition of the coagulation cascade through administration of tissue factor inhibitor and inactive factor VIIa reduced experimental intimal hyperplasia,\textsuperscript{81} but antithrombotic therapies did not appear to reduce restenosis.\textsuperscript{82,83} Inhibition of leukocyte migration through Mac-1 knockdown reduces experimental intimal hyperplasia.\textsuperscript{84}

The idea that the severity of injury determines the extent of intimal hyperplasia is supported by animal models, which have shown that the amount of intimal hyperplasia produced is proportional to the severity of the injury.\textsuperscript{69,85-87} De-endothelialization of rat carotid alone has been associated with increases in VSMC proliferation,\textsuperscript{85} but the response to mechanical stretch as well as de-endothelialization appears to be more powerful.\textsuperscript{85} This response is also illustrated in the pig carotid model, where two distinct injuries are found after balloon injury: the less severe medial dilation, and the more severe medial dissection with internal elastic lamina rupture.\textsuperscript{69} Medial dilation alone appears to have a less severe response, with markers of VSMC proliferation peaking relatively early, compared with medial dissection and internal elastic lamina rupture, which are associated with a prolonged elevation of greater magnitude.\textsuperscript{69} It appears there is a parallel between the markers of VSMC proliferation and neointimal hyperplasia, where the less severe injury was associated with an early plateau of neointimal hyperplasia formation compared with more severe injury, which resulted in an intimal area five times as large.\textsuperscript{69} There appears to be no difference in changes to the medial layer with the different injuries, and re-endothelialization is complete within the same time frame for both injuries.\textsuperscript{69}

Endothelial cells begin to cover the denuded artery at six weeks, and completely recover it by three months after intervention.\textsuperscript{72} They both migrate from the adjacent uninjured artery and derive from premature circulating cells of bone marrow origin.\textsuperscript{72} After intervention, the endothelial lining is disrupted and remains uncovered by endothelial cells for up to six weeks.\textsuperscript{72} From six weeks until three months some endothelium is regained, but it is not until around three months that endothelium wholly covers the artery wall.\textsuperscript{72}
1.4.1.2. **Migration and proliferation of VSMC**

Rodent models have provided insight into the response of VSMCs to the type of injury inflicted by percutaneous intervention. Schwartz *et al.* outline four phases of VSMC response and the variety of biochemical mediators of these responses (Table 1.1). Such a model is probably incomplete, and there is most likely some overlap in the biochemical mediators. However, these limitations are due, at least in part, to the complex and redundant nature of many cellular pathways. The first phase is characterized by proliferation of medial VSMCs, and begins 24 hours after injury. After this, smooth muscle cells migrate from the media into the intima, crossing the fragmented internal elastic lamina, constituting the second phase. The third and fourth phases are, respectively, initial VSMC proliferation and the continued heightened response to growth factors. The VSMCs comprising neointimal hyperplasia are polyclonal, and derive from a number of sources. VSMCs, both those resident in the intimal layer and those that migrate from the medial layer in response to inflammatory stimulation, are probably important. Additionally, it appears that intimal VSMC in neointimal hyperplasia can also be derived from the circulation. The extent of bone-marrow derived VSMCs is not known, with empirical studies suggesting a wide range of values. Studies of atherectomy specimens of human ISR, suggest that it could be between 2 and 29% of the cellular content of the neointima. Tanaka *et al.* found that this effect is variable with the severity of injury, with greater injury drawing a larger proportion of cells from the bone marrow. However, while human studies confirm that bone marrow cells appear to have a role in the pathogenesis of ISR, the bone marrow contribution is more modest in human studies than in small animal models. Interestingly, it appears that sirolimus-eluting DES may impair the mobilization of bone marrow cells. Bone marrow derived smooth muscle cells have been shown to be present in early plaque, but not late atherosclerosis or fibrous cap, and they may be pro-atherogenic, secreting the inflammatory cytokines monocyte chemotactic protein-1 and interleukin-6 and stimulating macrophage migration. Hence, while it appears that bone marrow derived cells make some contribution to vascular pathology, their role and the degree of their contribution are unclear. The maximum amount of VSMCs in the developing lesion occurs at two weeks, driven, at least partly, by increased proliferation. After the initial wave of VSMC apoptosis caused by mechanical injury, VSMCs migrating to the intima undergo
Table 1.1 Chemical mediators of vascular smooth muscle cell proliferation and migration

<table>
<thead>
<tr>
<th>Description</th>
<th>Mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>First wave (0 – 3 days)</td>
<td>bFGF, PDGF, Ang II</td>
</tr>
<tr>
<td>Replication of VSMCs within the media</td>
<td></td>
</tr>
<tr>
<td>Second wave (3 – 14 days)</td>
<td>bFGF, PDGF, Ang II</td>
</tr>
<tr>
<td>Migration of VSMCs from the media into the intima</td>
<td></td>
</tr>
<tr>
<td>Third wave (7 days – 1 month)</td>
<td>bFGF, Ang II, TGF-β</td>
</tr>
<tr>
<td>Proliferation of VSMCs within the neointima</td>
<td></td>
</tr>
<tr>
<td>Fourth wave (1 month – 3 months)</td>
<td>bFGF, Ang II, TGF-β</td>
</tr>
<tr>
<td>Prolonged and heightened response</td>
<td></td>
</tr>
</tbody>
</table>

Modified from Schwartz et al. Ang II = angiotensin II; bFGF = basic fibroblast growth factor; PDGF = platelet-derived growth factor; VSMC = vascular smooth muscle cells; TGF-β = transforming growth factor beta.

...apoptosis as well. The balance between proliferation and apoptosis of intimal cells shifts after about two weeks in the balloon injured rat iliac, with increased intimal apoptosis out to at least one month. The effects of growth factors on the behaviour of VSMCs in the various stages is probably linked to alterations in the cell cycle (Figure 1.2). VSMCs are generally in the quiescent state and administration of growth factors or mechanical injury stimulates the transition past G1 arrest. The cell cycle-controlling cyclin-dependent kinase inhibitors p27 and p21 appear to modulate the neointimal response. Knockdown of p27 exacerbates experimental vascular injury, with increased inflammatory cell infiltrate and neointimal hyperplasia. Conversely, up-regulation of p27 and p21 both result in attenuation of the neointimal response after balloon injury. In normal arteries, p21 expression is absent, but p27 appears to have constitutive expression, which probably keep VSMC in the quiescent state. Levels of p27 are down-regulated with arterial injury, concurrently as VSMC proliferation occurs.

1.4.1.3. Production of extra cellular matrix

Initially it was hypothesised that the balance between apoptosis and proliferation of VSMCs would determine the occurrence of ISR. This was plausible because VSMCs make up the majority of the neointima, and it appears that increased proliferation and resistance to
apoptosis in VSMCs predispose to ISR formation. However the cells in ISR lesions, at clinical presentation, are not actively proliferating. As the neointima matures, the expression of both p21 and p27 rise with cessation of VSMC proliferation, and there is a concurrent increase in TGF-β signalling and procollagen gene expression (Figure 1.2). Atherectomy samples of coronary ISR show that VSMCs in neointimal hyperplasia are synthetic. The cells are positive for α-actin filaments and have prominent intra-cytoplasmic organelles, suggesting a synthetic phenotype. This is in contrast to the contractile phenotype of VSMCs resident in the medial layer. The change in phenotype coinciding with the onset of proliferation has been termed “modulation”. Compared to the VSMCs of the medial layer, they have fewer microfilaments, smooth muscle myosin, desmin, caldesmon, α-actin, and have an extensive rough endoplasmic reticulum. The pattern of these changes suggests a loss of contractile and structural proteins, and a gain of protein synthesis machinery. There are over 147 genes differentially activated in intimal smooth muscle cells compared with medial smooth muscle cells. Concordant with the idea that VSMCs produce ECM, the 13 genes most highly expressed in developing neointima are involved with ECM production either directly (including collagens I, and III, and versican), or are thought to be involved indirectly. Furthermore, cultured smooth muscle cells taken from injured arteries synthesize ECM components in vitro. This suggests a
specialized role of intimal smooth muscle cells in the production of the ECM in which they are imbedded. Together they form a large part of the neointima. Hence, while the migration and proliferation of VSMCs may be an important mediator of neointimal hyperplasia, the production of ECM appears to be a determinant as well, and is mediated by VSMCs themselves.

At four weeks, the first ECM changes can be noted, with focal changes next to the struts of the stent. Over the next eight weeks inflammation subsides and \( \alpha \)-actin positive VSMCs migrate and begin to lay down ECM. At approximately 12 weeks, the ECM appears denser under light microscopy, with more bundles of fibres, which are organized in a circular fashion around the lumen. Interestingly, there appears to be a feedback mechanism whereby VSMC proliferation is inhibited by mature collagen through the up-regulation of cyclin-dependent kinase inhibitors p27 and p21, whereas non-crosslinked collagen fibrils stimulate the continued proliferation of VSMCs. The neointima ultimately develops three layers as time extends past three months. The luminal layer typically consists of between 15 and 30 layers of \( \alpha \)-actin positive VSMCs. The middle layer of the neointima is less cellular, with fewer VSMCs, but more ECM, whereas the outermost layer of the neointima is continuous with the media and VSMCs contained within the atherosclerotic plaque.

The composition of the ECM in the neointimal lesion has several characteristic proteins. Fibronectin, collagen I and III are ubiquitous. Histologically, there are two different patterns of ECM in the neointima; predominantly hyaluronan-rich loose connective tissue, containing VSMCs; and the relatively sparse acellular dense connective tissue, which is rich in collagens I and III. Areas rich in collagens also contain the proteoglycans, biglycan, and elastin. Versican associates with hyaluronan, and together they most likely play a number of roles. Cholesterol deposits do not typically characterise neointima lesions, and this, along with the abundance of the proteoglycan, hyaluronan, suggest that the neointima parallels early atherosclerotic lesions, but not mature ones. There are probably a number of roles for proteoglycans in the neointima, for example providing an attachment for inflammatory cells, providing an initial substrate for cellular migration, and trapping water in their hydrophyllic structure, which leads to tissue swelling, providing an initial structure upon which the ECM is formed. The proteoglycan-rich areas of restenotic tissue appear to get smaller with time and have fewer cells, perhaps through ongoing apoptosis. The result is that mature restenotic lesions are comprised of densely compacted
collagen with fewer cells\textsuperscript{106,116} and smaller amounts of versican and hyaluronan,\textsuperscript{116} although maturation may take up to 18 months.\textsuperscript{116}

\textbf{1.4.1.4. Additional mechanisms}

While neointimal hyperplasia is the predominant cause of ISR,\textsuperscript{51} the transmission of atherosclerotic plaque proximally or distally along the arterial wall may contribute to some cases of restenosis.\textsuperscript{117}

Allergic inflammation has an apparent role in ISR, with an association between metal-ion allergies and BMS restenosis.\textsuperscript{118} Rittersma \textit{et al.}\textsuperscript{119} compared atherectomy samples from patients who had developed restenosis after BMS implantation and balloon angioplasty alone. There were more eosinophils present in ISR specimens, apparently directly next to the stent struts.\textsuperscript{119} All the BMS utilized in the study by Rittersma \textit{et al.}\textsuperscript{119} were made from grade 316L stainless steel, containing iron, chromium, nickel, magnesium and molybdenum ions.\textsuperscript{120-122}

A number of studies have examined allergy patch tests to these ions in patients who have undergone treatment with BMS. Rates of positive tests ranged from 6.9\%\textsuperscript{121} to 19\%\textsuperscript{122} for nickel alone, and 7.6\% when all of the ions in grade 316L stainless steel were included.\textsuperscript{120} Two studies have detected a relationship between metal allergies and ISR. Koster \textit{et al.}\textsuperscript{120} found that of 131 patients who were undergoing angiography for suspected restenosis, all 10 (100\%) patients who also had metal ion allergies had ISR, compared to 69 (57\%) of patients without allergies, which was statistically significant. Saito \textit{et al.}\textsuperscript{122} studied 128 patients who had previous ISR after BMS, and at six months after repeated PCI, underwent metal ion patch testing. Of the 60 with ISR, a significantly higher proportion had nickel allergy (30\%), compared to 9\% of controls.\textsuperscript{122} Rates of positive reactions appeared to be higher for manganese and chromium as well, but these did not achieve statistical significance.\textsuperscript{122} A third study, attempting to prospectively confirm these relationships patch tested for nickel one day after BMS implantation. They found no association between nickel allergy and restenosis, as quantified by protocol angiography at six months, with ~30\% having restenosis in each group.\textsuperscript{121} They did not evaluate other metal ions.\textsuperscript{121} Thus, a number of studies have implicated metal allergies in the development of ISR, in at least a small proportion of patients. However, as Norgaz \textit{et al.}\textsuperscript{121} could not confirm these relationships with testing at the time of PCI, the allergy may take time to develop.

One group has evaluated pre-interventional levels of eosinophil activation, by assaying eosinophil cation protein, with BMS\textsuperscript{123} and DES.\textsuperscript{124} They found that levels of eosinophil
cation protein were higher in those developing the composite end point of cardiac death, non-fatal myocardial infarction and clinically-driven target lesion revascularisation at two years.\textsuperscript{123,124} In the study examining the relationship of eosinophil cation protein with DES,\textsuperscript{124} they found that the level of eosinophil cation protein was independent of clinical and angiographic variables, but did not present such an analysis in the paper describing patients undergoing BMS intervention.\textsuperscript{123} Thus it appears that allergy and eosinophilia may play a role in the development of restenosis.

### 1.4.2. Limitations of animal models

Animal models are the source of most of our insights into vascular repair. However, while animal models have been important in our understanding of vascular response to injury, they have limitations that need discussion. A large number of therapies based upon experiments on animal model experiments have ended up failing when applied to humans in a clinical context.\textsuperscript{125,126} Examples include angiotensin receptor antagonists, anti-oxidants, and anti-inflammatory drugs.\textsuperscript{127} More than 40 large clinical trials failed to show a reduction of restenosis with a number of different pharmacological agents, even though most had been shown to be effective in small animals.\textsuperscript{125} There are two categories that these fall into:

- Animals do not completely replicate human pathology. Hyperlipidaemic small animal models are limited in their reflection of atherosclerosis, generally displaying a phenotype characteristic of the monogenic syndromes causing hyperlipidaemia in humans rather than atherosclerosis.\textsuperscript{126} Secondly, the IEL of animals are often intact,\textsuperscript{92} whereas in humans, spontaneous fractures accumulate over years, which may allow VSMCs to migrate more freely. IEL fracture appears to be an important part of the development of both atherosclerosis\textsuperscript{128} and NIH,\textsuperscript{87} as evidenced by greater atherosclerotic lesion formation and neointimal proliferation when the IEL was disrupted.

- Different physiology of animals. Non-human primates are the most similar to humans, but logistic difficulties and high cost mean they are infrequently used. Smaller animals have different physiological systems, for example, dogs have different coagulation mechanisms,\textsuperscript{129} and animals heal in different time frames, with smaller animals tending to heal more quickly.\textsuperscript{130-133} Mice, rats and rabbits have much smaller coronary arteries, so iliac and femoral arteries, as well as the aorta, are used.\textsuperscript{129} There are differences in these arteries, for example, the iliac artery is an elastic artery, compared to the muscular nature of the coronary
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artery. One example from matrix metalloproteinase biology is that mice lack matrix metalloproteinase-1.\textsuperscript{134}

Because of these differences, we may conclude that while animal models provide important information in cardiovascular disease, we should be conservative when extrapolating our findings to humans.

1.4.3. Predictors of restenosis

In line with the inflammatory/response to injury genesis of ISR, clinical studies have demonstrated numerous variables that are associated with the development of ISR.\textsuperscript{135}

Lesion risk factors for restenosis include stenosis severity score;\textsuperscript{136} longer stent length;\textsuperscript{79,137} chronic total occlusions;\textsuperscript{46} smaller diameter of both stents;\textsuperscript{46,137} reference diameter;\textsuperscript{138,139} and increasing number of stents implanted.\textsuperscript{79} Mechanical and procedural factors have been associated with increased rates of restenosis, including the treatment of ostial lesions and bifurcations. Research into stent designs has revealed that stents with thinner struts and less metal contacting the vessel wall tend to provoke smaller hyperplastic responses.\textsuperscript{140,141} A mismatch between lesion and stent length have also been implicated as a risk factor, beyond that of the stent length alone, with increasing stent length:lesion length associated with greater risk of ISR.\textsuperscript{142} Sub-optimal stent expansion is also associated with higher risks for ISR.\textsuperscript{46} Soft plaque visible on intravascular ultrasound, defined as plaque appearing less dense than the adventitial layer, is associated with restenosis, which may be explained by greater compression of soft plaque during the PCI procedure.\textsuperscript{143}

In addition, Patient-related inflammatory factors appear to be associated with risk of restenosis, with diabetes mellitus posing a higher risk,\textsuperscript{46,144,145} while after adjustment for increasing age and complexity of disease, female sex appears to be protective from the development of ISR.\textsuperscript{44} Further evidence for endogenous risk includes higher rates of restenosis in previously restenosed lesions,\textsuperscript{146} and lesional interdependence.\textsuperscript{147} If a stented lesion developed\textsuperscript{148} ISR, then subsequent lesions within the same patient appeared to be twice as likely to develop ISR, when adjusted for confounding factors.\textsuperscript{147} This suggests that still unknown factors make significant contributions to the development of ISR. Further evidence of this is the bimodal nature of ISR narrowing.\textsuperscript{65,149} These studies\textsuperscript{65,149} found that while the range of narrowings of the initial lesions approximated a normal distribution, there appeared to be two sub-groups in the range of ISR narrowings. An aggressive response to stenting has been described,\textsuperscript{150} in which patients develop a diffuse restenosis which is more severe than the
original narrowing, and is associated with a presentation of myocardial infarction\textsuperscript{150} and this sub-group has an elevated risk for recurrent restenosis.\textsuperscript{151,152} These findings suggest the possibility of sub-groups of patients with an endogenous propensity to mounting a healing response, associated with higher risk for ISR.

1.4.4. Prevention of in-stent restenosis

A number of treatments for ISR have been designed, and their successes and failures have important implications for the development and revision of the pathobiology of ISR.

Prior to the introduction of the coronary stent, a number of interventions were posed as alternatives or adjuncts to angioplasty. Many of these methods included attempts at debulking of plaque by directional coronary atherectomy,\textsuperscript{153} rotational atherectomy\textsuperscript{154} and laser-assisted angioplasty.\textsuperscript{155} These techniques were aimed at maximizing the luminal area after intervention, however, patient outcomes were the same or worse with these devices,\textsuperscript{155,156} which may be secondary to the tissue trauma inherent in these techniques, and the consequent proliferative response of neointimal hyperplasia.\textsuperscript{153}

One area in which this is underscored, albeit perhaps in a more positive light, is the pathological response to coronary stenting. Intravascular ultrasound has revealed that the mechanisms of restenosis after angioplasty and coronary stenting differ.\textsuperscript{51} Restenosis after angioplasty is due to both inward remodelling of the vessel wall and neointimal hyperplasia,\textsuperscript{157,158} while restenosis after coronary stent implantation is primarily due to neointimal hyperplasia.\textsuperscript{51} The area encompassed by the stent struts does not change, and the decrease in lumen area is approximately equal to the increase in neointimal area.\textsuperscript{51} Hence, coronary stenting exacerbates neointimal hyperplasia, but the overall rates of restenosis with coronary stenting are lower than in angioplasty.\textsuperscript{38,39} This is consistent with the chronic inflammatory reaction at the stented site, initially characterised by thrombus and neutrophils,\textsuperscript{72} and later by lymphocytes directly next to the stent struts.\textsuperscript{79} Interleukin-8 and monocyte chemotactic protein-1 expression and leukocyte infiltration are prolonged with experimental stenting compared to balloon angioplasty alone.\textsuperscript{159}

ISR is fundamentally an inflammatory condition, and one obvious treatment option is corticosteroids, a powerful pharmacological class with many anti-inflammatory effects. While a meta-analysis of three randomized trials, comprising over 800 lesions, using a single bolus of methylprednisolone failed to show any benefit,\textsuperscript{160} subsequent trials utilizing higher doses of oral prednisone, and dexamethasone-eluting stents have shown reductions in both the
need for revascularisation and angiographic restenosis.\textsuperscript{161} Tranilast, an anti-inflammatory agent, failed to prevent either the composite outcome of adverse events, or any reduction of late in-stent loss by angiography or intravascular ultrasound.\textsuperscript{162}

1.4.5. Drug-eluting stent pathology

The first drug eluting stent, Cordis’ Cypher (Johnson & Johnson), was approved by the US Food and Drug Administration in 2003, followed closely by Boston Scientific’s Taxus. Both DES are designed to reduce neointimal hyperplasia by eluting either of the cytostatic drugs paclitaxel and sirolimus, which broadly inhibit cell function by targeting specific points in the cell cycle.\textsuperscript{52,163} Preclinical studies showed delayed endothelization with paclitaxel-eluting stenting, with an increase in inflammation, postulated to be from a reaction to the polymer.\textsuperscript{164}

The addition of polymer and cytostatic drugs onto a bare-metal stent frame appear to have implications for the pathobiology of treated lesions, as healing is impaired and extra foreign materials are present, both potentially causing problems.\textsuperscript{165} DES have been associated with delayed endothelialization,\textsuperscript{164,166-168} persistent incomplete endothelialization,\textsuperscript{169} a lack of neointimal coverage of stent struts,\textsuperscript{170} hypersensitivity reactions\textsuperscript{171} and stent thrombosis.\textsuperscript{172} Increased rates of stent thrombosis with DES compared to BMS were subsequently seen in a number of studies.\textsuperscript{62,173,174} However, at around 0.3 – 0.5% per year, the rates are low and it is not clear that this translates into an increase in mortality.\textsuperscript{175} In addition, their use in people with simple lesions appears to be safe; any increase in mortality seems to be restricted to use in patients with diabetes and “off label” use – long vessels, restenotic lesions, vein grafts and small arteries.\textsuperscript{173,176} Subsequently, registry data has suggested that rates of death over long-term follow up are lower with DES,\textsuperscript{177} but this is apparently due to indication bias, and comparison between DES-era and BMS-era mortality outcomes appear identical.\textsuperscript{178}

Several mechanisms have been proposed for the pro-thrombotic tendency associated with drug-eluting stents. Virchow’s triad states that thrombosis is caused by three factors: blood vessel wall alteration, abnormal flow and hypercoagulability.\textsuperscript{179} DES are associated with persistent incomplete re-endothelialization, with around 50% endothelial coverage by 40 months compared with mostly complete coverage in BMS.\textsuperscript{169} After 30 days, half of DES struts had deposits of fibrin, compared with one quarter of BMS struts.\textsuperscript{169} Beyond 60 days, the fibrin deposition appears to resolve from BMS struts while it remains present for at least 120 days with DES.\textsuperscript{169} Of 14 DES autopsy specimens with stent thrombosis, all had delayed
arterial healing, and three had no other risk factors, suggesting that arterial healing has an important role in DES stent thrombosis.\textsuperscript{169}

There has been concern that the polymers used in DES could be pro-coagulative via immune hypersensitivity.\textsuperscript{171} Stent thrombosis related to allergic inflammation with DES has been linked to the polymer coating.\textsuperscript{171,180} Different profiles of inflammatory cell reaction have been noticed in some cases of DES thrombosis examined at autopsy; there was an increase in eosinophils compared to no increase in more than 400 BMS lesions.\textsuperscript{171,181} Neither sirolimus or paclitaxel appear to be associated with hypersensitivity reactions,\textsuperscript{182} but reactions to polymers have been described.\textsuperscript{171} It is therefore likely that the polymer is causing the hypersensitivity reaction.

Outside of stent thrombosis, DES may cause other pathologies. It has been reported that sirolimus eluting stents are associated with endothelial dysfunction, with local paradoxical vasoconstriction during exercise.\textsuperscript{183} However, sublingual nitroglycerin, a non-endothelium dependent vasodilator, can still achieve vasodilation.\textsuperscript{183} These results suggest that sirolimus-eluting stenting may produce endothelial dysfunction.\textsuperscript{183} DES may also have an inhibitory effect on the formation of collateral coronary arteries.\textsuperscript{184} This could translate into a more severe pathology if an acute occlusion occurs in the stented artery, as the compensatory vessels are less developed and the likelihood of insufficient blood flow is increased.

### 1.4.6. Neo-atherosclerosis

Long-term follow up of patients who underwent PCI has revealed that, with time, the intimal hyperplasia within coronary stents undergoes atherosclerotic transformation, a condition labelled “neo-atherosclerosis”.\textsuperscript{185}

Nakazawa \textit{et al.}\textsuperscript{67} examined all autopsy specimens in their institution from patients who had undergone PCI with stent placement at least 30 days before death, with a median time from implantation of at least one year (n = 299). Neo-atherosclerosis, defined as the presence of foam cells or atheroma in neointimal tissue, was present in 31\% of lesions treated by DES and 16\% of lesions treated by BMS, despite the BMS being implanted, on average, for much longer times (median 721 [IQR 271 – 1,801] vs. 361 [172 – 540] days).\textsuperscript{67}

Neo-atherosclerotic lesions appeared to begin in the peri-strut area, as this was the location of foam cell accumulation.\textsuperscript{67} The median (IQR) time to appearance of neo-atherosclerotic lesions in pathological samples was 420 (361 – 683) days for DES but over
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five times as long for BMS, at 2,160 (1,800 – 2,880) days. In samples obtained less than two years after implantation, there was no evidence for neo-atherosclerosis in BMS, but 30% of DES had associated lesions. The longest intervals for which stents had been implanted were > 6 years for BMS and 2 – 6 years for DES, with rates of neo-atherosclerosis in each class around 40% at these late time points. The majority of BMS with associated neo-atherosclerosis had fibroatheromas in the neointimal tissue, with the remaining few having evidence of foam cells. In contrast, DES-associated neo-atherosclerosis showed an even distribution of fibroatheromas and less mature lesions. A small percentage of patients had evidence of plaque rupture associated with neo-atherosclerosis, with 4% of BMS and 1% of DES lesions having either thin cap fibroatheromas or plaque rupture. Clinical and patient factors associated with the presence of neo-atherosclerosis included younger age and initial unstable lesions, as well as the use of DES and the duration of implantation. These factors were independent of clinical and angiographic variables.

Yokoyama et al. performed serial angiography and angioscopy for over four years on 26 patients receiving BMS. They found that from between one to four years-post stenting, the prevalence of yellow plaque, plaque fissure/ulceration/intimal flap, and thrombus increased. Each feature was uncommon at one year-post stenting, but over half of lesions had evidence of yellow plaque at > 4 years and one third of patients had thrombus or surface irregularities. Furthermore, there was an increase in late re-narrowing associated with yellow plaque. The change in diameter stenosis from one year to four years follow up was nearly 20% in those with yellow plaque, but < 4% in those without. The authors were unable to confirm whether conventional risk factors were associated with the presence of yellow plaque.

Kang et al. utilized optical coherence tomography to examine 50 consecutive patients with DES ISR, aiming to characterise DES ISR tissue. The median duration of implantation was 32 (9 – 52) months. They found that 90% of DES had evidence of neo-atherosclerosis, with half having thin-cap fibroatheroma and evidence of rupture, and with higher rates in those with unstable compared to stable presentations. The detection of plaque rupture was nearly three times higher with optical coherence tomography than with angiography alone. Thus, the neointima associated with coronary stents appears to undergo atherosclerotic transformation in the years after stent implantation. Neo-atherosclerosis appears to form more rapidly with DES-treated lesions than with BMS-treated lesions, and may be an important contributor to DES restenosis. This could be consistent with an increased local inflammatory
reaction in response to DES implantation. However, Nakazawa et al.\textsuperscript{67} did not stain for eosinophils in their pathology series.

A key limitation of the studies by Nakazawa et al.\textsuperscript{67} and Kang et al.\textsuperscript{187} is selection bias, in that their samples are all from people who died after coronary stenting and had DES ISR, respectively. While around 30\% of patients in the series by Nakazawa et al.\textsuperscript{67} died from target-lesion related pathology, only around 5/299 (1.7\%) appear to have died directly from neo-atherosclerosis-related complications. Yamaji et al.\textsuperscript{188} describe 405 patients with >15 years of follow up after treatment with BMS in the early 1990s. They found that rates of target lesion revascularisation were highest in the first 14 months, at 16\%, and from that point up to four years, only an additional 2.5\% of patients underwent target lesion revascularisation. However, from 4 to 10 years, a further 8.5\% and from 10 to 15 years, an additional 9\% had target lesion revascularisation.\textsuperscript{188} Thus, rates of target lesion revascularisation per annum after the first year of follow up were approximately 0.8\% up to four years, which subsequently doubled to 1.4\% up to 10 years and 1.8\% at fifteen years. These compare to rates of 2.0 – 3.4\% for non-target lesion revascularisation (i.e. the rates attributable to the rest of the coronary tree).\textsuperscript{188} It appears that there continue to be events related to the stented lesion, with over half of all target lesion-related events over fifteen years occurring in the years after the initial 14 months.\textsuperscript{188} The proportion of all coronary events attributable to the target lesion over 15 years represented over one-third of all the future coronary events suffered, or 20\% occurring after the initial 14 months.\textsuperscript{188} Thus, the progression of neo-atherosclerotic lesions and neo-atherosclerotic plaque rupture may contribute to events over a long period of time, even if the rate is fairly low.

Aside from those identified by Nakazawa et al.,\textsuperscript{67} few specific risk factors for neo-atherosclerosis have been confirmed and no therapies have yet been suggested, although presumably these would be similar to the prevention and treatment of atherosclerotic lesions elsewhere. However, it was recently demonstrated that VSMCs expressing versican V3, which lacks chondroitin sulfate glycosaminoglycans, forms a compact neointima after balloon injury that is resistant to macrophage infiltration and lipid deposition.\textsuperscript{189} If a novel stent could deliver an agent that would lead to increased expression of versican V3, this may mitigate the late effects of neo-atherosclerosis.
1.5. **Biological markers**

The term biological marker, or biomarker, is defined as “a characteristic that is objectively measured and evaluated as an indicator of... pathogenic processes...”\(^{190}\) This is a broad definition and includes genetic, radiological, and physical variables as well as measurements of biochemical parameters in body tissues and fluids. Furthermore, the type of outcome of interest encompasses a wide spectrum of applications. For example they may assist in: 1. diagnosis, 2. selection of therapy, 3. monitoring disease progression, 4. detection of sub-clinical disease and 5. risk stratification.\(^{191}\) A risk factor is defined as a biomarker that is “in the causal pathway leading to the disease”, whereas a risk marker is a marker that is statistically “associated with the disease, but need not be causally linked” to the disease.\(^{190}\) It is estimated, when post-translational modification is taken into account, that there are at least 800 000 proteins circulating in the blood, and at present we can only measure a fraction of these.\(^{192}\) Approximately half of the protein in the plasma is albumin, which along with nine other proteins makes up 90%.\(^{192}\) The concentration difference between proteins at low and high concentrations – exemplified by albumin and cytokines – is enormous, with the concentration difference between albumin and interleukin-6 (IL-6) being approximately ten billion-fold.\(^{192}\) Many biomarkers have been investigated, with >70,000 papers on cardiovascular disease prognosis alone, but far fewer have penetrated into clinical practice.\(^{193}\) Several criteria for evaluating biomarkers have been suggested.\(^{191,194}\) Fundamentally, these are:

- Can the biomarker be measured in practice?
- Does the biomarker add new information?
- Will the biomarker assist in the management of patients?

To be useful, numerous conditions must be met. Good predictors have a positive risk-benefit ratio, are cost effective, and acceptable. The disease in question needs to pose significant morbidity and/or mortality, effective treatments for the disease must be available and there should be reason to apply the treatments to only some people. Finally, the biomarker must help to decide whether treatment is necessary over and above known risk factors.\(^{193}\) Ultimately, the role of prognostic biomarkers in determining patient management should be assessed in randomized control trials, where conventional therapy is compared to a biomarker-driven strategy.\(^{193}\) A number of biomarkers have been incorporated into clinical cardiology practice, including troponins I and T, and brain natriuretic peptide.\(^{195}\) These
markers were derived from an understanding of the underlying pathophysiology of myocardial ischaemia and heart failure, respectively.

Broadly speaking, two discovery strategies are used; gnostic candidate-based approaches\textsuperscript{192} and agnostic high throughput-based approaches.\textsuperscript{196} The former identifies putative factors based on knowledge of the underlying biology of the disease and measures relatively few variables in parallel.\textsuperscript{192} The latter attempts to measure the whole range of a category of variables (\textit{e.g.} all single nucleotide polymorphisms in the genome, in genome wide association studies), and adjusts the statistical analyses to account for the large number of parallel measurements.\textsuperscript{196}

From a biomarker discovery point of view, one important conceptual issue is the difference between the tissue compartments of the lesion, the draining blood and the peripheral venous circulation. In specific anatomical and physiological circumstances there may be other relevant compartments, \textit{e.g.} pericardial sac fluid, urine and cerebro-spinal fluid. While there may be a strong basic science background for the involvement of a putative biomarker in the disease process, this does not necessarily translate into a clinically effective marker. It may be helpful to ask “What is the source of this biomarker in this setting?” and “How does this alteration relate to the biological function?”

1.5.1. Pitfalls in investigating new circulating biological markers

There has been much continued interest in the development of biomarkers, but as the number of studies has proliferated,\textsuperscript{197} and many markers have failed to show clinical utility, scepticism towards the development of new biomarkers has developed.\textsuperscript{190} There are a number of reasons why these candidate biomarkers do not hold up in subsequent research, and are not shown to be clinically useful. These include statistical and study design biases, biomarkers being on the same casual chain as other risk markers and having overlapping association despite independence.

Some plasma proteins have many-fold variations in concentration in health and disease, for example high sensitivity C-reactive protein may increase from $50\mu g/L$ to $500mg/L$ during acute inflammation, a 10,000-fold increase.\textsuperscript{198} Large variation, as well as the possibility of release from multiple different body sources (\textit{e.g.} creatinine kinase may be released from myocardium and skeletal muscle),\textsuperscript{199} may lead to the situation where a biomarker is associated with the disease, but substantial overlap remains between cases and controls.\textsuperscript{200}
For a screening biomarker, the odds ratios associated with the highest and lowest quintile levels of the putative marker needs to be around 50,\(^{201}\) which requires that there be minimal overlap of biomarker levels. Furthermore, complex chronic diseases like coronary artery disease have multiple underlying causes, resulting in single causal factors being poor disease predictors.\(^{200}\)

Another limitation of biomarker studies is the study power. A recent systematic review of biomarkers associated with carotid intimal-medial thickness revealed that a lack of statistical power was one of the main reasons for not detecting an association, with over 60% of 28 reports linking high sensitivity C-reactive protein to carotid intimal-medial thickness being underpowered.\(^{202}\) A large component of this is probably the inherent variability of circulating markers, with significant intra- and inter-individual variability.\(^{198}\)

Negative biomarker studies are less likely to be published, and multiple biomarkers may be serially tested in the same population, without adjustment for multiple comparisons.\(^{203}\) This raises the rate of false positive findings, where there is no true association between the biomarker and disease, but a “statistically significant” result occurs by chance. If 20 biomarkers were tested, the likelihood that one would appear significant by chance is 64%.\(^{203}\)

Finally, biomarkers may duplicate information gained from clinical variables already assessed through history and physical examination.\(^{204}\) For example, biomarker measurements may reflect the same mechanisms as risk factors. e.g. a hypothetical risk marker reflects oxidative stress and inflammation, but this information may already be captured by enquiring about smoking history.

In order to resolve some of these problems, calls have been made for development of protocols for agnostic assessment of non-genetic markers\(^{196}\) and for registries of biomarkers.\(^{203}\) These suggestions have the potential to improve the quality of biomarker studies in general, by reducing the influence of publication bias and multiple hypothesis testing,\(^{196,203}\) and may improve adherence to standards for reporting novel biomarkers, including reporting in the context of established risk markers.\(^{205,206}\)

1.5.2. Role of receiver operating characteristic curves and reclassification in the evaluation of biomarkers for disease prediction

The statistical methods for evaluating prognostic markers have been evolving in recent years. It is important to examine the utility of a marker in the context of established, readily
available, risk markers. After the independent association between a marker and disease has been established, and multiple regression-adjustment performed for other risk factors, the overall contribution of the novel marker to disease prediction needs further analysis.

The two main methods that have been used for this have been discrimination, defined as the ability of a test to distinguish those who will develop disease from those who will not, and more recently, reclassification, defined as “the proportion of people moved into a different predefined treatment threshold”. Commonly used discrimination and reclassification measures include receiver operating characteristic (ROC) curves\textsuperscript{207} and net reclassification improvement (NRI).\textsuperscript{208}

ROC curves are plots of sensitivity by $(1 – \text{specificity})$ for all cut off points for a given variable (or variables), with the resultant area-under-the-curve (AUC or C-statistic) giving an indication of the ability of diagnostic accuracy.\textsuperscript{207} However, while ROC curves are a good test for evaluating diagnostic tests, they appear to have limitations in the evaluation of new markers for prospectively assigning risk.\textsuperscript{209} The first problem is that rank based tests are insensitive to clinically meaningful changes in risk prediction, for example, if a new marker changes an individuals’ estimated risk by 1%, the AUC changes the same as if their estimated risk had changed 10%.\textsuperscript{210} The second is that ROC curves test the discrimination of a model, while the calibration, or how closely the predicted probabilities reflect real world risk,\textsuperscript{211} is also important. Thirdly, when models are well calibrated, the AUC cannot reach unity due to theoretical limitations.\textsuperscript{212,213} Finally, the contribution of different variables to a complex disease may sum to greater than 100%.\textsuperscript{214} The reason for this is that many different constellations of risk factors may be sufficient to cause disease. Alternatively, multiple variables may be sufficient to cause a given disease, but are not necessarily required. Including calibration tests like the Hosmer-Lemeshow statistic to judge the goodness-of-fit, and a reclassification test like NRI appears to address these issues.\textsuperscript{215}

NRI appears to be a useful improvement on total reclassification, which increases regardless of whether the study participants being correctly reclassified or not.\textsuperscript{216} With the NRI those who are reclassified up and develop the disease are ‘correctly reclassified’, and those who are reclassified down are ‘incorrectly classified’. A similar tally is kept for the non-event group.

The net reclassification index (%) is calculated as:\textsuperscript{208}

Equation 1 Net reclassification index
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\[(\text{net increase of classification for cases / total number of cases}) + (\text{net decrease of classification for controls / total number of controls}) \] * 100%.

Because the contributions of cases and controls to the net reclassification index are computed separately, a different weighting can apply to each, depending on the composition of the study. This was illustrated by Polak et al.\textsuperscript{217} when they calculated the net reclassification index associated with the inclusion of maximum thickness of the internal carotid intima-media layer with the Framingham risk score. The net reclassification for cases was 5.8%, and for controls 1.8%, the sum of which is 7.6%.

The maximal value of the net reclassification index is 200%, when all cases are moved up a category, and all controls are moved down a category. The total number of participants correctly reclassified was 2.2%,\textsuperscript{217} as the outcome of interest (incident coronary artery disease) was relatively rare (~10%) and the biomarker performed more poorly in the control group. Hence, NRI may increase despite relatively low increases of total reclassification (and discrimination), if the inclusion of the novel marker reliably detects a rare outcome. An example of this is the inclusion of patient age in the Reynolds Risk Score (a cardiovascular risk score for the primary prediction of cardiovascular disease).\textsuperscript{215} While including age in the Reynolds Risk Score is associated with a modest absolute improvement in discrimination, with an increase of AUC of 3.7%, net reclassification improvement is a much larger 19.5%. However, the NRI has a number of down sides as well. Firstly, NRI may be inflated by changes in category with little clinical significance, such as people being shifted between low and moderate risk categories, with little shift in high risk prediction.\textsuperscript{218} Secondly, NRI has been widely misunderstood. Tzoulaki \textit{et al.}\textsuperscript{219} found that, of 51 studies using NRI to date, there was significant variation in methods and poor reporting of important outcomes associated with the NRI technique. While the goal of reclassification is to determine whether added variables improve upon what is achievable using established variables, only one-half of studies used a previously described model, even when validated models were available.\textsuperscript{219} Less than one third provided justification for the risk thresholds, and there was even variation in the risk thresholds used between similar studies.\textsuperscript{219} Only half assessed the calibration of the baseline model and baseline model plus new predictor models.\textsuperscript{219} Altogether, it appears that, so far in the literature there is poor reporting of NRI statistics and comparing novel markers to established scores.

NRI is sensitive to the number of categories, with a tendency to produce higher values of NRI with more categories.\textsuperscript{210} However, if each of the categories is meaningful from a
clinical perspective, this is not necessarily a problem, although it is probably important to be aware of the effect. The final limitation is that there are no known clinically significant values of NRI. Whether a given NRI is clinically significant probably varies with the outcome of interest and how difficult it is to implement the novel marker. This would most likely be examined as a cost-effectiveness analysis, determining the overall benefit gained from including the intervention, relative to the cost of introducing it.

1.5.3. Examples of novel ISR biomarkers

Many biomarkers have been linked to the development of ISR (Table 1.2). The broad range of putative markers includes variables from multiple different systems, reflecting the broad range of systems involved in the pathogenesis of ISR. These include markers of haemostasis and coagulation, cytokines and inflammatory mediators, circulating cells, cell binding proteins, and markers of endothelial function.

Although many markers have been investigated for ISR, none are currently used in clinical practice. Many of the studies investigating biomarkers for ISR have been small, and only powered to detected changes in late lumen loss on follow up angiography\cite{220-227} rather than the development of ISR. Alternatively, some small studies have taken measurements directly from the coronary sinus after PCI\cite{221,223,227,228}, which may be more sensitive to changes in biological markers than systemic measurements. Furthermore, many of these initial observations have not been subject to replication studies\cite{220,223,224,226,227,229-243}.

Two biomarkers that have been subjected to large studies with encouraging results are high sensitivity C-reactive protein\cite{244} and bilirubin\cite{245}. A total of nine studies of pre-interventional high-sensitivity C-reactive protein levels were included in a meta-analysis by Ferrante et al.\cite{244} with a total of 2,747 patients, who developed 187 events. All patients received bare-metal stents and the outcome was determined by protocol angiography. The authors found that there was a relationship between high-sensitivity C-reactive protein levels
Table 1.2 Examples of novel ISR biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th># of studies</th>
<th>Total n</th>
<th>Cases n</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adiponectin&lt;sup&gt;225,246-248&lt;/sup&gt;</td>
<td>4</td>
<td>387</td>
<td>106</td>
<td>lower in 3/4</td>
</tr>
<tr>
<td>tumour necrosis factor-α&lt;sup&gt;222,229,249,250&lt;/sup&gt;</td>
<td>3</td>
<td>374</td>
<td>63</td>
<td>higher in 2/4</td>
</tr>
<tr>
<td>lymphocyte function-associated antigen-1&lt;sup&gt;220&lt;/sup&gt;</td>
<td>1</td>
<td>31</td>
<td>4</td>
<td>higher</td>
</tr>
<tr>
<td>sulfatide&lt;sup&gt;223&lt;/sup&gt;</td>
<td>1</td>
<td>21</td>
<td>9*</td>
<td>higher</td>
</tr>
<tr>
<td>macrophage-1 antigen&lt;sup&gt;220,221,230&lt;/sup&gt;</td>
<td>3</td>
<td>145</td>
<td>33</td>
<td>higher in 2/3</td>
</tr>
<tr>
<td>Pentraxin&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;227&lt;/sup&gt;</td>
<td>1</td>
<td>20</td>
<td>6</td>
<td>higher</td>
</tr>
<tr>
<td>circulating monocytes&lt;sup&gt;251,252&lt;/sup&gt;</td>
<td>2</td>
<td>192</td>
<td>44</td>
<td>higher in 2/2</td>
</tr>
<tr>
<td>monocyte chemotactic protein-1&lt;sup&gt;2,80,253&lt;/sup&gt;</td>
<td>2</td>
<td>93</td>
<td>33</td>
<td>higher in 1/2</td>
</tr>
<tr>
<td>interleukin-10&lt;sup&gt;249,250,254&lt;/sup&gt;</td>
<td>3</td>
<td>390</td>
<td>61</td>
<td>lower in 1/3</td>
</tr>
<tr>
<td>interleukin-8&lt;sup&gt;229&lt;/sup&gt;</td>
<td>1</td>
<td>40</td>
<td>6</td>
<td>higher</td>
</tr>
<tr>
<td>interleukin-6&lt;sup&gt;228,229,250&lt;/sup&gt;</td>
<td>3</td>
<td>308</td>
<td>56</td>
<td>higher in 2/3</td>
</tr>
<tr>
<td>eosinophil cation protein&lt;sup&gt;123,124&lt;/sup&gt;</td>
<td>2</td>
<td>310</td>
<td>40</td>
<td>higher in 2/2</td>
</tr>
<tr>
<td>chemokine ligand 5&lt;sup&gt;230&lt;/sup&gt;</td>
<td>1</td>
<td>52</td>
<td>16</td>
<td>higher</td>
</tr>
<tr>
<td>high sensitivity C-reactive protein&lt;sup&gt;244,#&lt;/sup&gt;</td>
<td>9</td>
<td>2747</td>
<td>187</td>
<td>OR 1.6‡</td>
</tr>
<tr>
<td>bilirubin&lt;sup&gt;245&lt;/sup&gt;</td>
<td>1</td>
<td>1076</td>
<td>341</td>
<td>lower</td>
</tr>
<tr>
<td>soluble CD40 ligand&lt;sup&gt;231,255&lt;/sup&gt;</td>
<td>2</td>
<td>156</td>
<td>39</td>
<td>higher in 2/2</td>
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<tr>
<td>CD40&lt;sup&gt;231&lt;/sup&gt;</td>
<td>1</td>
<td>120</td>
<td>29</td>
<td>higher</td>
</tr>
<tr>
<td>paraoxonase&lt;sup&gt;232&lt;/sup&gt;</td>
<td>1</td>
<td>60</td>
<td>31</td>
<td>lower</td>
</tr>
<tr>
<td>arylesterase&lt;sup&gt;232&lt;/sup&gt;</td>
<td>1</td>
<td>60</td>
<td>31</td>
<td>lower</td>
</tr>
<tr>
<td>γ-glutamyl transferase activity&lt;sup&gt;233&lt;/sup&gt;</td>
<td>1</td>
<td>60</td>
<td>60</td>
<td>higher</td>
</tr>
<tr>
<td>vascular cell adhesion molecule-1&lt;sup&gt;224&lt;/sup&gt;</td>
<td>1</td>
<td>15</td>
<td>6</td>
<td>higher</td>
</tr>
<tr>
<td><strong>Haemostatic/Coagulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fibrinogen&lt;sup&gt;235&lt;/sup&gt;</td>
<td>1</td>
<td>390</td>
<td>93</td>
<td>higher</td>
</tr>
<tr>
<td>fibrinogen-positive platelets&lt;sup&gt;236&lt;/sup&gt;</td>
<td>1</td>
<td>50</td>
<td>11</td>
<td>higher</td>
</tr>
<tr>
<td>tissue factor&lt;sup&gt;237&lt;/sup&gt;</td>
<td>1</td>
<td>36</td>
<td>10</td>
<td>higher</td>
</tr>
<tr>
<td>urokinase&lt;sup&gt;238&lt;/sup&gt;</td>
<td>1</td>
<td>159</td>
<td>37</td>
<td>higher</td>
</tr>
<tr>
<td>plasminogen activator inhibition-1&lt;sup&gt;238,256-258&lt;/sup&gt;</td>
<td>4</td>
<td>422</td>
<td>90</td>
<td>&amp;frac1{3}, &amp;frac1{1}</td>
</tr>
<tr>
<td>heparin cofactor II&lt;sup&gt;239&lt;/sup&gt;</td>
<td>1</td>
<td>134</td>
<td>30</td>
<td>lower</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sLOX-1&lt;sup&gt;240&lt;/sup&gt;</td>
<td>1</td>
<td>210</td>
<td>27</td>
<td>higher</td>
</tr>
<tr>
<td>oxidised low-density lipoprotein&lt;sup&gt;259,260&lt;/sup&gt;</td>
<td>2</td>
<td>243</td>
<td>70</td>
<td>higher in 1/2</td>
</tr>
<tr>
<td>asymmetric dimethylarginine&lt;sup&gt;234&lt;/sup&gt;</td>
<td>1</td>
<td>105</td>
<td>27</td>
<td>higher</td>
</tr>
<tr>
<td>flow mediated dilatation&lt;sup&gt;261-263&lt;/sup&gt;</td>
<td>3</td>
<td>377</td>
<td>86</td>
<td>lower in 3/3</td>
</tr>
<tr>
<td>soluble RAGE&lt;sup&gt;264,265&lt;/sup&gt;</td>
<td>1</td>
<td>238</td>
<td>97</td>
<td>higher in 2/2</td>
</tr>
<tr>
<td>N-terminal pro-brain natriuretic peptide&lt;sup&gt;241&lt;/sup&gt;</td>
<td>1</td>
<td>249</td>
<td>92</td>
<td>higher</td>
</tr>
<tr>
<td>aldosterone&lt;sup&gt;242&lt;/sup&gt;</td>
<td>1</td>
<td>156</td>
<td>42</td>
<td>higher</td>
</tr>
<tr>
<td>CD34+ cells&lt;sup&gt;226&lt;/sup&gt;</td>
<td>1</td>
<td>17</td>
<td>7</td>
<td>higher</td>
</tr>
<tr>
<td>pregnancy-associated plasma protein A&lt;sup&gt;243&lt;/sup&gt;</td>
<td>1</td>
<td>184</td>
<td>64</td>
<td>higher</td>
</tr>
</tbody>
</table>

*Not given in original publication. †Meta-analysis of 9 studies reporting pre-interventional high sensitivity C-reactive protein and angiographic restenosis. ‡For elevated levels of high sensitivity C-reactive protein, (95% CI 1.2 – 2.1). CD = cluster of differentiation; RAGE = receptor for advanced glycation end products; sLOX-1 = soluble lectin-like oxidized low density lipoprotein receptor-1.
before intervention and the development of ISR, with an odds ratio of 1.6 (95% CI 1.2 – 2.1) for the presence of elevated high sensitivity C-reactive protein, after adjustment for clinical and angiographic variables.\textsuperscript{244} However, there was evidence of heterogeneity between studies and publication bias.\textsuperscript{244} The $I^2$ (the percentage of variability not explained by chance) was 45%, $p = 0.07$, and it appeared that studies demonstrating smaller effect sizes may have been less likely to be published.\textsuperscript{244} Therefore, the relationship between high sensitivity C-reactive protein and ISR may be more modest than the result in this study. The authors did not report an association between high sensitivity C-reactive protein and late loss.\textsuperscript{244}

Bilirubin was measured before bare metal stent implantation in 1,076 patients in a single study by Kuwano et al.\textsuperscript{245} A total of 341 patients developed the outcome of ISR, quantified by protocol angiography.\textsuperscript{245} Levels of bilirubin were higher before intervention in the patients who subsequently developed ISR, independently of clinical and angiographic variables.\textsuperscript{245} The relationship between bilirubin and ISR was also independent of suspected liver injury.\textsuperscript{245} High sensitivity C-reactive protein was not included in this study.\textsuperscript{245}

However, while the above studies by Kuwano et al.\textsuperscript{245} and Ferrante et al.\textsuperscript{244} strongly suggest that the measurements of bilirubin and high sensitivity C-reactive protein before intervention are predictive of ISR development, no studies have examined whether these molecules afford any incremental value upon that given by clinical and demographic variables alone. Furthermore, there is likely to be overlap between many of the inflammatory variables that have been associated with ISR. It is unclear which inflammatory marker, or combinations of markers, best capture the inflammatory component of ISR development.

Overall, even though there are some encouraging studies linking biomarkers to ISR, their impact has been limited. While many may have potential clinical use, often no further research has been done into their utility, and no biomarkers are currently used clinically.
1.6. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a subfamily of the metzincin superfamily of endogenous proteinases.\textsuperscript{266} The metzincin superfamily are evolutionarily ancient, with prototypic members present across the spectrum of the animal kingdom.\textsuperscript{267} Many are characterised by the amino acid sequence HExxHxxGxxH/D, at the active site, where two histidines bind Zn\textsuperscript{2+} ions and glutamate donates a hydrogen ion in order to cleave the target peptide bond.\textsuperscript{268} Across the different kingdoms of life metzincins participate in a wide variety of interactions including cell- and protein- interactions, and signal transduction, with specific sub-families having a degree of specificity.\textsuperscript{268} The structural homology of metzincins across plant and animal species suggests that they arose at least 3.5 billion years ago, before the Cambrian explosion.\textsuperscript{269} MMPs are likewise conserved across a number of living kingdoms.\textsuperscript{268} There are multiple MMPs, having some degree of species specificity, which developed during early vertebrate evolution.\textsuperscript{270} The main role of MMPs across all species appears to be targeting ECM components for proteolysis.\textsuperscript{268} Individual MMPs are largely similar in terms of structure, but variation in modular components determines substrate specificity and relates to function.\textsuperscript{269} It is likely that a single, primordial MMP gene was duplicated and resulting divergence led to the observed diversity of structure and function between different members of the MMP family.\textsuperscript{267}

1.6.1. Matrix metalloproteinases structure and function

There are 24 members of the MMP family in humans,\textsuperscript{267} and all have the following features in common: MMPs break down extra cellular matrix; have Zn\textsuperscript{2+} in their active site; require Ca\textsuperscript{2+} to function; function at neutral pH; most are secreted in an inactive pro-form; and they are inhibited by a family of endogenous inhibitors, the tissue inhibitor of matrix metalloproteinases (TIMPs).\textsuperscript{271} The inhibition of MMPs appears to be the main function of TIMPs,\textsuperscript{272} but they have other functions, which will be discussed below. The MMP family may be broken into four groups on the basis of structural and substrate specificity: collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs).\textsuperscript{266} Typical structural features are displayed in Figure 1.3.

Collagenases include MMPs-1, -8, and -13, and break down interstitial collagens I, II and III. The gelatinase subgroup is comprised of MMP-2 and MMP-9. They derive their name from the fact that they are good at cleaving denatured collagen, or gelatin. They have a fibronectin-like domain near the active site that can bind to matrix targets, notably the
basement membrane. This may facilitate interactions with fibronectin that are important for the activation of MMPs-2 and -9. MMP-2 degrades collagens I, II, III, and they both degrade collagen IV, an important part of the basement membrane. The stromelysins are MMP-3, -10 and -11. These are structurally similar to the gelatinases, but have a broader range of specificities. MT-MMPs are bound to cell membranes via a transmembrane linker domain. They are capable of degrading collagens I, II and III as well as being involved in the activation of other MMPs. Hemopexin-like domains are common structural features amongst vertebrate MMPs, and appear to be involved with substrate binding. The pro-peptide inhibits the enzymatic activity of MMPs, and will be discussed in further detail below. A comparison of a selection of MMPs involved in vascular disease is displayed in Table 1.3.

![Figure 1.3 Structural overview of matrix metalloproteinases](#)

Adapted from Das et al. and Massova et al.

In normal physiology, MMPs are involved in embryo development, wound healing, parturition, and bone remodelling. MMP-9 is required to liberate cells from the bone marrow niche. However, when dysregulated, or subject to abnormal stressors, MMPs can play a role in pathology. Two examples of conditions that MMPs have been associated
Table 1.3 Overview of selected MMPs involved in vascular disease

<table>
<thead>
<tr>
<th>MMP</th>
<th>Chromosome</th>
<th>Molecular weight (kDa)</th>
<th>Collagen substrates</th>
<th>Other substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pro-</td>
<td>Active</td>
<td></td>
</tr>
<tr>
<td>Collagenases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-8</td>
<td>11q21-q22</td>
<td>75</td>
<td>58</td>
<td>I, II, III, V, VII, VIII, X</td>
</tr>
<tr>
<td>MMP-13</td>
<td>11q22</td>
<td>60</td>
<td>48</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Gelatinases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>20q12-q13</td>
<td>92</td>
<td>86</td>
<td>IV, V, VII, X, XIV</td>
</tr>
<tr>
<td>Stromelysins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>11q22</td>
<td>57</td>
<td>45</td>
<td>II, III, IV, IX, X, XI</td>
</tr>
<tr>
<td>Matrilysins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>11q21-q22</td>
<td>28</td>
<td>19</td>
<td>IV, X</td>
</tr>
<tr>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>14q11-q12</td>
<td>66</td>
<td>56</td>
<td>I, II, III</td>
</tr>
<tr>
<td>(MT-MMP-1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Modified from Raffeto et al.\textsuperscript{275} and Chakraborti et al.\textsuperscript{283}

with are the development of cancer metastases by breakdown of the basement membrane, allowing tumour migration and invasion,\textsuperscript{284} and arthritis, where MMPs have a role in facilitating the pathological breakdown of cartilage.\textsuperscript{285}
1.6.2. Regulation of MMPs

The active state of MMPs appears to be necessary for their biological activity. In order to regulate their activity, MMPs are both targeted to cellular and ECM proteins (e.g. MT-MMPs bound to cell membrane, and to ECM via the fibronectin- and hemopexin-like domains), and their activity is controlled at three levels. The first is translation, or the production of enzyme by the cell from mRNA. The MMP family is divided on its response to different signals. A number of MMPs are secreted in response to growth factor mediators of remodelling, vascular stress, injury, inflammation and reactive oxygen species. On the other hand, MMP-2 appears to be unique in that it is not induced by inflammatory cytokines, and consequently has a more stable expression in vivo. Most MMPs are secreted upon translation, but in neutrophils, MMP-8 and -9 have an extra level of regulation, where they are stored separately in specific granules and are able to be released upon activation of the neutrophil.

1.6.2.1. Translational regulation of MMPs

MMP function is regulated at the transcription level, with signal-dependent production and inhibition of MMP and TIMP genes. MMP-2 is constitutively expressed, and MMPs-1, -3, -8, -9 and -14 are inducible. Members of the matrix metalloproteinase family are regarded as inflammatory mediators, as many are responsive to inflammatory signals, for example, MMP-9 release is stimulated by IL-8. On the basis of gene promoter elements, MMP genes can be classified into three groups. The first is the TATA box/AP(activator protein)-1 positive genes, the second is the TATA box positive/AP-1 negative genes and finally the TATA box/AP-1 negative genes (Figure 1.4). Clearly, multiple regulatory elements are involved in determining the production of MMPs. This scheme predicts that the transcription of Group 1 MMPs should be stimulated by inflammatory mediators such as interleukin-1 and tumour necrosis factor α, as these cytokines interact with TATA box and AP-1 elements. This is consistent with empirical evidence. However, further diversity may also be possible through the precise genetic structure of each element. The production of MMP and TIMP microRNA occurs 4 – 8 hours after stimulation, suggesting that MMP and TIMP genes are targets of regulation by the so-called “immediate-early response” genes, which are activated immediately after stimulation, and appear to co-ordinate complex responses to stimuli. It is probably in this way that MMPs are
Figure 1.4 Regulatory elements in matrix metalloproteinase gene promoter regions
Adapted from Yan and Boyd, Westermarck and Kahari, Clark et al., and Fanjul-Fernandez et al.

AP-1 = activator protein-1; AP-2 = activator protein-2; C/EBP-β = CCAAT/enhancer binding protein-β; GC = Sp-1-binding site; PEA = polyoma enhancer A binding protein-3; SPRE = stromelysin-platelet derived growth factor responsive element; TATA = TATA box; Tcf-4 = T-cell factor-4/β-catenin-binding site; TIE = transforming growth factor-β inhibitory element.

linked to the activity of various pathways that coordinate responses to injury, inflammation and growth, like mitogen-activated protein kinases, enabling appropriate breakdown of ECM.
1.6.2.2. Activation of MMPs from latent form

The second level of regulation of MMPs is activation from the pro-form. All known MMPs are activated by a “cysteine switch” mechanism, where a cysteine residue complexes with the Zn$^{2+}$ ion in the active site and prevents the action of the enzyme. When the interaction between the cysteine residue and the Zn$^{2+}$ ion is disrupted, the enzyme is activated. This can be accomplished by a number of methods, all of which remove, modify or expose the cysteine residue in some way. This stops another cysteine in the conserved PRCGxPD sequence from interacting with the Zn$^{2+}$ ion in the active site, thus allowing it to interact with water and catalyze reactions. The activation may occur intracellularly, at the cellular surface, or extracellularly.

Currently, a number of enzymes and biological molecules have been identified as playing a role in the activation of MMPs. This includes oxidative compounds, and a range of proteolytic enzymes, including other MMPs, and the serine proteinases chymase and urokinase-plasminogen activator. MMP-2 is known to be activated by the membrane-type MMPs, and MMP-9 is activated by both MMP-3 and MMP-3-independent mechanisms. In vitro, activation of MMPs may be performed by the addition of $p$-aminophenylmercuric acetate (APMA), and by detergents (e.g. sodium dodecyl sulfate). All of the above mechanisms appear to act via interruption of the cysteine switch, removing the cysteine complexing the catalytic Zn$^{2+}$ ion at the active site. Numerous, and possibly redundant, pathways may be involved in MMP activation in vivo (Figure 1.5), and the relative importance of each has not been established. However, at least with respect to the vascular system, the activation cascade of urokinase, plasmin and then MMP activation appears to be important, with inhibition of this pathway by genetic knockout of urokinase and plasminogen resulting in altered MMP activity.

1.6.2.3. Regulation of MMP activity by enzymatic inhibition

The third level of regulation is the endogenous inhibition by the TIMPs which are two-domain proteins, with a number of roles in normal physiology and disease. All TIMPs appear to block MMP activity by steric hindrance, fully occupying the active site in non-covalent 1:1 complexes. The primary role of TIMPs appears to be the regulation of MMP activity by binding non-covalently to the target, inhibiting functioning of the active site by interacting with the Zn$^{2+}$.
the diversity of MMPs, duplication of TIMP genes explains the diversity seen today. TIMP-1 appears to have arisen first, then TIMP-3, with TIMPs-2 and -4 evolving most recently.\textsuperscript{272} Each TIMP inhibits most MMPs tested so far, although TIMP-1 appears to be a poor inhibitor of the membrane-type MMPs.\textsuperscript{266} TIMP-3 has a wider specificity, and is able to inhibit non-MMP metzincins.\textsuperscript{266,316} Notably, while mice with knockout of \textit{TIMP1} or \textit{TIMP2} genes do not display obvious abnormalities,\textsuperscript{266} \textit{TIMP3} knockout results in emphysematous changes.\textsuperscript{317}

![Figure 1.5 Putative activation cascade of matrix metalloproteinases](image)

*TIMPs also appear to have other roles apart from MMP inhibition, including mitogenic and anti-apoptotic effects on a number of different cell types,\textsuperscript{323} and can bind to MMPs in non-inhibitory complexes.\textsuperscript{272} TIMP-2 binds to the C-terminal domain of MMP-2, and this complex allows MMP-2 to be activated at the cellular surface, by the membrane-type MMPs.\textsuperscript{324}

\subsection*{1.6.3. Additional functions of MMPs}

ECM is not just a structural scaffold, but biological molecules are also sequestered in the three-dimensional structure, and are released upon degradation. By facilitating
degradation and the release of biological molecules, MMPs alter cellular functioning. An example of this is the anti-angiogenic product formed when MMP-9 cleaves collagen type IV, inhibiting tumour growth in mice. MMPs also contribute to the regulation of cytokine function, by proteolytically cleaving the ligand-binding domain of the membrane receptor of the specific cytokine, a process called “shedding”.

1.6.4. MMP assaying methods

As established, there are multiple members of the MMP and TIMP families, and each MMP may be present in multiple isoforms (Figure 1.6). One of the most popular activity assays has been zymography. This technique is based on the knowledge that activation of MMPs is associated with a loss of about 10kDa. Therefore, when sorted by molecular weight using electrophoresis, it is thought that the activity of various MMPs in biological samples can be inferred. However, during the zymography procedure, pro-MMPs are activated by 4-aminophenyl mercuric acetate (APMA) and reversibly denatured by treatment with sodium dodecyl sulfate, which also remove bound TIMPs. By virtue of its mode of action, it is clear that zymographic results represent soluble protein, not that bound to ECM. Furthermore, while the gelatin-based zymography used for MMP-2 and -9 is sensitive to small amounts of enzyme, this is not true for some of the substrates required for other MMPs.

An enzyme-linked immunosorbent assay (ELISA) technique that specifically measures the activity of individual MMP isoforms has been described. This assay functions by capturing specific MMPs with autoantibodies, then the activity of the MMP cleaves the pro-sequence of a modified urokinase, which in turn acts upon a quenched chromogenic substrate, which is quantified by spectrophotometry. When this result is compared to a standard curve created by serial dilution of a known amount of MMP, the concentration of MMP in the sample can be calculated. In describing this technique, Hanemaaijer et al. compared a standard zymographic technique utilizing 125I-labelled gelatin degradation to the modified urokinase-based ELISA activity assay head to head in their ability to detect MMP-9 levels in the saliva of patients with Sjögren’s syndrome. While the zymographic technique was able to detect elevations of both the endogenous active and the total form, neither of these reached statistical significance. In light of the fact that the process of sample preparation for zymography interferes with TIMP-binding, the difference in results in the above study may represent reduced study power due to increased variability in active MMP-9 measurements.
Introduction

Figure 1.6 Isoforms of matrix metalloproteinases
Pro-enzyme and active enzyme are determined largely by proteolytic cleavage, however some activity is possible with a conformational change despite the pro-sequence being intact. MMP-2 and -9 can be bound by TIMPs while in the pro-enzyme form, but it appears that other MMPs cannot. Latent enzyme is comprised of MMP in the inactive pro-form and active enzyme bound by TIMP.

Evidence for this includes correlation between the two measurements from another study ($r^2 = 0.45, p = 0.015$)\textsuperscript{334} being relatively modest, given that both measurements are reportedly measuring the same variable.

A further limitation of zymography may be the reliance on exogenous activation by APMA to measure the pro-form. When pro-MMP-9 is activated by APMA, the resulting total MMP-9 concentration measurement by activity ELISA is higher than the pro-measurement by conventional ELISA.\textsuperscript{335} While this may be due to measurement of both the inactive fraction and the endogenous active fraction, the endogenous active fraction is typically substantially lower than the pro-fraction,\textsuperscript{336-338} which would be too small to account for the discrepancy. This indicates that APMA activation may also disrupt MMP complexes with TIMPs or other
Introduction

plasma or matrix components. Many current commercial assays using this technique also have little cross reactivity to TIMP-1 and -2 bound enzyme, and negligible cross reactivity to other MMP isoforms.\textsuperscript{339,340} One point on MMP assaying methods should be clarified. In this thesis the following terminology is used regarding the isoform of MMPs measured in the literature. The “pro-form” indicates the zymogen of the specific MMP, and is measured by ELISA detecting the MMP and pro-region, or by assessing the larger MMP bands on zymography and confirming the presence of MMPs by APMA and substrate degradation. The pro-form ELISA may or may not cross react with active MMP and TIMP-1 bound MMP, whereas zymography would distinguish between these permutations. “Active” forms are measured by activity ELISA, where a specific antibody captures the MMP and the resultant action on a substrate that produces a coloured product, or by zymography where migration in an electrophoresis gel and subsequent digestion of substrate allows quantification of MMP function. Both methods allow direct quantification of the functional activity. When an activity ELISA is used in conjunction with activation of pro-forms by APMA, the result has been termed the “total” form. This is misleading because it ignores the fact that some MMPs are bound to TIMP, which is not detectable by activity ELISA. This has also been termed the “latent” fraction.\textsuperscript{333} Some conventional ELISAs detect both pro- and active MMP isoforms, and in this thesis these measurements have also been labelled “total”.

Most MMPs in vivo are in the pro-form, so the “total” fraction would be expected to be similar to the pro-fraction, or the total MMP antigen levels if the assay does not distinguish pro- from active. However, the distinction is that the “total” form is capable of activity after being activated and may depend on the amount of TIMP available to dynamically interact with the specific MMP. The “pro-” form, when measured by ELISA, reflects only the specific MMP antigen and not the function. For example TIMP-2 cannot bind to inactive MMP-9 whereas TIMP-1 can.\textsuperscript{307} The distinction of “total” from “pro-” may be important in this example. However, in the biochemistry literature “latent” is used synonymously with “pro-”.\textsuperscript{341} For the sake of clarity, whenever referring to the inactive enzyme protein this thesis uses the term “pro-”, and when referring to the fraction of MMP that is capable of enzymatic activity after experimental activation, or to ELISA capturing both the active and pro-form MMPs, uses “total”.
1.6.5. Matrix metalloproteinases in neointimal hyperplasia and coronary stent restenosis

MMPs have previously been implicated in various areas of human cardiovascular pathophysiology, and preclinical studies have implicated MMPs in vascular response to injury. A rise in MMP expression is seen with vascular damage, and VSMCs release MMP to break down connective tissue in order to allow cellular migration. Early work done on MMP modulation during PCI showed some success at reducing intimal hyperplasia in animal models. More recently, changes in MMP expression have been seen in humans undergoing coronary stent implantation.

This section will provide an overview of the evidence supporting a role for MMPs in the pathogenesis of ISR, and how they could have utility as biological markers of ISR.

1.6.6. Putative roles for the MMP family in neointimal hyperplasia

It has been hypothesised that MMPs play a number of roles in the development of neointimal hyperplasia, centred on assisting the role of VSMC. A summary of the proposed roles by Newby involves promotion of:

1. basement membrane degradation,
2. phenotypic modulation of VSMC,
3. migration of VSMC,
4. release of sequestered growth factors from ECM, and
5. proliferation of VSMC.

It is suggested that MMPs undergo sequential up-regulation in the development of intimal thickening. MMP-2 is constitutively expressed in VSMC, but both MMPs-2 and -9 are up-regulated in response to balloon injury in the first phase. Expression of the gelatinases in the medial layer most likely facilitates the beginning of neointimal development by changing the local cellular and extracellular environment. MMP-2 and MMP-9 may be able to degrade the basement membrane, permitting the migration of VSMC out of the medial compartment. Interactions between VSMC and the ECM could be interrupted by MMP lysis of surface integrins and cadherins. Whereas quiescent, contractile VSMC bind mature matrix components like fibrillar collagen and laminins, MMP activity may produce growth-stimulating collagen fragments.
As VSMC migrate down the cytokine gradient into the injured intimal layer, the same chemotactic signals elicit MMP release, which facilitates the goal of repairing the intima.

Inflammatory cytokines expressed after arterial injury stimulate a second phase of MMP up-regulation, with IL-1 and PDGF further up-regulating MMP-9, but also recruiting MMPs-1, -3, -9, -14. Together, this complement of MMPs are probably able to degrade most ECM components, including various proteoglycans, collagens and the internal elastic lamina (Table 1.3), removing physical boundaries which prevent cellular migration.

Sequestered growth factors trapped in collagen and proteoglycan matricies are released on MMP digestion of these components. However, the release and activation of sequestered growth factors may also an additional role in releasing sequestered fibrogenic cytokines, like TGF-β. MMP-9 can then activate the latent TGF-β. This may stimulate the production of TIMPs, which in turn inhibits MMP activity, limiting the cascade described above and preventing uncontrolled ECM destruction. MT-MMP-1 (MMP-14) appears to have a role in TGF-β-mediated fibrosis, with MT-MMP-1 over-expression resulting in multiple steps of the TGF-β signalling pathway being up-regulated. Perhaps in a similar way, Lemaitre et al. demonstrated that enhanced MMP-9 expression, combined with TIMP-1 knockout, led to increased collagen deposition of both types I and III collagen, which was associated with increased TGF-β activation. Finally, the cell cycle stabilizing effects of TIMPs may also moderate the action of VMSCs by locking them in G0, the quiescent phase, via up-regulation of the cell-cycle protein p27 (Figure 1.2).

MMPs-2, -3 and -9, as well as TIMPs-1 and -2, all appear to be important for neointimal development based on gene knockout mouse models (Table 1.4). Experimental knockout of MMPs-2, -3 or -9, or an over-expression of TIMPs-1 or -2, result in decreased neointimal volumes, suggesting that perhaps VSMCs get trapped amongst ECM components which the remaining complement of MMPs cannot digest. Additionally, it appears that there is significant cross-activation of MMP species (Figure 1.5). For example, activation of MMP-9 by MMP-3 appears to be important for the development of intimal hyperplasia.

Inhibition of matrix metalloproteinases impairs smooth muscle cell migration in mice, rats, rabbits, micropigs, pigs and cynomolgus monkeys. However, different animals respond differently. Matrix metalloproteinase
Table 1.4 Effects of MMP inhibition and gene-alteration on mouse models of arterial injury

<table>
<thead>
<tr>
<th>Experimental MMP alteration</th>
<th>Effect on intimal hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-selective MMP inhibition</td>
<td>decreased or delayed</td>
</tr>
<tr>
<td>TIMP-1 over-expression</td>
<td>decreased</td>
</tr>
<tr>
<td>TIMP-1 decrease</td>
<td>increased</td>
</tr>
<tr>
<td>TIMP-2 over-expression</td>
<td>decreased&lt;sup&gt;374&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIMP-2 decrease</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-1 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-1 decrease</td>
<td>ND&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-2 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-2 deficiency</td>
<td>decreased</td>
</tr>
<tr>
<td>MMP-3 over-expression</td>
<td>decreased&lt;sup&gt;375&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-3 deficiency</td>
<td>delayed or decreased&lt;sup&gt;362&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-7 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-7 deficiency</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-8 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-8 deficiency</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-9 over-expression</td>
<td>no change&lt;sup&gt;376&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-9 deficiency</td>
<td>decrease&lt;sup&gt;377,362&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-14 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-14 deficiency</td>
<td>ND</td>
</tr>
</tbody>
</table>

Modified from Kuzuya <i>et al.</i>,<sup>378</sup> and Janssens <i>et al.</i>,<sup>379</sup>
ND = Not done.
<sup>*</sup>Mice do not appear to have an MMP-1 gene.

Inhibitors have been shown to decrease neointimal hyperplasia in a dose dependent manner in human arteries and veins, with a concomitant reduction of gelatinases.<sup>380-382</sup> However, broad-spectrum pharmacological MMP inhibition appears to result in only delayed neointimal development, at least in non-murine models. The “late catchup” seen in larger animals appears to be due to the increased proliferation of VSMCs resident within the neointimal layer.<sup>342</sup>This could be related to increased proliferation, or to repopulation of the neointima by bone marrow cells. Once VSMC are present in the neointimal layer, a third phase of MMP expression may develop, where MMPs are involved in processing the maturing neointimal matrix and ECM compaction. Evidence for this includes continuing high levels, of at least the gelatinases, up to two months after injury,<sup>383</sup> long after initial cellular migration has finished. Additionally, late neointimal modelling in mouse knockout models appears to be impaired. Over-expression of MMP-3 appears to decrease intimal hyperplasia.<sup>375</sup> This late phase of MMP expression may be involved in compaction of ECM performed by these MMPs. It has been demonstrated that lack of MMP-9 results in decreased collagen compaction,<sup>377</sup> but it has not been directly demonstrated for MMP-3.
1.6.7. Experimental evidence of MMP expression after injury.

In human saphenous veins, both of the gelatinases (MMP-2 and MMP-9) rise within hours of experimental injury. MMP-9 is expressed particularly highly in co-localization with proliferating intimal SMC, and is widespread throughout the media as well. MMP-1 and MMP-3 are also up-regulated, but to a much smaller degree, and injury to SMC also causes them to express MMPs -1 and -3. TIMPs -1, -2 and -3 also rise, and each TIMP has a distinctive pattern of expression. Initially, TIMP-2 is expressed in the first few days after injury, diffusely spread throughout the media and adventitial ECM and cells. At two weeks, TIMP-2 is greatly increased in the NI cells, and slightly increased in the media and adventitia. TIMP-3 is expressed in small amounts, as early as day two, spread through the ECM of the media and adventitia, and at 14 days, again in small amounts, in the neointima, and remains in the media. TIMP-1 begins to be expressed by at least two weeks after injury, and is localised in the forming intima, contained within endosomes in SMC and endothelial cells. After two weeks, TIMP-3 is present at low concentration in the neointima, and is expressed most in the luminal side of the media. These changes probably mitigate the effect of increased MMP activity, in order to prevent uncontrolled breakdown.

These results can also be seen in arterial tissue after experimental injury to animals. The gelatinases (MMPs-2 and -9) rise with injury to small, and large, animal arteries. Other MMPs that increase after experimental injury to animals include MT-MMP-1 (MMP-14), MMP-3, MMP-12, and MMP-13.

MMPs have been shown to rise along the length of the stented segment of a ApoE null mouse aorta using in vivo fluorescent molecular tomography, a non-invasive biological imaging technique, using a pan-MMP sensor most specific for the active endogenous forms MMP-9 and -13. This was confirmed by realtime polymerase chain reaction, showing approximately a three-fold increase in MMP-2 mRNA, and a two-fold increase in MMP-9 and -13. Furthermore, this same model was used to show a reduced MMP elevation in non-atherosclerotic mice, which was also associated with reduced intimal hyperplasia.

Of particular interest, MMP-9 was present from one to six days, with MMP-9 mRNA co-localized with VSMC, but was not found two weeks after injury. This timeframe is consistent with the migration of VSMC in this model. The expression of both MMP-9 and MMP-2 over the first eight days correlated with the severity of injury, which is known to strongly associated with the development of NIH. This suggests a strong relationship
Introduction

between both MMPs and the development of NIH. However, while changes can be noted in arterial tissue, it does not necessarily follow that corresponding changes will be able to be detected in the circulation.

Total MMP-9 is acutely elevated in the coronary sinus with balloon inflation, and Ge et al. found that MMP-9 concentrations up to seven days post-procedure are associated with ISR. Also, the concentration of MMP-2 sampled from the coronary sinus four hours after PCI was correlated with ISR in 29 patients studied by Hojo et al., as was the peripheral circulation concentration after one day in 48 patients; although MMP-1 did not change significantly. These findings confirm the role of MMPs in human ISR, and parallel the association of MMP concentration and degree of NIH found in animal injuries. In both animals and humans, MMP-2 and -9 at least are increased, with time profiles appearing to have a two-phase response. Levels initially remain high for around a week after arterial injury, coinciding with the influx of VSMCs into the developing neointima. Secondly, levels appear to be high from one week up to at least two months. This may represent the expression of MMPs during ECM remodelling, and may tie in with the observation that MMP-9 in particular appears to be important for ECM compaction. It seems likely that the levels decrease over time, as MMP-9 and TIMP-1 do appear to be expressed in restenotic lesions at clinical presentation.

1.6.8. Matrix metalloproteinases as clinical predictors of in-stent restenosis

Some work has already suggested that pre-interventional MMPs may have a predictive role in ISR. However, all studies to date have been limited by either sample size, or MMP assaying technique. Serum pro-MMP-9 appears to be associated with angiographic late loss of acute gain but not binary restenosis in a study by Ge et al. Ye et al. found that levels of total (i.e. protein available for activation) MMP-9 before intervention were associated with the need for any revascularizations after coronary stenting and CABG, but no difference in levels of MMP-2 or -3. A study by Hojo et al. found that levels of both pro- and active MMP-2 rose in the coronary sinus after percutaneous coronary intervention, and appeared to be correlated with late loss, assessed by angiography at six months. A Russian-language study by Zemlianskaia et al. appears to show pre-intervention levels of MMP-2 and MMP-9 predicting ISR. While some preliminary work has been done on pre-interventional MMPs and ISR, all studies are limited by small sample sizes, and the studies examined various different forms of MMPs, with only one study including a measurement of MMP activity.
1.7. Involvement of MMPs in atherosclerosis and atherothrombosis

The first stage of atherosclerosis is intimal thickening in response to damaging stimuli, and the process is probably similar to the involvement of MMPs in the response to the experimental and therapeutic injury described above, although at an attenuated level. The mechanisms of MMP expression through constitutive expression, VSMC migration and phenotypic migration are probably not specific to balloon/stenting injury, therefore the formation of intimal thickening in response to atherogenic stimuli may be expected to proceed through similar mechanisms to those described above. However, the development of the additional pathological features of atherosclerotic plaques, including the involvement of macrophages and foam cells in producing the fatty streak, the formation of a lipid core and fibrous cap, and finally the breakdown of the cap and plaque rupture, also appear to involve MMPs.\textsuperscript{21}

There has been much interest in the role of MMPs in the progression of atherosclerosis and complications such as plaque rupture since the observations of Henney \textit{et al.},\textsuperscript{397} and Galis \textit{et al.},\textsuperscript{363} that MMP expression was localised in the shoulder of unstable plaques in human pathological specimens. This has been taken as MMPs potentially being the prime movers of plaque rupture, with research focussing on whether MMPs could aid cardiovascular disease prediction, either by using circulating measurements\textsuperscript{398-401} or MMP specific tags with imaging techniques,\textsuperscript{402,403} and the proposition that MMP blockade could mitigate plaque rupture altogether.\textsuperscript{404}

1.7.1. Putative roles of MMPs in atherosclerosis and atherothrombosis

Much of the evidence that MMPs are involved in atherosclerosis and atherothrombosis has been observational:

1. macrophage activation and infiltration,
2. fibrous cap formation, analogous to neointimal hyperplasia, and
3. breakdown of plaque shoulder.

Like the sequential activation of MMPs in neointimal hyperplasia associated with VSMC, a similar process may occur in atherosclerosis development, but associated with macrophages and foam cells.\textsuperscript{21} Normal arteries express MMP-2, both at the cellular surface, and inside quiescent VSMC, as well as expressing MMP-3.\textsuperscript{131} Both MMPs-2 and -3 are mostly expressed in the adventitial layer.\textsuperscript{131} Attachment of monocytes to activated...
endothelium and activation of Mac-1 may stimulate inflammatory cells to produce MMP-9. Early atherosclerotic lesions appear to have small amounts of MMPs-2 and -9, with high levels in more advanced lesions. TIMP-1 knockout leads to increased macrophage infiltration, presumably by unopposed proteolysis. In contrast to the apparent dependency of MMP-9 activity on the presence of MMP-3, MMP-9 does not appear to be dependent on activation by MMP-2. Presumably assisted by the gelatinases, macrophages migrate towards sources of inflammation such as oxidised LDL, aided by the signalling from cytokines to produce MMP-9 and the MT-MMPs. Contact with lymphocytes via the CD40 receptor stimulates a broader range of MMP elaboration, including MMPs-1, -3, -8, and -9. The transformation of macrophages into foam cells appears to provoke further production of some of the same MMPs, including MMPs-1, -2, -3, and MMP-14. Presumably signals released from foam cells result in the recruitment of VSMC, which lay down the fibrous cap through similar mechanisms to the development of neointimal hyperplasia, though in an attenuated manner due to the chronic, low-grade nature of this stimulus.

Progression towards plaque rupture probably occurs through an imbalance in the production of the fibrous cap and the breakdown of ECM at the plaque shoulder. MMPs released from macrophages are the prime culprit for these changes. Again, mouse genetic studies have shed light on the roles of MMPs during these processes (Table 1.5).

One of the interesting observations is that both over-expression and knockout of TIMP-1 may reduce atherosclerosis. Rouis et al. administered a TIMP-1 adenoviral vector to ApoE -/- mice, causing an elevation of TIMP-1 expression for around one month. This resulted in a marked reduction in mean lesion size compared to all other groups. This may have been mediated through reduction of macrophage infiltration, as no Mac-1 staining was present in the TIMP-1 treated animals. On the other hand, Silence et al. knocked out the TIMP-1 gene from ApoE -/- mice. Staining for Mac-3 demonstrated the presence of macrophages in the plaques of TIMP-1 -/- mice, with smaller plaques but a greater lipid content.
Table 1.5 Effect of MMP inhibition and gene-alteration on mouse models of atherosclerosis

<table>
<thead>
<tr>
<th>Experimental MMP alteration</th>
<th>Effect on atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-selective MMP inhibition</td>
<td>increased plaque area, unstable(^{418})</td>
</tr>
<tr>
<td>TIMP-1 over-expression</td>
<td>reduced plaque area(^{417})</td>
</tr>
<tr>
<td>TIMP-1 decrease</td>
<td>reduced plaque area(^{409})</td>
</tr>
<tr>
<td>TIMP-2 over-expression</td>
<td>no change(^{360})</td>
</tr>
<tr>
<td>TIMP-2 decrease</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-1 over-expression</td>
<td>reduced plaque area(^{419})</td>
</tr>
<tr>
<td>MMP-1 decrease</td>
<td>ND*</td>
</tr>
<tr>
<td>MMP-2 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-2 deficiency</td>
<td>decreased plaque area(^{410})</td>
</tr>
<tr>
<td>MMP-3 over-expression</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-3 deficiency</td>
<td>increased plaque area(^{420})</td>
</tr>
<tr>
<td>MMP-7 over-expression</td>
<td>increased VSMC, size and stability unchanged (^{420})</td>
</tr>
<tr>
<td>MMP-7 deficiency</td>
<td>plaque rupture;† increased collagen deposition‡</td>
</tr>
<tr>
<td>MMP-9 over-expression</td>
<td>decreased collagen and macrophage content,(^{422})</td>
</tr>
<tr>
<td>MMP-9 deficiency</td>
<td>enlarged plaque area(^{420})</td>
</tr>
<tr>
<td>MMP-12 over-expression</td>
<td>no change,(^{422}) smaller plaques (^{420})</td>
</tr>
<tr>
<td>MMP-12 deficiency</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-13 over-expression</td>
<td>increased atherosclerosis (^{423})</td>
</tr>
<tr>
<td>MMP-13 deficiency</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-14 over-expression</td>
<td>increased collagen deposition(^{424})</td>
</tr>
<tr>
<td>MMP-14 deficiency§</td>
<td>ND</td>
</tr>
</tbody>
</table>

Modified from Kuzuya et al.,\(^{378}\) Fanjul-Fernandez et al.\(^{267}\) and Newby.\(^{425}\)

ND = Not done.

*Mice do not appear to have an MMP-1 gene.
†Few instances of plaque rupture were present with wild-type MMP9,\(^{426, 427}\) but widespread with auto-activating G100L substitution in MMP9 gene.\(^{426}\)
‡Only in combination with TIMP-1 knockout.\(^{360}\)
§MMP-14 deficiency is fatal in the neo-natal period, this study only knocked out MMP-14 from the bone marrow.\(^{424}\)
However, the simple model of a balance of MMP activity and TIMP inhibition being necessary for homeostasis may be inadequate. Rather, it could be that at different times during the development of atherosclerosis, net proteolytic activity could be either detrimental or beneficial in retarding the development of atherosclerosis. An example might be the necessity of MMP action in the formation of the intimal thickening of the fibrous cap \(^{362}\) – this is probably an adaptive function. Conversely, if TIMP levels were inappropriately low in the shoulder of a vulnerable plaque, this could have disastrous consequences for the individual. Consistent with this idea, non-selective MMP inhibition appeared to have a detrimental effect on experimental atherosclerosis.\(^{418}\) Whereas broad inhibition of MMPs appears to have a favourable effect on reducing intimal hyperplasia (even if only temporarily), administration of a synthetic inhibitor of predominantly MMPs-2, -9, -12, -8 and -13 to ApoE \(-/-\) mice fed an atherogenic diet did not protect the mice from the development of atherosclerosis.\(^{418}\) In fact, it appeared that there may have been worse atherosclerotic disease amongst the mice on the MMP-inhibitor treatment, with a greater plaque area and smaller populations of VMSC.\(^{418}\) However, these changes were not observed at all time points, increasing the likelihood that these findings were due to chance. Nevertheless, it appears that broad-spectrum MMP inhibition does not protect against atherosclerosis.

In the series of mouse knockout studies of MMPs in atherosclerosis there have been apparently contradictory studies.\(^{420,422}\) Luttun \textit{et al.}\(^{422}\) and Johnson \textit{et al.}\(^{420}\) both examined the role of MMPs-9 and -12 in the production of atherosclerosis, in ApoE \(-/-\) mice. However, each study produced different results for the two MMPs. Luttun \textit{et al.}\(^{422}\) found that \textit{MMP9} knockout produced smaller plaques, with fewer infiltrating macrophages and less evidence of rupture. Johnson \textit{et al.}\(^{420}\) found that \textit{MMP9} knockout resulted in increased plaque area, macrophage content, and the number of buried layers, suggestive of intraplaque haemorrhage and instability. Similarly, Luttun \textit{et al.}\(^{422}\) found that \textit{MMP12} knockout had no effect on plaque size overall, decreased macrophage infiltration and the prevalence of plaque rupture; whereas Johnson \textit{et al.} found decreased plaque area, buried layers and macrophages. The reason for these discrepancies is unclear, as both groups used similar animals and protocols, except that Johnson \textit{et al.}\(^{420}\) examined brachiocephalic plaques and Luttun \textit{et al.}\(^{422}\) studied the aorta. Concurrent with the experimental atherosclerosis produced in the model of Luttun \textit{et al.},\(^{422}\) knockout of both \textit{MMP9} and \textit{MMP12} led to reduced ectasia of the associated aorta, which may have altered the natural history of atherosclerosis development.
1.7.2. Human studies of MMPs in atherosclerosis and atherothrombosis

In humans with atherosclerotic disease, circulating levels of a number of MMPs have been reported to be higher. Total MMP-9 levels in people with coronary artery disease are around 110% higher than that of age-matched healthy controls.\textsuperscript{428,429} Similarly, total-MMP-2 was 25% higher in patients with coronary artery disease.\textsuperscript{429} Atherosclerosis in other areas also appears to have an impact on circulating MMPs, with peripheral arterial disease being associated with increased levels of total-MMP-9 and TIMP-1,\textsuperscript{430} pro-MMPs-1, -3 and -7 being higher in those with increased intimal medial thickness,\textsuperscript{431} and pro-MMP-9 and TIMP-1 being associated with carotid stenosis.\textsuperscript{432}

Increased MMP levels have been observed in acute coronary syndrome compared to both healthy controls and patients with stable angina. Compared to healthy, vascular disease-free controls, MMP-2 and MMP-9 levels are increased in the peripheral circulation,\textsuperscript{429,433} and MMP-9 and TIMP-1 in the coronary circulation, during acute coronary syndrome.\textsuperscript{434} Atherosclerosis is a systemic disease, and it could be expected that a number of sources contribute the production of circulating MMPs. A study by Inoue \textit{et al.}\textsuperscript{435} found that both MMP-1 and MMP-3 were approximately doubled in the coronary sinus at presentation with acute coronary syndrome, but no change in aortic levels compared to healthy controls. This finding implicates a coronary source, at least in the setting of acute myocardial ischaemia.

Circulating MMPs may also be correlated with disease burden and prognosis. Levels of pro-MMP-9 were higher in the circulation of patients who developed further cardiovascular events.\textsuperscript{398} Patients with critical ischaemia due to peripheral artery disease had MMP-9 and TIMP-1 levels that were significantly higher than those with intermittent claudication.\textsuperscript{430} Levels of pro-MMP-9 appear to be related to plaque morphology, with echolucent plaques – which probably represent lipid-rich atheroma – being associated with higher levels of pro-MMP-9 than those without visible plaques.\textsuperscript{436}

1.8. Altered expression of MMPs with other conditions

The circulating levels of MMPs deriving from other physiological and pathological processes are a source of potential confounding for the use of MMPs as biomarkers for ISR. We do know that a number of conditions do increase the plasma concentration of MMPs, so this warrants discussion.
1.8.1.1. **MMP expression in myocardial dysfunction**

The ECM orientates cardiomyocytes and provides the structural strength of the heart, and altered ECM appears to be a key component of myocardial dysfunction.\(^{437}\) MMP-2 is up-regulated with heart failure in clinical studies,\(^{438,440}\) and is correlated with established markers of heart failure.\(^{438,441}\) Both circulating MMP-9 and -3 appear to be associated with left ventricular dysfunction shortly after myocardial infarction.\(^{442,443}\) A rat model of heart failure through coronary ligation demonstrated a varying profile of MMPs-2, -8, -9, -13 and -14, as well as TIMPs-1 and -2 with time, continuing to evolve up to four months after ligation.\(^{444}\)

Diastolic dysfunction is it is an under recognised phenomenon and pre-symptomatic diastolic dysfunction is common, and strongly associated with long-term mortality.\(^{445}\) MMPs appear to be differentially expressed in both animal and human studies of diastolic dysfunction.\(^{446-448}\) Martos *et al.* demonstrated that pro-MMPs-2 and -9 increase with more severe diastolic dysfunction. However, this appeared to be restricted to those with established diastolic heart failure, with little difference between categories in those with pre-symptomatic dysfunction.\(^{448}\)

1.8.1.2. **Other conditions associated with alterations in MMP levels**

Other variables, which may alter plasma MMP levels, include cancer,\(^{449,450}\) gender,\(^{428}\) ethnicity,\(^{428}\) fitness,\(^{428}\) age,\(^{428,451}\) medications,\(^{452-454}\) obesity,\(^{455}\) surgery,\(^{456}\) asthma,\(^{457}\) liver fibrosis,\(^{458}\) and rheumatoid arthritis.\(^{459}\) Circulating MMPs are elevated in patients with aortic aneurysm,\(^{460-463}\) and appear to return to baseline after surgical resection.\(^{461,462}\) A number of MMPs are altered in the myocardium and circulation in heart failure.\(^{437}\)

Hence, many variables and co-existing conditions may be present in people undergoing coronary stenting, and should be considered when proposing circulating MMPs as markers of ISR.
1.9. Aims of this study

The specific aims and *a priori* hypotheses are included below. Overall, this thesis aimed to examine the idea that:

“the active forms of matrix metalloproteinases have a pivotal role in the development and progression of cardiovascular disease, including ISR, and that this is reflected by altered circulating levels, when accurately measured”

**Aims:**

1) to examine the circulating active levels of the candidate MMPs-1, -2, -3 and -9, as well as TIMP-1 in participants with a history of ISR.

2) to establish whether the measurement of active MMP levels provides incremental benefit to conventional risk factors in the prediction of ISR.

3) to replicate any associations between active MMPs in a prospective study, taking blood samples prior to intervention. The primary hypotheses were that active MMP-9 and TIMP-1 would be elevated prior to intervention amongst those who would develop ISR compared to those who would not.

4) to describe the profiles of MMP activity during the natural history of ISR development. The primary hypotheses were that active MMP-9 and TIMP-1 levels would be elevated at three months after intervention amongst those developing ISR compared to those who were not developing ISR. That active MMP-9 and TIMP-1 would be increased from baseline amongst those developing ISR compared to those not developing ISR was included as a secondary outcome.

5) to study circulating levels of the active fraction of MMPs and the development of non-ISR cardiac events. The primary hypothesis was that those who went on to develop non-ISR events would have elevated active MMP-9 and TIMP-1 at all time points. That active MMP-9 and TIMP-1 would be elevated prior to presentation amongst those developing non-ISR events was included as a secondary outcome.

6) to establish whether active MMPs are associated with myocardial remodelling, particularly diastolic dysfunction, after interventional treatment for coronary artery disease.

To investigate these hypotheses, firstly the plasma levels of candidate MMPs will be correlated with previous development of ISR in a case control study of asymptomatic patients. This will allow us to gain an insight into a panel of candidate MMPs and their potential associations in people with a history of coronary stenting, with and without restenosis. This
study design will allow sufficient power for multiple comparisons to be made, and to compare the findings in the context of established risk factors.

A prospective cohort study will then be recruited, taking blood samples prior to intervention, and assessing the relationship between the plasma levels and clinical outcome after one year. This will confirm whether the MMPs highlighted in the retrospective study have utility in the pre-interventional prediction of ISR. Serial blood samples will afford the opportunity to describe changes in plasma MMPs during the natural history of ISR, over a six month period after stenting. This will allow a comparison of whether the change in plasma profiles of MMP activity differs between those who develop ISR and those who do not.

As numerous potential confounders arise at the time of coronary intervention, including acute myocardial necrosis and ventricular remodelling, the relationship between these variables and the activity of MMPs will be examined, both prior to intervention, and serially over six months. Finally, the level of MMP activity will be assessed with respect to the development of non-ISR coronary events, both prior to the index intervention, and in the months leading up to clinical presentation.
2. Methods and materials

2.1. Overview of studies

Two groups of clinical patients were recruited for the studies in this thesis. These are referred to as the “retrospective” and the “prospective” studies. The retrospective study had a case-control design, which enrolled patients with confirmed coronary artery disease and a history of either percutaneous coronary intervention with stent placement or ISR. All patients were asymptomatic for at least six months at the time of recruitment. Because of the nature of the case-control recruitment there was a disproportionately large group of cases, and this study was used for hypothesis generation about which active MMP markers may have a role in the prediction of ISR. The prospective study was a cohort study, enrolling patients as they underwent percutaneous coronary intervention with stent placement. Participants were then followed for one year with phone call interviews and serial blood samples. Cases were then selected from those who had clinically-driven, angiographically-proven ISR, or other cardiac events. This study was then analysed with a case-cohort design. While this design yields fewer cases, it is methodologically stronger for associating biomarkers with disease and allows serial measurement of biomarkers.

Within this chapter, the methods for all of the included studies are described. First, the recruitment and sampling methodology for each of the studies is detailed, along with a brief description of the biochemical assays used in each study.

This is followed by the description of how demographic and clinical characteristics were recorded from participants in all studies, and how coronary angiography was interpreted in each study. Then the blood sample preparation and biochemical assay methods common to both studies are presented. Finally, the statistical analyses are described.

2.2. Retrospective study

2.2.1. Retrospective study patient recruitment

From February 2003 through December 2004, patients were recruited from clinical coronary angiography records at Dunedin Public Hospital. Patients who had undergone PCI from January 1999 to February 2002 were included. All patients had angiographically proven
coronary artery disease. Controls were those who received bare-metal stents for de novo coronary artery disease and at the time of recruitment had been angina-free for >1 year after their stent was placed. Cases were recruited from those who received a bare-metal stent for coronary artery disease and subsequently presented with symptoms of ISR, which was then angiographically-proven as being due to a stenosis >50% in the stented segment. Such patients then underwent further treatment as per standard practices, and were free of coronary artery disease symptoms and cardiovascular events for ≥ 6 months at the time of recruitment. All patients gave written, informed consent as a part of recruitment, and the study was conducted with the approval of the Lower South Ethics committee (Appendix 11.1).

2.2.2. Retrospective study sample collection

Samples of peripheral venous whole blood were taken and placed in 2 x 4mL EDTA (ethylenediaminetetraacetic acid) tubes and 1 x 4mL heparin tube. Blood was collected by both the Dunedin hospital venepuncture service and experienced research staff in the wider regional community. Samples collected from the wider Otago-Southland region bloods were centrifuged and frozen the same day they were collected.

2.2.3. Retrospective study biochemical assays

Endogenous active MMPs-1, -2, -3 and -9 were measured in heparin samples using the Biotrak Activity Assay system (RPN 2629, 2631, 2639, and RPN 2634 respectively, GE Healthcare Life Sciences). Pro-MMP-9 and TIMP-1 were measured by the conventional Biotrak Assay system ELISA (products RPN 2614 and RPN 2611, respectively) in EDTA samples.

2.3. Prospective study

2.3.1. Prospective study patient recruitment and follow up

From February 2007 till February 2010, a prospective cohort of patients was recruited from Dunedin Public Hospital, after angiography confirmed the indication for coronary stent placement. In contrast to other studies, patients with acute coronary syndrome and acute myocardial infarction were included. Patients were eligible for the study if they were to receive bare-metal coronary stent(s) for de novo lesions. Patients were ineligible for the study for any of the following reasons: they were to receive any drug-eluting stents, were
undergoing PCI for ISR lesions, had heart failure, were under the age of 20 years, or had other comorbidities that were likely to restrict expected life length to less than one year (e.g. stage IV cancer, end stage respiratory disease).

Written informed consent was obtained from all patients and the study was conducted with the approval of the Lower South Ethics committee (see Appendix 11.2). Participants were followed at three, six and 12 months after intervention with a phone call interview and blood sampling. Interview questions included whether new or recurrent angina had occurred, the emergence of adverse events including revascularisation, myocardial infarction, stroke, cardiac death, and changes to prescribed medication (Appendix 11.2.5). Clinical events were then confirmed by examining patient hospital records. Subjects were considered to be clinically free of symptomatic ISR if they remained free of angina for more than 12 months after their initial stent placement. Control subjects were randomly selected from this clinically asymptomatic group, stratified by initial clinical presentation (three controls to one ISR case). Random numbers were obtained from http://www.random.org/ integers/. Based on an estimated 40% difference in active MMP-9 levels between ISR and controls in the retrospective study, an estimated 30 ISR cases would have to be included for 80% power to detect a similar difference when matched three-to-one.

For the comparison of active MMP-9 and TIMP-1 in the months prior to presentation with incident cardiovascular (non-ISR) events (Chapter 8), the last time point before the event developed was identified for each case, and compared to the matching time point for controls. The controls were selected by two methods. The first analysis included controls selected at random, and the second analysis included controls matched for index clinical presentation and diastolic dysfunction category. Up to three controls were selected for each case.

2.3.2. Prospective study sample collection

Baseline samples of whole blood were taken at index and again at re-presentation with clinical events necessitating angiography. Samples were drawn from the peripheral circulation through the intra-arterial sheath (either femoral or radial artery). All samples were processed within 60 minutes. Blood was placed in 2 x 4mL EDTA tube, and 1x 4mL heparin tube.

Follow up blood samples were drawn from a peripheral vein, at the Dunedin Public Hospital venepuncture service or in the wider regional community, either at a local community laboratory (Southern Community Laboratories Ltd.) or a general practitioner’s
office. Samples were stored at 4°C and transported from the wider regional community to the central Southern Community Laboratory in Dunedin Public Hospital, and from there they were then transferred to the Vascular Research Group laboratory.

2.3.3. Prospective study biochemical assays

Endogenous active MMP-9 was measured in heparin samples using the Biotrak Activity Assay System (RPN 2634, GE Healthcare Life Sciences), and TIMP-1 was measured by the conventional Biotrak Assay system ELISA (RPN 2611) in EDTA samples.

2.4. Demographics and clinical risk factors

A questionnaire obtaining baseline demographic information was administered during the initial interview, and clinical information was gathered from hospital notes and laboratory database. Detailed demographic information including anthropometrics, cardiovascular disease risk factors, vascular disease history and medication were recorded (for the retrospective recruitment see Appendix 11.1, for the prospective recruitment see Appendix 11.2). For the retrospective study, medications represented those used at recruitment; for the prospective study they represented medications being prescribed at time of admission. One smoking pack year was defined as 20 cigarettes (one pack) per day for 1 year or if loose tobacco was used, the following formula was applied:

Equation 2 Formula for pack years from loose tobacco

"12.5 grams per week x (1/7) x number of years smoked = pack years"

In the prospective study, transthoracic echocardiograms (GE/VingMed Vivid-6 system, GE Healthcare, Milwaukee, USA) were performed within two weeks of intervention, and ejection fraction was calculated by the Simpson’s Biplane method.466,467

A sub-set of patients with preserved left ventricular systolic function with ejection fraction ≥45% underwent Doppler examination with analyses subsequently performed off-line by an experienced cardiologist who was blinded to MMP measurements (Dr. John Chu, Senior Lecturer and Consultant Cardiologist, Department of Medicine, University of Otago). Two-dimensional echocardiography imaging, conventional and tissue Doppler imaging (TDI) ultrasound measurements were obtained. All measurements were taken according to the guidelines of the American Society of Echocardiography468-470 and were averaged over five measurements on different cardiac cycles. Left ventricular mass in grams was derived from left ventricular linear dimensions by the following formula:
Equation 3 Calculation for left ventricular mass by echocardiography

$$\text{LV mass} = 0.8 \times \left(1.04 \times (\text{LVIDd} + \text{PWTd} + \text{SWTd})^3 - (\text{LVIDd})^3\right) + 0.6$$

where LVIDd, PWTd and SWTd were left ventricular internal dimension at end diastole, posterior wall thickness at end diastole and septal wall thickness at end diastole, respectively. The following conventional pulsed Doppler measurements were obtained in the apical view with a cursor at the mitral valve inflow and pulmonary vein: maximal early (E) and late (A) transmitral velocities in diastole, (from which the E/A ratio was calculated), and E-wave deceleration time. Isovolumic relaxation time was measured in the apical 4-chamber view by continuous-wave Doppler placed between the mitral inflow area and the left ventricular outflow tract. The ratio of transmitral flow velocity to annular velocity ($E/E'$), and the left ventricular filling index were calculated. Diastolic dysfunction (DD) was graded as mild (impaired relaxation), mild-moderate (impaired relaxation), moderate (pseudonormal pattern), and severe (restrictive filling) using the Canadian Consensus Classification.

2.5. Angiographic analysis

In the retrospective study, angiographic analysis was performed by visual assessment by an interventional cardiologist. In the prospective study measurements were performed with quantitative coronary angiography (Camtronics Medical Systems, CRS-PCT-GEMNET; Version 3.1.04.10), by an interventional cardiologist or catheterization laboratory radiographer.

Quantitative coronary angiography was performed on the culprit lesion with analysis of three end diastolic frames. The minimum lumen diameter (MLD), lesion length and reference vessel diameter were measured in the single plane worst-view of the lesion and percent diameter stenosis calculated. Coronary artery disease was quantified by the number of vessels with >50% stenoses by visual assessment. The modified American College of Cardiology/American Heart Association (ACC/AHA) classification was used to categorise the morphology of baseline coronary lesions. Angiographic variables included were the number of sites stented, and diameter and length of stents. The restenosis pattern was categorized by the Mehran classification, using the angiographic view with the most severe appearance (Appendix 11.4), and number of ISR lesions was recorded. Additional angiographic information was recorded in those with restenosis including MLD, length of lesion, reference diameter of the vessel, percent diameter stenosis of ISR lesions as...
well as time to recurrence of symptoms. The dates of admission, and of PCI were recorded, as were the clinical presentation and echocardiography results (Appendix 11.2.5).

The severity of CAD was assessed by the number of coronary vessels that were diseased. Which arterial branches were designated as being major depended on whether the coronary circulation was right- or left- dominant. If the circulation was left-dominant, then the three arteries were (1) left-anterior descending, (2) proximal left circumflex and marginal branches, and (3) distal left circumflex. If the circulation was right-dominant, then the three arteries were (1) right coronary, (2) left anterior descending, and (3) left circumflex. A balanced circulation was categorised the same as a right-dominant circulation. In the setting of left main stem disease, if the circulation was right-dominant, it was classified as two-vessel disease, and if it was left-dominant, it was classified as three-vessel disease.

The author performed validation of the quantitative coronary angiography by double reading thirty lesions on randomly selected angiograms. Because different frames from the same lesion may give different measurements, the percent stenosis for each lesion was averaged over three measurements at end diastole and compared to those determined during a separate session utilizing the same frames. Each assessment was blinded to patient outcome and MMP results, and studies were read in a random order (random numbers were obtained from http://www.random.org/integers/). The mean difference between the first and second measurement was 0.1%. The standard deviation of percent stenosis was 2.7%, and there was no evidence for an influence of absolute percent stenosis on measurement difference (Figure 2.1). The SD for the first individual measurement from each session was 7.6%. This degree of precision is similar to that of other published studies which range from 3.6% for a clinical study with averaged measurements, or 3.6 – 8.3% for single measurements. Studies of earlier quantitative systems describe precisions of 8.7 – 18.4% compared to the true lumens of plexiglass models, whereas contemporary systems appear to be capable of <5% repeatability under similar circumstances. All subjects included in the ISR group had angiographic restenosis, which was confirmed by their treating cardiologist, and clinically driven revascularisation. An interventional cardiologist or catheterization laboratory radiographer reviewed the angiograms of patients who developed ISR. Time to ISR, the number of ISR lesions and the percent stenosis were also recorded (Appendix 8). Those who suffered from ISR during follow up had blood drawn at angiographic confirmation, and underwent another year of follow up, again with three phone call interviews and blood samples.
A Bland-Altman plot is the difference between two measurements plotted by the average of the absolute measurement, and allows an unbiased assessment of agreement. Percent stenosis was determined by quantitative coronary angiography. The percent stenosis was averaged over three readings and compared to a separate reading utilizing the same frames, but during a separate session. The assessor was blinded to patient outcome and MMP results.
2.6. Definition of ISR events

ISR was defined as having clinical symptoms suggestive of restenosis within one year of initial PCI, subsequently confirmed by angiography, with a diameter stenosis of ≥ 50% of the vessel reference diameter at the site of the previously treated lesion in ≥ 1 projections.

2.7. Definition of non-ISRA cardiac events

Non-ISRA cardiac events were defined as the symptoms, or occurrence, of any of the following during 12 months after the index procedure: stable angina; unstable angina; NSTEMI; STEMI; stent thrombosis or cardiac death, unless the criteria for ISR were met. Stable angina was defined as a clinical diagnosis of a syndrome characterised by “discomfort in the chest, jaw, shoulder, back, or arms, typically elicited by exertion or emotional stress and relieved by rest or nitroglycerin”. Unstable angina was defined as the onset of severe or frequent (≥ 3 episodes per day) angina over two months, or the development of angina at rest, without increases in troponin T. Myocardial infarction was defined as an increase in troponin T according to local hospital standards. ST-elevation myocardial infarction was defined as the occurrence of a myocardial infarction with associated ST-segment elevation or new-onset left bundle branch block. Non-ST-elevation myocardial infarction was defined as the occurrence of a myocardial infarction with the absence of ST-segment elevation or new-onset left bundle branch block. All deaths in the study cohort were identified by searching the National Health Index (NHI) database and death certificates were obtained for all patients who died during the follow up period. Cardiac death was defined as all death related to “a cardiac diagnosis, a complication of a [cardiac] procedure, treatment for a complication of the [cardiac] procedure, or an unexplained cause”.

Stent thrombosis was defined according to the Academic Research Consortium definition, with “Definite” indicating presentation with acute coronary syndrome and either angiographic or autopsy evidence of thrombosis or occlusion. “Probable” indicated unexplained deaths within 30 days, or acute myocardial infarction without angiographic confirmation, corresponding to the territory of the stented lesion. “Possible” stent thrombosis was defined as all unexplained deaths at least 30 days after PCI.

Target lesion revascularisation was defined as management of the non-ISRA cardiac event that included revascularisation of the initially treated artery segment, between 5mm proximal and distal to the stent margin. Non-target lesion revascularisation was defined as
management of the non-ISR cardiac event that included an intervention for revascularisation of a coronary artery, but did not include the initially treated artery segment (between 5mm proximal and distal to the stent margin). Medical therapy was defined as management of the non-ISR cardiac event that did not include an intervention for revascularisation.

2.8. Sample Analysis

2.8.1. Plasma separation

Whole blood samples were centrifuged at 4°C and 3000 rpm for 8 minutes. Plasma from all anticoagulant tubes was then stored at -80°C, while red blood cells and buffy coat from the EDTA samples were reconstituted with normal saline, and were stored at -20°C. All samples remained frozen at their respective temperatures until analysis.

2.8.2. MMP and TIMP assays

Endogenous active MMPs-1, -2, -3 and -9 were measured in heparin samples using the Biotrak Activity Assay System (RPN 2629, 2631, 2639, and RPN 2634 respectively, GE Healthcare Life Sciences). This system measures the active isoform of the specific matrix metalloproteinase. Furthermore, by activating the endogenous enzyme with APMA, the level of latent enzyme (the pro-form and the active form not bound to TIMPs) is detected. Throughout this thesis the plasma MMP concentrations reported are for endogenous free active levels (no APMA added to the sample) unless stated otherwise.

The mechanism of the assays involves MMP-capturing antibodies, which, when bound, do not inhibit activity. The antibodies in each assay are specific for a single MMP isoform with minimal cross reactivity. The presence of captured MMPs leads to enzymatic cleavage of the labelled substrate. Cleavage of the substrate results in increased substrate fluorescence. The amount of fluorescence produced is proportional to the concentration of the captured active MMP, which can therefore be quantified by spectrophotometry (Figure 2.2).
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Figure 2.2 Mechanism of action of the MMP activity ELISAs
From RPN2639 Matrix Metalloproteinase-3 (MMP-3) Biotrak™ Activity Assay System, GE Healthcare™. The inactive MMP may be activated by addition of APMA, and after washing to remove non-bound MMP, the resulting enzyme activity cleaves a known amount of a proprietary modified urokinase and a fluorescently tagged substrate. The urokinase then acts on the substrate, un-quieting the fluorescent properties, and producing fluorescence proportionally to the amount of MMP in the well.

The MMP-1 (RPN 2629), MMP-2 (RPN 2631) and MMP-9 (RPN 2634) activity assays each use a specific mouse anti-MMP capture antibody, attached directly to the assay wells on the supplied plates. In contrast, the MMP-3 activity assay (RPN 2639) has an extra step. A linking goat anti-mouse antibody is attached to the assay well, which binds a mouse-anti MMP-3 antibody, which captures active MMP-3 from the plasma sample. The plates were then washed with phosphate buffer and unknown samples were added to individual wells and incubated overnight at 2-8°C. In order to create the standard, or detect the latent (all enzyme not bound to TIMP) MMP fraction, APMA was added. APMA was not added when the free endogenous active form was assessed. After incubation the plate was washed, and a reconstituted proprietary substrate, S-2444™ and a modified urokinase were added. The bound active MMPs cleaved the modified urokinase, thereby activating it, which in turn cleaved the substrate resulting in increased fluorescence. The plates were then incubated for 1 hour at 37°C and the absorbance measured in a spectrophotometer at a wavelength of 405 nm.

These assays work by similar mechanisms, differing mainly by antibody specificity. Kits contained (1) 12 x 8 well strips coated with antibodies (RPN 2639 contained goat anti-
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Mouse F(ab)_2 and RPN 2629, 2631 and 2634 contained anti-MMP-1, -2 and -9 respectively; (2) assay buffer which, when diluted with distilled water, gives 50 mM Tris-HCl buffer (pH 7.6), containing 1.5 mM Sodium Chloride, 0.5 mM Calcium Chloride, 1 μM Zinc Chloride, 0.01% (v/v) BRIJ™ 35 and 1 unit/ml Heparin; (3) an isoform-specific standard, with 32 ng of the respective pro-MMP lyophilized enzyme in the assay buffer; (4) APMA in a powder form; (5) lyophilized S-2444™ substrate which is reconstituted in the assay buffer; (6) 100 μl of a concentrated solution of the detection enzyme, a modified urokinase, in the assay buffer; (7) a wash buffer with 12.5 mL phosphate buffer concentrate which gives 0.01 M Sodium Phosphate buffer, pH 7.0, containing 0.05% Tween™ 20 when diluted with distilled water. In addition, RP 2639 contained (8) a lyophilized mouse anti-MMP-3, which was reconstituted in the assay buffer.

The absorbance was correlated to concentration by comparing to a standard curve and a blank that were run concurrently with the sample assays. The standard curve was created by serially diluting the known concentrations of the inactive specific MMP supplied, which was subsequently activated by the addition of APMA. Examples of standard curves are provided in Figure 2.3.

Pro-MMP-9 and total TIMP-1 were measured by conventional ELISA (product RPN 2614 and RPN 2611) in EDTA samples. Both assays capture their respective protein with a specific antibody attached to the plate, then, after washing, bind another antibody with horseradish peroxidise as a detection label. A substrate which reacts with horseradish peroxidise is added, which then produces a coloured product in proportion to the amount of horseradish peroxidase, and therefore the amount of pro-MMP-9 or TIMP-1, respectively. The plates were then incubated for 30 minutes at 20ºC. The reaction was then stopped with 100 μL of 1 M sulfuric acid and the absorbance measured in a spectrophotometer at a wavelength of 450 nm.

These kits contain (1) a plate with 12x8 well strips coated with their respective anti-protein antibodies; (2) an assay buffer with 10 mL of phosphate buffer, the pro-MMP-9 kit has a 0.01M phosphate buffer at a pH of 7.0, with 100mM sodium chloride, 1% (w/v) bovine serum albumin and 10mM EDTA, and the TIMP-1 kit has 0.1 M phosphate buffer at a pH of 7.5, with 0.9% (w/v) sodium chloride, 0.1% (w/v) bovine serum albumin and 0.1% Tween™ 20; (3) a standard with lyophilized protein, the pro-MMP-9 kit contains 64 ng of human pro-MMP-9, and the TIMP-1 kit contains 100 ng TIMP-1. Both were reconstituted in the assay buffer; (4) a lyophilized antibody conjugated with horseradish peroxidase. On
dilution, the concentrations are 0.01M phosphate buffer at pH 7.0 with 0.05% Tween™20 for the pro-MMP-9 kit and 0.01M phosphate buffer at pH 7.5 with 0.05% Tween™20 for the TIMP-1 assay; (6) a solution of 3, 3’, 5, 5’-Tetramethylbenzidine / Hydrogen Peroxide which is ready for use.

A standard curve was created by serially diluting known concentrations of each protein, and the assayed concentration is determined by comparing the absorbance of the sample with those of the standard curve. The ratio of pro-MMP-9 to active enzyme indicated the proportion of zymogen activation. The average coefficient of variance for both activity and ELISA assays was <5.5%.

While the ELISA activity assays were specific to the stated MMP isoforms, the company did report some degree of cross-reactivity to the isoform-TIMP-1 complex. There was negligible cross-reactivity to other MMPs. While not explicitly stated, this is presumably after the MMP-TIMP complex is activated by APMA, as the assays are dependent on the activity of the MMP cleaving a colour-forming substrate. Active MMPs inhibited by TIMP would not have the ability to enzymatically cleave a substrate while bound. The reported cross-reactivities to the MMP:TIMP-1 complex for each activity assay were: MMP-1: 12.5%, MMP-2: 21%, MMP-3: 38%, MMP-9: 22%. Furthermore, a number of kits were cross-reactive to TIMP-2 complexes (active MMP-2/TIMP-2: 8.6%, active MMP-9/TIMP-2: 7%), and pro-MMP complexes (proMMP-2/TIMP-2: 43%, pro-MMP-3/TIMP-1: 38%, pro-MMP-3/TIMP-2: 38%, pro-MMP-9/TIMP-1: 39%). The lyophilized antibody is reconstituted in the assay buffer; (5) wash buffer, with 12.5 mL phosphate buffer concentrate. For all ELISA assays, the standard curve and blank were run concurrently with the sample assays. All standards were performed in duplicate. Of the 60 unknown samples on each plate, 20 were run as duplicates. Cases and controls were evenly distributed over each plate, and both were evenly represented amongst the samples run in duplicate. Unknown samples were diluted to ensure that all measures were conducted within the linear phase of the standard curve (Figure 2.3).
Figure 2.3 Examples of standard curves for pro-MMP-9 ELISA and MMP-9 activity assay
2.8.3. Justification of MMP activity assay strategy

The main two ways that MMP activity has been assayed in the literature are zymography and activity ELISA. Zymography may over-represent the active fraction of MMPs by interruption of the MMP:TIMP complex, and auto-activation during sample preparation. Zymographic measurements suggest that approximately one-quarter\textsuperscript{362} to one-half\textsuperscript{490} of MMP-9 is present in the active form. A representative gelatin zymograph is displayed in Figure 2.4. Conversely, less than 10\% of MMP-9 is functionally active in plasma when assessed through ELISA-based methods.\textsuperscript{333,464} A third way of measuring MMP activity that has been used in the literature is calculating the ratio between circulating inactive/TIMP-bound MMPs and TIMPs, each quantified by conventional ELISA.\textsuperscript{491-499} However, the relationship between the ratio of pro-MMP:TIMP antigen and MMP activity has not been empirically established. Therefore, as a part of this thesis, a direct measurement of MMP-9 activity performed with activity ELISA was compared to the ratio of pro-MMP-9:TIMP-1.

Participants in the retrospective study were included. Pro-MMP-9 and total TIMP-1 were measured by conventional ELISA (product RPN 2614 and RPN 2611, GE Healthcare Life Sciences) in EDTA plasma. Active MMP-9 was measured in heparin plasma samples using the Biotrak Activity Assay System (RPN 2634, GE Healthcare Life Sciences). Both the pro-MMP-9 and the TIMP-1 assays were fully cross-reactive with pro-MMP-9/TIMP-1 complexes. The pro-MMP-9 assay did not cross react with active MMP-9.

The mean age was 63.0 (SD 9.4) and 219 (74.2\%) were male. The median (interquartile ranges) of pro-MMP-9 and active MMP-9 were 23.3 (16.5 – 39.0) and 1.7 (1.2 – 2.5) ng/mL, respectively. The mean concentration of TIMP-1 was 236.8 ng/mL (SD 58.7). The median (interquartile range) of pro-MMP-9/TIMP-1 ratio was 0.10 (0.07 – 0.18). There was a weak negative correlation between the ratio of pro-MMP-9/TIMP-1 ratio and the concentration of active MMP-9 (Figure 2.5A). When age, sex and history of clinical events were entered into the model this finding remained significant, but explained little of the variation of active MMP-9. This indicates that larger ratios of pro-MMP-9/TIMP-1 are associated with less MMP-9 activity, but the weak correlation suggests pro-MMP-9/TIMP-1 ratio is a poor surrogate for active MMP-9.
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Figure 2.4 Representative gelatin zymograph of abdominal aortic aneurysm tissue. Abdominal aortic aneurysm tissue separated into intimal, medial and adventitial layers. Pro- and active bands of MMPs-2 and -9 are identified based on characteristic molecular weights. The majority of both active MMPs-2 and -9 appear to be present in the active form, in all layers. Reproduced with permission from Assoc Prof Greg Jones, Vascular Research Group, University of Otago.
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Figure 2.5 Association between circulating active MMP-9 and pro-MMP-9/TIMP-1 ratio, pro-MMP-9 and TIMP-1

A. Association between active MMP-9 and pro-MMP-9/TIMP-1 ratio.
B. Association between active MMP-9 and pro-MMP-9.
C. Association between active MMP-9 and TIMP-1.

Results are Spearman’s correlation coefficient.
There were modest relationships between individual measurements of active MMP-9 with both pro-MMP-9 and TIMP-1 (Figure 2.5B and C). The pro-MMP-9 was inversely related to active MMP-9, with higher levels of pro-MMP-9 being associated with lower levels of active MMP-9. The correlations between active MMP-9 and pro-MMP-9 and TIMP-1 remained significant after adjustment for age, sex and history of clinical events, but pro-MMP-9 and TIMP-1 explained little of the variation in active MMP-9.

There was a very strong linear relationship between pro-MMP-9 and pro-MMP-9/TIMP-1 ratio ($r = 0.95$). As expected, there was a negative relationship between TIMP-1 and the pro-MMP-9/TIMP-1 relationship ($r = -0.38$). However, in contrast to the strong relationship between pro-MMP-9 and pro-MMP-9/TIMP-1 ratio, there was only a moderate correlation between TIMP-1 and pro-MMP-9/TIMP-1 ratio. Both of the associations between pro-MMP-9, TIMP-1 and pro-MMP-9/TIMP-1 ratio remained significant after adjustment for age, sex and history of cardiovascular events. Similar results were obtained for all analyses by repeating each comparison in the control and events groups.

The main finding of this analysis was that the pro-MMP-9/TIMP-1 ratio was weakly and inversely related with direct measure of circulating active MMP-9. This is opposite to the interpretation of many authors in the literature, who commonly assume that increased pro-MMP/TIMP ratios are an indication of increased MMP activity.491,493,494,496-499

One possible explanation for the negative correlation between the pro- and active forms of MMP-9 is that the pro-MMP-9 antigen available for capture is consumed when MMP-9 is activated. The pro-MMP-9 ELISA assay that we used was able to detect both free and TIMP-bound pro-MMP-9, but was not cross-reactive to active MMP-9. In contrast to the poor association between MMP-9 activity ELISA measurements and the pro-MMP-9:TIMP-1 ratio, the correlation between activity ELISA and zymographic measurements of active MMP-9 appear to be relatively more robust ($r^2 = 0.45$, $p = 0.015$).334 While this analysis was limited by not including zymographic measurements of active MMP-9, the main implication for pro-MMP-9/TIMP-1 appears to be a poor surrogate for active MMP-9, and active MMP-9 should be measured directly.

There is no external frame of reference for measuring MMP activity in biological tissues, so there is no gold standard for measuring MMP activity. However, activity ELISA may have a number of advantages over zymographic analysis: activity ELISA measurements (1) preserve the impact of the endogenous inhibitors; (2) specifically identify the MMP
isoform being assayed; (3) give quantitative results of the concentration of active endogenous enzyme; and (4) may have a greater sensitivity than standard zymography-based methods.

A further issue in the assessment of plasma MMPs is the role of anticoagulant used for sample collection. Anticoagulants that have been investigated include heparin, citrate, EDTA and serum (with and without clot activator). While most work has been done utilizing zymography and pro-form ELISA, two studies have looked at the impact of anticoagulants on the activity ELISAs used in this thesis. A priori criteria for laboratory methods in biomarker studies might include necessary are that measurements are (1) repeatable; (2) not biased towards cases or controls; and (3) strongly correlated with in vivo conditions.

It appears that each of the major anticoagulants that have been used for assaying MMPs (EDTA, citrate, heparin) have an effect on at least some MMP and TIMP species, with apparent concentrations of paired samples increasing or decreasing. Both EDTA and citrate are metal ion-chelators, with targets that include Zn\(^{2+}\) and Ca\(^{2+}\), which are required for MMP activity. Heparin and heparin-related molecules also appear to alter MMP biology. Heparan sulfate has an endogenous role as an ECM constituent, and appears to accentuate the activity of at least some MMPs, namely MMPs-1, -7 and -13.

Heparin has previously been shown to both bind MMP-1, and increase its activity. Pro- and zymographic activity of MMP-9 in paired samples of citrate and heparin plasma have good agreement (r = 0.8 and 0.7, respectively). Two studies have examined the effect of anticoagulants on endogenous activity with activity ELISA measurements. Serum levels of active MMPs-2 and -9 were comparable to citrate, EDTA gave higher levels, and heparin lower levels. When serum was collected, and levels of active MMPs-2 and -9 assayed, before and after the administration of anticoagulants, EDTA greatly increased the levels of both active MMPs-2 and -9; heparin reduced levels somewhat; and citrate did not appear to alter their activity.

Changes in the assayed concentration of MMPs with increasing time from blood storage to centrifugation have also been demonstrated. It appears that both the use of serum and heparinised plasma is associated with rising apparent concentration of MMPs over longer duration from blood collection to centrifugation. After two hours at room temperature, the assayed concentration of pro-MMP-9 was 200% from baseline, and 500% after 24 hours at 4°C. This effect does not seem to extend to the use of EDTA or citrate,
as both citrate and EDTA were associated with a ~50% increase of pro-MMP-9 after 24 hours at 4°C, although this did not reach significance in the citrate samples (due to increased variability). Pro-MMP-2 and TIMP-1 appear to be stable in heparinised plasma over this time period.

In this thesis, pro-MMP-9 and TIMP-1 were measured in EDTA, as this appears to provide stable levels of pro-MMP-9 with increasing duration of blood collection to centrifugation, and TIMP-1 appears to be stable regardless of strategy. Heparin was used to assay active MMPs-1, -2, -3 and -9, as while heparin gives similar readings to citrate, high levels of citrate may reduce MMP activity. While the use of heparin as the anticoagulant for all of these MMPs has not been validated for activity ELISA, heparin and citrate appear to give similar readings of the pro-forms of these MMPs.

2.8.4. Other Tests

Urea and creatinine were analysed by the Dunedin Hospital Medical Laboratory using routine laboratory technique (Roche Modular ACN 418 & 690, Roche Diagnostics) at the time of hospital admission and creatinine clearance was estimated by the Cockcroft-Gault method. Results were taken from the day before intervention, from patient notes or the clinical database.

EDTA plasma samples were analyzed for high sensitivity C-reactive protein (Roche, Tina-quant high sensitivity latex assay) and lipid profiles (Roche, enzymatic-colorimetric methods), performed by Mr. Ashley Duncan and Mrs. Michelle Harper, Department of Human Nutrition, University of Otago. Levels of high sensitive C-reactive protein > 10 mg/L were excluded due to presumed acute inflammation. This cut off point represents the 99th centile of the normal population, and has been recommended as indicating acute inflammation. This cut off point has been utilized in other studies.

2.9. Statistical Analysis

StatView version 5.01 (SAS Institute) and Stata 10.1 (StataCorp) were used to perform statistical analysis. The distribution of continuous variables (kurtosis and skewness) was assessed. Results were given as mean and standard deviation, except for non-normally distributed variables, which were expressed as medians and interquartile ranges (IQR). Categorical variables were expressed as the number of observations and the column percentage. Odds ratios were expressed with 95% confidence intervals.
Continuous variables were analyzed with either the Mann-Whitney U-test or Student’s t-test. ANOVA and Kruskall-Wallis were used for assessing continuous and ordinal variables over multiple groups. Within-subject deltas were calculated as “second measurement – initial measurement”. When deltas were being compared, ANCOVA adjusting the second variable for the first were also performed as a sensitivity analysis. Bonferroni-Dunn correction was utilized when multiple non-hypothesis driven comparisons were done. Chi² test was used for comparison of binary variables and for testing trends across groups, except where a cell had less than five observations, in which case Fischer’s exact test was used.

Multiple logistic regression was used to evaluate the independence of variables with confounding factors. Care was taken to avoid incorrectly adjusting for common consequences of the outcome (e.g. if the outcome of interest were more likely to receive medication because they were developing the outcome, then the presence of the medication would become a surrogate of the outcome. Variables displaying this effect have been termed “colliders”, and may obscure a real relationship). A stepwise model was used to choose variables for inclusion in the regression model, identifying those with significant ($p < 0.05$) or suggestive ($p < 0.15$) associations, as well as those known to be associated with the outcome from examining the literature. Non-normally distributed variables were transformed by natural logarithm and the distribution of the product was checked before entering into regression models. Residuals were examined for deviance from normal distribution to check that assumptions were met. For analyses in which the assumption for independence of samples were not met (i.e. samples from the same individual were included in the analysis), logistic regression with clustering for the identifying variable of each individual participant was utilized.

In all instances where random numbers were used they were obtained from http://www.random.org/integers/. This system utilizes randomness from atmospheric noise. When duplicate numbers were given, the second number was discarded and the next in the list was used.

2.9.1. Receiver-operator curve statistics

Receiver operating characteristic (ROC) curves are widely used in the cardiovascular literature. A ROC curve is a plot of the sensitivity over (1 – specificity), for all cut-off values of a diagnostic test. The area underneath the curve (AUC) is related to the diagnostic accuracy, with 0.5 being random chance, or 50% of the cases having higher scores than the
controls and 1.0 being perfect accuracy, or 100% of the cases having higher scores than the controls.\textsuperscript{207} Maximizing the AUC is one strategy that has been used to assess combinations of variables for use in risk classification.\textsuperscript{207}

mROC (Unité de biostatistiques, CRLC Val d’Aurelle, V1.0) was used to perform multiple receiver-operator characteristic curves simultaneously.\textsuperscript{517} This software uses a non-parametric, empirical model to generate the AUC,\textsuperscript{517} which is useful, as protein biomarkers may not fit the assumptions for logistic regression, that is (1) linearity, (2) constant variance, (3) normality and (4) independence.

ROC curves were used to identify cut off points for biological markers with maximum sensitivity and specificity. Additionally, ROC curves were used to assess the contribution of novel markers to overall discrimination, by deriving the AUC for a group of contemporary markers both with and without the novel marker. Formal hypothesis testing was performed to examine the differences between ROC curves.

\subsection*{2.9.2. Risk model and reclassification analysis}

MMP variables were incorporated into previously derived scores for the prediction of ISR of bare-metal stents. Two scores were utilized, both derived from the Prevention of RESTenosis with Tranilast and its Outcomes trial.\textsuperscript{162} The first was a ready-made score described by Singh \textit{et al.}\textsuperscript{518} which will be referred to as the “PRESTO score” (Table 2.1). However, because of limitations of the PRESTO score, a further score was derived from the multivariate $\beta$ coefficients associated with ischaemic target vessel revascularisation also described in the PRESTO study.\textsuperscript{519} This second score was designated the ITVR (Ischaemic Target Vessel Revascularisation) score (Table 2.2). It has previously been shown that ISR is the predominant driving factor for target vessel revascularisation.\textsuperscript{520}

The predictive value of the clinical and demographic variables from the retrospective study, and the two previously described scores were investigated through a number of statistics. Area under the curve for the scores was calculated using mROC. Bayesian Information Criteria, model $R^2$ and model classification were derived from regression analyses performed with StatView 5.01 and Stata 10.1. Net reclassification index\textsuperscript{208} was calculated across quartiles of risk scores.
### Table 2.1 Modified PRESTO risk score for ISR

<table>
<thead>
<tr>
<th>Variable</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC/AHA type C</td>
<td>3</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2</td>
</tr>
<tr>
<td>Female sex</td>
<td>1</td>
</tr>
<tr>
<td>Lesion length &gt;20mm</td>
<td>4</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>2</td>
</tr>
<tr>
<td>Vessel size</td>
<td></td>
</tr>
<tr>
<td>&gt; 4.0 mm (reference)</td>
<td>0</td>
</tr>
<tr>
<td>3.5 – 4.0 mm</td>
<td>1</td>
</tr>
<tr>
<td>3.0 – 3.5 mm</td>
<td>2</td>
</tr>
<tr>
<td>&lt; 3.0 mm</td>
<td>3</td>
</tr>
<tr>
<td>Maximum score</td>
<td>15</td>
</tr>
</tbody>
</table>

Not included: previous PCI (score 2), unstable angina (score 1). Modified from Singh et al.\textsuperscript{518}

### Table 2.2 ITVR risk score for ISR

<table>
<thead>
<tr>
<th>Variable</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>10</td>
</tr>
<tr>
<td>Age, per year (for every 10y)</td>
<td>-1</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1</td>
</tr>
<tr>
<td>Lesion length &gt;20mm</td>
<td>2</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>2</td>
</tr>
<tr>
<td>Multiple lesions treated</td>
<td>4</td>
</tr>
<tr>
<td>Maximum score</td>
<td>9</td>
</tr>
</tbody>
</table>

Modified from Singh et al.\textsuperscript{519}
Calculation of the net reclassification index requires meaningful risk categories (i.e., risk levels above which clinical management is changed) to be defined. No formal risk categories for clinical decision-making have been proposed in managing the risk of restenosis, so analysis of reclassification was performed using quartiles of the calculated risk scores. As the size of this study was small, and the aim was to test new biomarkers in the context of a previously established model rather than to develop a risk model, the data set was not split into training and validation groups.

Net reclassification improvement (NRI) was calculated by:

Equation 4 Formula for net reclassification improvement

\[
\text{NRI \%} = \left[ \text{proportion (up | disease)} - \text{proportion (down | disease)} \right] - \left[ \text{proportion (up | no disease)} - \text{proportion (down | no disease)} \right] \times 100\%
\]

From Pencina et al.\textsuperscript{208} Where “proportion” indicates the fraction of the study population, “up” and “down” indicate movement of an individual to higher or lower risk category, and “disease” and “no disease” indicate the presence and absence of the disease of interest.

The Hosmer-Lemeshow statistic was calculated to determine if there was significant deviation of subgroup risk from that predicted by the scores. Non-significant \( p \) values indicate a good model fit for the derived scores.
3. Association of MMP markers with a history of ISR

3.1. Introduction

In-stent restenosis (ISR) is one of the main factors limiting percutaneous coronary intervention (PCI). ISR causes morbidity due to further symptoms and the necessity for further intervention, with a low, but significant rate of serious complications. A previous case control study was conducted by the Vascular Research Group comparing coronary ISR with non-ISR stent recipients found that plasma active MMP-9 was significantly associated with a history of ISR.521

The MMP family has 23 members in humans, and a number of these isoforms have been linked to the vascular response to injury and intimal development.30 These include MMPs-1, -2, -3 and -9,131,383,386 as well as the endogenous inhibitors, the TIMPs, of which TIMP-1 is the most highly expressed in the response to injury.387 As part of this doctoral project, the size of the study group recruited by the Otago Vascular Research Group was expanded, and additional analysis for other active MMP isoforms was conducted.

Commercial ELISA-based activity assays are available for a number of MMPs. Prior to this, MMP activity has been assessed by zymography. However, during the preparation of samples for MMP zymography, complexed TIMPs are removed. Hence, this technique may result in imprecise measurements of the active endogenous fraction. The aim of this study was to determine (1) whether the endogenous active forms of MMP-1, -2, -3 and -9, the pro-form of MMP-9, and TIMP-1 are associated with a history of ISR, (2) whether there is a biological gradient, with higher levels of MMPs in those with multiple ISR, (3) exploratory analyses associating the levels of MMPs with other cardiovascular variables.
3.2. Results

3.2.1. Study population characteristics

In all, 303 patients were included in this study. There were 152 who had a history of ISR (cases), and 151 with a history of PCI without further symptoms at one year (controls). Those with a history of ISR had significantly increased waist circumference and body mass index, high-sensitivity C-reactive protein, decreased HDL-cholesterol and were more likely to be receiving long acting nitrates and calcium channel antagonists (Table 3.1).

Between the ISR and control group there were no significant differences in age, sex, hypertension, smoking history, history of dyslipidaemia, triglycerides, total cholesterol, LDL-cholesterol, diabetes mellitus or renal function.

Patients with ISR had longer total stent length, smaller average stent diameter, and more sites stented. They were more likely to have triple vessel disease and a greater proportion of single-worst lesions classified as type C by the American College of Cardiology/American Heart Association (ACC/AHA) (Table 3.2). There was no significant difference between rates of ISR between those with double vessel and single vessel disease, and subsequently the groups with single and double vessel disease were combined. Similarly, there was no difference between ACC/AHA scores A, B1 or B2, but all were significantly different to ACC/AHA C. Subsequently, A, B1 and B2 were combined. Of the 54 participants with diabetes, 51 had type 2 diabetes.

Among the 152 patients with a history of ISR, there were 28 patients with multiple lesion ISR (124 patients had a history of a single ISR lesion). Similarly to the comparisons of ISR alone with controls, those with multiple ISR had significantly lower HDL-cholesterol and were more likely to be prescribed calcium channel antagonists and long-acting nitrates than those without ISR (Table 3.3).

When comparing patients with multiple ISR to those without ISR, there were no significant differences with respect to sex, age, waist, BMI, hypertension, smoking history, history of dyslipidaemia, total cholesterol, triglycerides, LDL-cholesterol, diabetes, or impaired renal function. Patients with multiple ISR had significantly higher rates of triple vessel disease, longer total stent length, smaller average stent diameter, more sites stented, and more lesions classified ACC/AHA score C compared to those without ISR (Table 3.4).
Table 3.1 Demographic profile of retrospective study participants

<table>
<thead>
<tr>
<th></th>
<th>CAD with stent n = 151</th>
<th>ISR n = 152</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64.0 ± 9.3</td>
<td>62.1 ± 9.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Sex, male</td>
<td>107 (79.9%)</td>
<td>112 (73.7%)</td>
<td>0.67</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>96.9 ± 11.4</td>
<td>100.5 ± 10.9</td>
<td>0.03</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.9 ± 4.3</td>
<td>29.3 ± 4.3</td>
<td>0.008</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.39 ± 0.99</td>
<td>4.43 ± 1.00</td>
<td>0.75</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.96 ± 1.17</td>
<td>2.23 ± 1.27</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.26 ± 0.80</td>
<td>2.34 ± 0.85</td>
<td>0.37</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.23 ± 0.41</td>
<td>1.07 ± 0.27</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>hs-CRP, mg/L*</td>
<td>1.9 (1.1 – 3.4)</td>
<td>2.3 (1.1 – 4.2)</td>
<td>0.008</td>
</tr>
<tr>
<td>Hypertension</td>
<td>66 (43.7%)</td>
<td>70 (46.1%)</td>
<td>0.73</td>
</tr>
<tr>
<td>Diabetes</td>
<td>22 (14.6%)</td>
<td>32 (21.1%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Pack years</td>
<td>10 (0 – 30.0)</td>
<td>11.3 (0 – 32.0)</td>
<td>0.50</td>
</tr>
<tr>
<td>eCrCl &lt; 60mL/min</td>
<td>39 (25.8%)</td>
<td>41 (27.0%)</td>
<td>0.57</td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td>70 (46.4%)</td>
<td>87 (57.2%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>102 (67.5%)</td>
<td>115 (75.7%)</td>
<td>0.27</td>
</tr>
<tr>
<td>Ca²⁺ antagonist</td>
<td>25 (16.6%)</td>
<td>52 (34.2%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Statin</td>
<td>134 (88.7%)</td>
<td>138 (90.8%)</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td>27 (17.9%)</td>
<td>67 (44.1%)</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Results are expressed as means ± one standard deviation, median (range), or number (percentage). ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; BMI = body mass index; eCrCl = estimated creatinine clearance, Cockraft-Gault model; HDL = High-density lipoprotein; hs-CRP = high-sensitivity C-reactive protein.

*High sensitivity C-reactive protein levels > 10 mg/L excluded (n = 27) due to presumed acute inflammation.
Table 3.2 Association of pre-interventional angiographic variables by outcome

<table>
<thead>
<tr>
<th>Variable</th>
<th>CAD with stent n = 151</th>
<th>ISR n = 152</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD severity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1VD</td>
<td>66 (43.7%)</td>
<td>50 (32.9%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2VD</td>
<td>53 (34.9%)</td>
<td>50 (32.9%)</td>
<td></td>
</tr>
<tr>
<td>3VD</td>
<td>32 (21.2%)</td>
<td>52 (34.2%)*</td>
<td></td>
</tr>
<tr>
<td>Average stent diameter, mm</td>
<td>3.15 ± 0.49</td>
<td>3.00 ± 0.45</td>
<td>0.008</td>
</tr>
<tr>
<td>Number of sites stented</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 0.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total stent length, mm</td>
<td>22.7 ± 12.8</td>
<td>30.6 ± 17.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Average stent length, mm</td>
<td>18.6 ± 9.0</td>
<td>22.0 ± 12.9</td>
<td>0.01</td>
</tr>
<tr>
<td>ACC/AHA score (%)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A</td>
<td>23 (15.2%)</td>
<td>14 (9.2%)</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>56 (37.1%)</td>
<td>50 (32.9%)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>48 (31.8%)</td>
<td>28 (18.4%)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>24 (15.9%)</td>
<td>60 (39.5%)*</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as means ± one standard deviation, or number (percentage).

*p < 0.01 versus 1 and 2VD combined. †p < 0.0001 versus ACC/AHA scores A, B1 and B2 combined.
Table 3.3 Demographic profile of retrospective study participants by number of ISR lesions

<table>
<thead>
<tr>
<th></th>
<th>No ISR lesions n = 151</th>
<th>One ISR lesion n = 124</th>
<th>Multiple ISR lesions n = 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63.6 ± 9.4</td>
<td>62.2 ± 9.4</td>
<td>63.2 ± 9.1</td>
</tr>
<tr>
<td>Sex, male</td>
<td>116 (76.8%)</td>
<td>83 (66.9%)</td>
<td>20 (71.4%)</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>96.8 ± 11.2</td>
<td>101.2 ± 11.2†</td>
<td>98.7 ± 10.4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.9 ± 4.2</td>
<td>29.6 ± 4.6†</td>
<td>28.5 ± 3.4</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.38 ± 0.97</td>
<td>4.43 ± 1.04</td>
<td>4.51 ± 0.95</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.96 ± 1.15</td>
<td>2.26 ± 1.37</td>
<td>2.27 ± 1.03</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.2 ± 0.78</td>
<td>2.32 ± 0.87</td>
<td>2.41 ± 0.88</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.22 ± 0.41</td>
<td>1.08 ± 0.27†</td>
<td>1.07 ± 0.29†</td>
</tr>
<tr>
<td>hs-CRP, mg/L§</td>
<td>1.8 (1.1 – 3.3)</td>
<td>2.3 (1.4 – 4.3)*</td>
<td>2.4 (1.5 – 4.1)*</td>
</tr>
<tr>
<td>Hypertension</td>
<td>72 (47.7%)</td>
<td>55 (44.4%)</td>
<td>9 (32.1%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>23 (15.2%)</td>
<td>23 (18.5%)</td>
<td>8 (28.6%)</td>
</tr>
<tr>
<td>Pack years</td>
<td>10.0 (0 – 30.0)</td>
<td>10.0 (0 – 30.0)</td>
<td>16.5 (0 – 34.0)</td>
</tr>
<tr>
<td>eCrCl &lt; 60mL/min</td>
<td>39 (25.8%)</td>
<td>22 (17.7%)</td>
<td>8 (28.6%)</td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td>75 (49.7%)</td>
<td>66 (53.2%)</td>
<td>16 (57.1%)</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>102 (67.6%)</td>
<td>94 (75.8%)</td>
<td>21 (75.0%)</td>
</tr>
<tr>
<td>Ca²⁺ antagonist</td>
<td>25 (16.6%)</td>
<td>40 (32.3%)†</td>
<td>12 (42.9%)†</td>
</tr>
<tr>
<td>Statin</td>
<td>134 (88.7%)</td>
<td>111 (89.5%)</td>
<td>27 (96.4%)</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td>27 (17.9%)</td>
<td>56 (45.2%)‡</td>
<td>11 (39.3%)‡</td>
</tr>
</tbody>
</table>

Results are expressed as means ± one standard deviation, median (range) or percentages. *p < 0.05 versus no ISR lesions; †p < 0.1 versus no ISR lesions; ‡p < 0.001 versus no ISR lesions. §High sensitivity C-reactive protein values > 10 ng/L excluded (n = 27) due to presumed acute inflammation. ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; BMI = body mass index; eCrCl = estimated creatinine clearance, Cockcroft-Gault model; HDL = High-density lipoprotein; hs-CRP = high-sensitivity C-reactive protein.
Table 3.4 Clinical and angiographic predictors for retrospective study participants by number of ISR lesions

<table>
<thead>
<tr>
<th></th>
<th>No ISR lesions n = 151</th>
<th>One ISR lesion n = 124</th>
<th>Multiple ISR lesions n = 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD severity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1VD</td>
<td>66 (43.7%)</td>
<td>45 (36.3%)</td>
<td>6 (21.4%)</td>
</tr>
<tr>
<td>2VD</td>
<td>53 (35.1%)</td>
<td>38 (30.6%)</td>
<td>11 (39.3%)</td>
</tr>
<tr>
<td>3VD</td>
<td>32 (21.2%)</td>
<td>41 (33.1%)</td>
<td>11 (39.3%)</td>
</tr>
<tr>
<td>Average stent diameter, mm</td>
<td>3.15 ± 0.49</td>
<td>3.03 ± 0.48*</td>
<td>2.90 ± 0.26‡</td>
</tr>
<tr>
<td>Number of sites stented</td>
<td>1.3 ± 0.6</td>
<td>1.4 ± 0.5</td>
<td>2.3 ± 0.5‡</td>
</tr>
<tr>
<td>Total stent length, mm</td>
<td>22.7 ± 12.8</td>
<td>27.5 ± 15.6</td>
<td>41.6 ± 16.8‡</td>
</tr>
<tr>
<td>Average stent length, mm</td>
<td>18.6 ± 9.0</td>
<td>22.8 ± 14.1†</td>
<td>19.0 ± 5.8</td>
</tr>
<tr>
<td>ACC/AHA score (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>23 (15.2%)</td>
<td>10 (8.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>B1</td>
<td>56 (37.1%)</td>
<td>48 (38.7%)</td>
<td>9 (32.1%)</td>
</tr>
<tr>
<td>B2</td>
<td>48 (31.8%)</td>
<td>21 (16.9%)</td>
<td>5 (17.9%)</td>
</tr>
<tr>
<td>C</td>
<td>24 (15.9%)</td>
<td>45 (36.3%)</td>
<td>14 (50.0%)</td>
</tr>
</tbody>
</table>

Results are expressed as means ± one standard deviation, or percentages.
*p < 0.05 vs. no ISR lesions; †p < 0.01 vs. no ISR lesions; ‡p < 0.0001 vs. both no ISR lesions and one ISR lesion. CAD = coronary artery disease; VD = vessels diseased.
Additionally, those with multiple ISR lesions also had more stented lesions, smaller diameter and longer total stent length compared to those with single ISR. However, the association with longer total stent length was probably explained by an increased number of stented lesions, because the average stent length (*i.e.* total stent length divided by the number of stents placed) was not increased relative to the group with a single ISR lesion.

### 3.2.2. Circulating levels of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases-1 in those with a history of in-stent restenosis

The associations between plasma levels of MMP markers and a history of ISR are displayed in Table 3.5. Levels of both active MMPs-3 and -9, and TIMP-1 were significantly higher in patients with a history of ISR compared with the control group. Levels of active MMP-2 were significantly lower in the ISR group compared with controls. There was a trend towards lower levels of pro-MMP-9, and higher levels of active MMP-1 in those who had ISR compared to controls, but this did not reach statistical significance. There was also an increase in the ratio of active MMP-9/pro-MMP-9 in the ISR group.

The ratio between active MMPs and TIMP-1 is not given in this study, because the activity assays used in this study depended on the endogenous activity of the captured MMP to cleave a colour-producing substrate and were not cross-reactive. However, there was no difference to the above results when the ratio was calculated.

For logistic regression analysis, the natural logarithm of MMP variables was taken. Active MMP-3 and TIMP-1 were associated with an increase in odds of ~50% for each standard deviation increase in the MMP marker. Active MMP-9 was associated with a greater than two-fold increase in odds for each standard deviation increase in circulating level. Clinical and demographic variables that were significant on univariate analysis and in the literature were included in a logistic regression model predicting a history of ISR. When active MMPs-3 and -9 and TIMP-1 were each introduced into the model, they remained significantly associated with ISR (Table 3.6). The variables included in the model were age, sex, diabetes, waist circumference, high sensitivity C-reactive protein, coronary artery disease severity, ACC/AHA score, stent characteristics, number of sites stented, and impaired renal function. There was no evidence for poor model fit. The adjusted odds ratios suggest that active MMPs-3 and -9, and TIMP-1 have even more powerful relationships with ISR than on univariate analysis. The multivariate odds ratios for standard deviation increases of TIMP-1
Table 3.5 Circulating levels of matrix metalloproteinases with a history of ISR

<table>
<thead>
<tr>
<th></th>
<th>CAD with stent n = 151</th>
<th>ISR n = 152</th>
<th>p value</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1, ng/mL</td>
<td>2.2 (1.0 – 4.4)</td>
<td>2.9 (1.4 – 4.8)</td>
<td>0.07</td>
<td>0.56 (0.50 – 0.63)</td>
</tr>
<tr>
<td>Active MMP-2, ng/mL</td>
<td>16.2 (11.0 – 19.8)</td>
<td>13.5 (9.2 – 18.7)</td>
<td>0.03</td>
<td>0.57 (0.51 – 0.64)</td>
</tr>
<tr>
<td>Active MMP-3, ng/mL</td>
<td>2.4 (1.4 – 4.9)</td>
<td>3.9 (1.8 – 7.7)</td>
<td>&lt;0.0001</td>
<td>0.62 (0.56 – 0.69)</td>
</tr>
<tr>
<td>Active MMP-9, ng/mL</td>
<td>1.4 (0.9 – 1.9)</td>
<td>2.2 (1.5 – 2.8)</td>
<td>&lt;0.0001</td>
<td>0.71 (0.65 – 0.76)</td>
</tr>
<tr>
<td>Pro-MMP-9, ng/mL</td>
<td>24.2 (17.1 – 37.7)</td>
<td>21.3 (14.7 – 37.1)</td>
<td>0.06</td>
<td>0.56 (0.50 – 0.62)</td>
</tr>
<tr>
<td>TIMP-1, ng/mL</td>
<td>225.6 ± 52.9</td>
<td>248.5 ± 62.2</td>
<td>0.0007</td>
<td>0.61 (0.55 – 0.67)</td>
</tr>
<tr>
<td>Ratio of pro-MMP-9/TIMP-1</td>
<td>0.11 (0.07 – 0.18)</td>
<td>0.09 (0.06 – 0.16)</td>
<td>0.03</td>
<td>0.58 (0.52 – 0.65)</td>
</tr>
<tr>
<td>Ratio of active MMP-9/pro-MMP-9</td>
<td>0.05 (0.02 – 0.09)</td>
<td>0.09 (0.04 – 0.15)</td>
<td>&lt;0.0001</td>
<td>0.66 (0.60 – 0.72)</td>
</tr>
</tbody>
</table>

Results are expressed as median (interquartile range) and area under the curve (95% CI). Associations with TIMP-1 are mean ± standard deviation. The area under the curve for markers with negative relationships with ISR (i.e. active MMP-2, pro-MMP-9 and the pro-MMP-9/TIMP-1 ratio) were reversed, giving an area under the curve > 0.50, to facilitate direct comparison with other the other markers.
## Table 3.6 Univariate and multivariate association of MMP variables with ISR

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1, ng/mL</td>
<td>1.32 (1.03 – 1.68)</td>
<td>1.32 (0.96 – 1.80)</td>
</tr>
<tr>
<td>Active MMP-2, ng/mL</td>
<td>0.80 (0.64 – 1.02)</td>
<td>0.81 (0.60 – 1.09)</td>
</tr>
<tr>
<td>Active MMP-3, ng/mL</td>
<td>1.56 (1.22 – 2.00)†</td>
<td>1.71 (1.23 – 2.37)†</td>
</tr>
<tr>
<td>Active MMP-9, ng/mL</td>
<td>2.29 (1.71 – 3.08)‡</td>
<td>2.38 (1.65 – 3.45)‡</td>
</tr>
<tr>
<td>Pro-MMP-9, ng/mL</td>
<td>0.87 (0.69 – 1.10)</td>
<td>0.78 (0.58 – 1.06)</td>
</tr>
<tr>
<td>TIMP-1, ng/mL</td>
<td>1.50 (1.18 – 1.92)†</td>
<td>1.69 (1.19 – 2.42)†</td>
</tr>
<tr>
<td>Active MMP-1, ≥ 2 ng/mL</td>
<td>1.41 (0.89 – 2.28)</td>
<td>1.45 (0.79 – 2.64)</td>
</tr>
<tr>
<td>Active MMP-2, ≥ 15 ng/mL</td>
<td>0.55 (0.35 – 0.89)*</td>
<td>0.62 (0.34 – 1.14)</td>
</tr>
<tr>
<td>Active MMP-3, ≥ 3 ng/mL</td>
<td>2.57 (1.57 – 4.14)‡</td>
<td>3.09 (1.65 – 5.77)†</td>
</tr>
<tr>
<td>Active MMP-9, ≥ 2 ng/mL</td>
<td>4.63 (2.83 – 7.61)‡</td>
<td>4.17 (2.21 – 7.88)‡</td>
</tr>
<tr>
<td>Pro-MMP-9, ≥ 35 ng/mL</td>
<td>0.91 (0.55 – 1.48)</td>
<td>0.88 (0.42 – 1.65)</td>
</tr>
<tr>
<td>TIMP-1, ≥ 220 ng/mL</td>
<td>2.16 (1.35 – 3.46)†</td>
<td>2.82 (1.44 – 5.55)†</td>
</tr>
</tbody>
</table>

Adjusted model includes age, sex, diabetes, waist circumference, high sensitivity C-reactive protein, coronary artery disease severity, ACC/AHA score, stent characteristics, number of sites stented, and impaired renal function. All associations with continuous MMP variables are first transformed by logarithm, and each association is for a one standard deviation increase.

*p < 0.05; †p < 0.01; ‡p < 0.0001, all for ISR vs. stent.

There was no evidence of poor model fit.
and active MMP-3 were ~1.7 and nearly 2.4 for active MMP-9. While active MMP-2 was significantly lower in those with ISR on univariate analysis, this observation did not remain statistically significant when adjusting for clinical and demographic variables. Although active MMP-1 and pro-MMP-9 had suggestive associations with ISR on univariate analysis, and this was not the case when adjusted for clinical and demographic variables.

The optimal cut offs for the MMP markers were selected by identifying the point of maximum sensitivity and specificity using ROC curves. The sensitivity and specificity for ISR and the number of cases and controls identified by each cut off point are shown in Table 3.7. The cut off point for active MMP-9 gave the best specificity, and was elevated in only ~25% of controls, whereas the TIMP-1 and active MMP-3 cut off points gave better sensitivity. The different properties of these markers may allow additive benefit in the prediction of ISR by adding combinations of these markers. This is tested in Chapter 4.

Other markers gave more modest sensitivity and specificity. While the pro-MMP-9 cut off point appears to be associated with a relatively high level of specificity, pro-MMP-9 does not give any discrimination (i.e. elevated levels are present in ~30% of both cases and controls).

The logistic regression associations of optimum MMP cut offs are displayed in Table 3.6. Elevated levels of active MMPs-3 and -9, and TIMP-1 were associated with strong odds ratios, and these remained strong and significant on multivariate analysis. An elevated level of active MMP-2 appeared to be protective on univariate analysis, with approximately a halved risk, but this was not sustained on multivariate adjustment. When considered as cut offs, neither active MMP-1 nor pro-MMP-9 were associated with a history of ISR. The relationships of MMP markers with a history of multiple ISR are displayed in Table 3.8. Both active MMP-3 and -9 had highly significant dose-responses with an increasing number of ISR lesions. Levels of both active MMP-3 and -9 were significantly higher in those who had a history of a single ISR lesion compared to those with no ISR, and furthermore, there were significantly higher levels in the multiple ISR group compared with the single ISR and the control group. TIMP-1 levels trended towards being higher with an increasing number of lesions, but there was no significant difference between the multiple ISR group and the single ISR group. There was no difference in the level of active MMP-1 with a single ISR lesion, but the multiple ISR group had a higher level than those with no ISR. Active MMP-2 appeared to have a decreasing trend with more ISR lesions, but this only reached statistical significance between the single ISR and non-ISR groups. There was no difference between pro-MMP-9 levels in those who had a history of a multiple or single ISR when compared to
Table 3.7 Sensitivity, specificity and area under the curve for MMP cut off points

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>CAD with stent n = 151</th>
<th>ISR n = 152</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1, ≥ 2 ng/mL</td>
<td>0.55</td>
<td>0.59</td>
<td>77 (51.0%)</td>
<td>87 (57.2%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Active MMP-2, ≥ 15 ng/mL</td>
<td>0.59</td>
<td>0.56</td>
<td>82 (54.3%)</td>
<td>59 (38.8%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Active MMP-3, ≥ 3 ng/mL</td>
<td>0.63</td>
<td>0.61</td>
<td>58 (38.4%)</td>
<td>89 (58.6%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Active MMP-9, ≥ 2 ng/mL</td>
<td>0.60</td>
<td>0.78</td>
<td>36 (23.8%)</td>
<td>90 (59.2%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pro-MMP-9, ≥ 35 ng/mL</td>
<td>0.31</td>
<td>0.70</td>
<td>48 (31.8%)</td>
<td>43 (28.3%)</td>
<td>0.69</td>
</tr>
<tr>
<td>TIMP-1, ≥ 220 ng/mL</td>
<td>0.66</td>
<td>0.53</td>
<td>73 (48.3%)</td>
<td>97 (63.8%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Results are sensitivity, specificity and number (percentage) of each group with levels above the specified cut off.
Results by Chi-square test.
### Table 3.8 Association of MMPs with multiple ISR

<table>
<thead>
<tr>
<th></th>
<th>No ISR lesions (n = 151)</th>
<th>One ISR lesion (n = 124)</th>
<th>Multiple ISR lesions (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active MMP-1, ng/mL</strong></td>
<td>2.2 (1.0 – 4.4)</td>
<td>2.5 (0.9 – 4.1)</td>
<td>3.6 (1.8 – 5.3)*</td>
</tr>
<tr>
<td><strong>Active MMP-2, ng/mL</strong></td>
<td>16.2 (11.0 – 19.8)</td>
<td>13.9 (9.2 – 18.6)*</td>
<td>12.6 (8.0 – 17.3)</td>
</tr>
<tr>
<td><strong>Active MMP-3, ng/mL</strong></td>
<td>2.4 (1.4 – 4.9)</td>
<td>3.8 (1.2 – 6.4)†</td>
<td>6.1 (3.2 – 9.1)†‡</td>
</tr>
<tr>
<td><strong>Active MMP-9, ng/mL</strong></td>
<td>1.4 (0.9 – 1.9)</td>
<td>2.1 (1.4 – 2.8)†</td>
<td>2.5 (1.8 – 3.2)†‡</td>
</tr>
<tr>
<td><strong>Pro-MMP-9, ng/mL</strong></td>
<td>24.2 (17.1 – 37.7)</td>
<td>21.1 (8.9 – 33.3)</td>
<td>22.7 (12.3 – 33.4)</td>
</tr>
<tr>
<td><strong>TIMP-1, ng/mL</strong></td>
<td>225.6 ± 52.9</td>
<td>245.8 ± 55.8*</td>
<td>261.1 ± 87.0*</td>
</tr>
</tbody>
</table>

Results presented as median (interquartile range) and mean ± standard deviation.

* *p < 0.05 vs. no ISR lesions, †p < 0.001 vs. no ISR lesion, ‡p < 0.05 vs. one ISR lesion. Continuous variables compared by Mann Whitney-U.
controls. There was no association between Mehran score and MMP markers, regardless of whether MMP markers were considered as continuous variables or as the specific cut-offs.

3.2.3. Association of matrix metalloproteinases and tissue inhibitor of metalloproteinases-1 with cardiovascular disease variables

The relationships between MMP levels and clinical and inflammatory markers are displayed in Table 3.9 and Table 3.10. The difference between categorical variables are displayed in Table 3.9 and associations with continuous variables are displayed in Table 3.10. While multiple comparisons were made, no statistical correction was performed. Instead, these results are considered as hypothesis generating.

TIMP-1 levels were significantly associated with coronary artery disease severity, with a dose-response of increasing levels with double- and triple-vessel disease. TIMP-1 was also elevated in patients with a history of hypertension, renal impairment or diabetes mellitus. The associations between TIMP-1 and coronary artery disease severity, hypertension, diabetes mellitus and renal impairment all remained significant on adjustment for age, sex and history of ISR, indicating that TIMP-1 may be independently associated with these conditions. The association of TIMP-1 with hypertension was independent of anti-hypertensive medication, and the association with diabetes was independent of renin/angiotensin system antagonists. There was no association between any MMP variable and the number of antihypertensive medications.

Active MMP-2 was correlated with increasing HDL-cholesterol concentrations but was lower in those with more extensive smoking histories. The association with HDL-cholesterol remained significant after adjustment for age, sex and history of ISR, but the association with smoking history did not. Active MMP-3 was associated with decreasing HDL-cholesterol and an increased number of stents. The association of active MMP-3 with HDL-cholesterol remained significant after adjustment for age, sex and history of ISR, but the association with number of stented sites appeared to be a surrogate measure for ISR. Active MMP-9 was higher in those with an increased number of stents, and smaller average diameter of stents, but these correlations appeared to be surrogates for the presence of ISR, and were no longer significant after adjustment. Pro-MMP-9 was higher in those with a more extensive smoking history, but this did not remain significant when adjusted for age, sex and history of ISR. TIMP-1 levels were higher in those who had more stented sites, but this was not significant...
### Table 3.9 Association of MMPs with nominal clinical and angiographic variables

<table>
<thead>
<tr>
<th></th>
<th>Active MMP-1</th>
<th>Active MMP-2</th>
<th>Active MMP-3</th>
<th>Active MMP-9</th>
<th>Pro-MMP-9</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAD severity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>†</td>
</tr>
<tr>
<td>1VD, n = 115</td>
<td>2.3 (1.0 – 4.1)</td>
<td>14.7 (10.1 – 18.2)</td>
<td>3.2 (1.5 – 6.0)</td>
<td>1.7 (1.1 – 2.4)</td>
<td>26.0 (17.6 – 41.4)</td>
<td>226.8 ± 52.0</td>
</tr>
<tr>
<td>2VD, n = 103</td>
<td>3.1 (1.2 – 5.3)</td>
<td>16.4 (10.7 – 20.7)</td>
<td>2.8 (1.7 – 5.2)</td>
<td>1.8 (1.0 – 2.7)</td>
<td>20.1 (15.4 – 34.1)</td>
<td>235.1 ± 57.8</td>
</tr>
<tr>
<td>3VD, n = 84</td>
<td>2.1 (1.1 – 4.0)</td>
<td>14.2 (8.9 – 18.7)</td>
<td>3.2 (1.7 – 6.8)</td>
<td>1.9 (1.2 – 2.6)</td>
<td>24.2 (16.3 – 52.0)</td>
<td>255.8 ± 66.4</td>
</tr>
<tr>
<td><strong>ACC/AHA score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>†</td>
</tr>
<tr>
<td>A, n = 29</td>
<td>2.3 (0.9 – 3.2)</td>
<td>14.0 (10.6 – 20.0)</td>
<td>2.6 (1.4 – 4.7)</td>
<td>1.3 (0.6 – 2.7)</td>
<td>21.3 (13.9 – 55.0)</td>
<td>225.3 ± 57.4</td>
</tr>
<tr>
<td>B1, n = 108</td>
<td>2.3 (1.2 – 4.6)</td>
<td>13.8 (8.8 – 18.4)</td>
<td>3.0 (1.7 – 6.4)</td>
<td>1.7 (1.2 – 2.6)</td>
<td>26.0 (17.0 – 48.7)</td>
<td>240.7 ± 57.5</td>
</tr>
<tr>
<td>B2, n = 77</td>
<td>2.2 (0.6 – 4.3)</td>
<td>15.1 (9.5 – 18.1)</td>
<td>2.8 (1.3 – 5.9)</td>
<td>1.6 (1.0 – 2.3)</td>
<td>20.6 (15.9 – 36.4)</td>
<td>241.1 ± 57.5</td>
</tr>
<tr>
<td>C, n = 82</td>
<td>3.2 (1.3 – 5.0)</td>
<td>16.0 (11.3 – 20.1)</td>
<td>3.5 (1.7 – 7.3)</td>
<td>1.9 (1.2 – 2.5)</td>
<td>22.4 (15.8 – 36.0)</td>
<td>235.2 ± 62.1</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>†</td>
</tr>
<tr>
<td>Yes, n = 166</td>
<td>2.7 (1.4 – 4.5)</td>
<td>15.1 (10.0 – 19.4)</td>
<td>3.0 (1.6 – 6.1)</td>
<td>1.7 (1.1 – 2.5)</td>
<td>26.0 (16.1 – 40.1)</td>
<td>247.5 ± 63.7</td>
</tr>
<tr>
<td>No, n = 136</td>
<td>2.2 (1.0 – 4.5)</td>
<td>14.6 (10.0 – 18.7)</td>
<td>3.2 (1.6 – 6.2)</td>
<td>1.7 (1.2 – 2.6)</td>
<td>22.3 (16.6 – 38.0)</td>
<td>228.8 ± 52.9</td>
</tr>
<tr>
<td><strong>eCrCl &lt; 60mL/min</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>†</td>
</tr>
<tr>
<td>Yes, n = 69</td>
<td>2.3 (1.1 – 4.1)</td>
<td>14.9 (10.7 – 19.6)</td>
<td>3.0 (1.4 – 6.0)</td>
<td>1.7 (1.0 – 2.5)</td>
<td>22.3 (16.5 – 39.0)</td>
<td>264.4 ± 70.6</td>
</tr>
<tr>
<td>No, n = 223</td>
<td>2.9 (1.2 – 5.4)</td>
<td>14.8 (9.1 – 20.3)</td>
<td>3.3 (1.8 – 7.8)</td>
<td>1.7 (1.3 – 2.7)</td>
<td>28.4 (16.6 – 43.2)</td>
<td>227.9 ± 52.2</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>†</td>
</tr>
<tr>
<td>Yes, n = 249</td>
<td>2.5 (1.5 – 4.0)</td>
<td>12.8 (9.2 – 18.6)</td>
<td>3.1 (1.9 – 6.1)</td>
<td>1.7 (1.2 – 2.6)</td>
<td>20.4 (15.4 – 34.4)</td>
<td>268.3 ± 73.3</td>
</tr>
<tr>
<td>No, n = 54</td>
<td>2.3 (1.1 – 4.5)</td>
<td>15.3 (10.6 – 19.2)</td>
<td>3.1 (1.5 – 6.2)</td>
<td>1.9 (1.2 – 2.5)</td>
<td>23.3 (16.5 – 39.0)</td>
<td>230.2 ± 53.0</td>
</tr>
</tbody>
</table>

Results are median (interquartile range) and mean (standard deviation).
*Pink indicates a trend (p <0.1); †blue indicates statistically significant (p < 0.05); and ‡red indicates highly statistically significant (p < 0.0001).
ACC/AHA = modified American College of Cardiology/American Heart Association; CAD = coronary artery disease; eCrCl = estimated creatinine clearance, Cockroft-Gault method.
Association of MMP markers with a history of ISR

Table 3.10 Association of MMP variables with continuous clinical and angiographic variables

<table>
<thead>
<tr>
<th></th>
<th>Active MMP-1</th>
<th>Active MMP-2</th>
<th>Active MMP-3</th>
<th>Active MMP-9</th>
<th>Pro-MMP-9</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average stent diameter, mm</td>
<td>-0.026</td>
<td>-0.045</td>
<td>-0.13*</td>
<td>-0.17†</td>
<td>0.014</td>
<td>-0.013</td>
</tr>
<tr>
<td>Total stent length, mm</td>
<td>0.0035</td>
<td>0.036</td>
<td>0.072</td>
<td>0.053</td>
<td>-0.0031</td>
<td>0.054</td>
</tr>
<tr>
<td>Number of sites stented</td>
<td>0.10</td>
<td>0.03</td>
<td>0.16†</td>
<td>0.14†</td>
<td>0.065</td>
<td>0.19‡</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>-0.14*</td>
<td>0.069</td>
<td>-0.12*</td>
<td>-0.0057</td>
<td>0.028</td>
<td>-0.062</td>
</tr>
<tr>
<td>Triglycerides, mmol/</td>
<td>0.0034</td>
<td>-0.054</td>
<td>0.029</td>
<td>0.12*</td>
<td>0.053</td>
<td>0.16†</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>-0.097</td>
<td>-0.0013</td>
<td>-0.064</td>
<td>-0.028</td>
<td>-0.033</td>
<td>-0.070</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>-0.12*</td>
<td>0.25‡</td>
<td>-0.24‡</td>
<td>-0.016</td>
<td>-0.045</td>
<td>-0.26‡</td>
</tr>
<tr>
<td>Pack years</td>
<td>0.026</td>
<td>-0.20‡</td>
<td>0.045</td>
<td>0.057</td>
<td>0.15†</td>
<td>0.04</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>-0.056</td>
<td>-0.13*</td>
<td>0.049</td>
<td>-0.025</td>
<td>0.076</td>
<td>0.15†</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>0.11*</td>
<td>-0.015</td>
<td>0.018</td>
<td>0.075</td>
<td>0.045</td>
<td>0.24‡</td>
</tr>
</tbody>
</table>

Results are presented as Spearman’s rho.

Pink colouring indicates a correlation coefficient with an absolute value of < 0.15; blue indicates a correlation with an absolute value between >0.15 and 0.3. Only variables with a minimum p value of 0.1 are coloured. HDL = high density lipoprotein; hs-CRP = high sensitivity C-reactive protein; LDL = low density lipoprotein.

*p value < 0.1; † p value < 0.05; ‡ p value < 0.001.
after adjusting for ISR. However, TIMP-1 was elevated with increasing total cholesterol, waist circumference and high sensitivity C-reactive protein, and lower with increasing HDL-cholesterol. These associations of TIMP-1 with lipid and inflammatory measures were independent of age, sex and history of ISR, indicating that these may be real relationships.

The associations between MMP markers and prescribed medication at time of recruitment are displayed in Table 3.11. Participants who were taking angiotensin converting enzyme inhibitors or angiotensin II receptor blockers had higher levels of TIMP-1, and lower levels of pro-MMP-9. The association with TIMP-1 was not significant when adjusted for age, sex and history of ISR. However, pro-MMP-9 levels appeared to be lower in participants with diabetes mellitus (although this association did not achieve statistical significance). After diabetes was included in the model there was no association between pro-MMP-9 and ACE-I/ARB. Prescription of calcium channel antagonists was associated with higher levels of active MMPs-1 and -9, and lower levels of active MMP-2. When adjusted for age, sex and history of ISR, only the association between Ca$^{2+}$ channel blockers and higher active MMP-9 remained significant. There was no interaction between prescription of calcium channel antagonists and history of patient-reported hypertension with active MMP-9. Furthermore, the association between active MMP-9 and Ca$^{2+}$ channel blockers remained significant after adjusting for the number of antihypertensive medications.

Both active MMPs-1 and -3 were lower in people not taking statins, and this remained significant after adjusting for age, sex and history of ISR. Only a minority of participants were not on statins (n = 22, 7.3%). They were not different in terms of age or sex and there was no association with ISR. Those on statins had lower total cholesterol (4.33 ± 0.83 vs. 5.52 ± 1.40 mmol/L, $p < 0.0001$) and lower LDL-cholesterol (2.2 ± 0.73 vs. 3.51 ± 1.06 mmol/L, $p < 0.0001$), but there was no difference in triglycerides, HDL-cholesterol or high sensitivity C-reactive protein. There was no interaction between statin therapy patient-reported history of dyslipidaemia and concentrations of active MMPs-3 and -9. Those who were taking long-acting nitrates had higher levels of TIMP-1 and a trend towards higher active MMP-3, but these comparisons were not significant after adjustment for age, sex and history of ISR.
### Table 3.11 Association of MMP variables with prescribed medications

<table>
<thead>
<tr>
<th>Medication</th>
<th>Active MMP-1</th>
<th>Active MMP-2</th>
<th>Active MMP-3</th>
<th>Active MMP-9</th>
<th>Pro-MMP-9</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACE-I or ARB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n = 157</td>
<td>2.4 (1.4 – 4.4)</td>
<td>15.7 (9.7 – 19.2)</td>
<td>3.4 (1.7 – 6.4)</td>
<td>1.7 (1.2 – 2.6)</td>
<td>20.4 (15.4 – 34.4)</td>
<td>246.0 ± 68.0</td>
</tr>
<tr>
<td>No, n = 128</td>
<td>2.6 (1.0 – 4.5)</td>
<td>14.2 (10.1 – 18.5)</td>
<td>2.8 (1.4 – 5.7)</td>
<td>1.9 (1.2 – 2.5)</td>
<td>26.3 (16.9 – 49.3)</td>
<td>230.5 ± 45.4</td>
</tr>
<tr>
<td><strong>Beta blocker</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n = 217</td>
<td>2.4 (1.2 – 4.5)</td>
<td>14.6 (9.9 – 19.2)</td>
<td>3.2 (1.7 – 6.5)</td>
<td>1.7 (1.1 – 2.5)</td>
<td>24.2 (15.8 – 39.3)</td>
<td>239.6 ± 59.7</td>
</tr>
<tr>
<td>No, n = 78</td>
<td>2.2 (1.1 – 4.2)</td>
<td>14.8 (10.4 – 18.0)</td>
<td>2.8 (1.3 – 5.2)</td>
<td>1.7 (1.2 – 2.5)</td>
<td>22.4 (17.1 – 38.6)</td>
<td>232.8 ± 57.1</td>
</tr>
<tr>
<td><strong>Ca(^{2+}) antagonist</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n = 183</td>
<td>3.4 (1.5 – 5.4)</td>
<td>12.2 (8.8 – 17.6)</td>
<td>3.4 (1.7 – 6.1)</td>
<td>2.4 (1.5 – 3.0)</td>
<td>24.3 (15.6 – 52.7)</td>
<td>243.7 ± 59.5</td>
</tr>
<tr>
<td>No, n = 77</td>
<td>2.3 (1.2 – 4.4)</td>
<td>14.9 (10.4 – 19.2)</td>
<td>3.0 (1.5 – 6.5)</td>
<td>1.7 (1.2 – 2.5)</td>
<td>21.6 (15.8 – 37.1)</td>
<td>242.7 ± 57.6</td>
</tr>
<tr>
<td><strong>Statin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n = 272</td>
<td>2.6 (1.2 – 4.7)</td>
<td>14.8 (10.1 – 19.2)</td>
<td>3.2 (1.7 – 6.2)</td>
<td>1.8 (1.2 – 2.6)</td>
<td>23.6 (16.3 – 38.6)</td>
<td>237.5 ± 58.1</td>
</tr>
<tr>
<td>No, n = 22</td>
<td>1.3 (0.6 – 2.0)</td>
<td>14.1 (9.5 – 16.6)</td>
<td>1.8 (1.1 – 3.4)</td>
<td>1.4 (1.2 – 2.1)</td>
<td>24.0 (16.1 – 54.0)</td>
<td>247.6 ± 67.3</td>
</tr>
<tr>
<td><strong>Long-acting nitrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes, n = 94</td>
<td>2.6 (1.4 – 4.4)</td>
<td>14.3 (8.8 – 18.1)</td>
<td>3.7 (1.8 – 6.8)</td>
<td>1.9 (1.3 – 2.7)</td>
<td>20.8 (14.7 – 39.1)</td>
<td>255.2 ± 69.3</td>
</tr>
<tr>
<td>No, n = 171</td>
<td>2.6 (1.2 – 4.8)</td>
<td>15.0 (10.1 – 19.2)</td>
<td>2.9 (1.5 – 5.8)</td>
<td>1.9 (1.2 – 2.6)</td>
<td>23.3 (16.6 – 38.1)</td>
<td>233.0 ± 50.7</td>
</tr>
</tbody>
</table>

Results are median (interquartile range) and mean (standard deviation).

*Pink indicates a trend \( p < 0.1\); †blue indicates statistically significant \( p < 0.05\); and ‡red indicates highly statistically significant \( p < 0.01\).
3.3. Discussion

This study evaluated the circulating levels of a panel of active MMPs and TIMP-1 in asymptomatic patients who had been treated for in-stent restenosis compared to patients who developed no further symptoms after initial bare-metal coronary stent placement, in an attempt to link alterations of these proteins to a history of ISR. The main findings were that the circulating concentrations of active MMPs-3, and -9 and TIMP-1 were strongly elevated in asymptomatic patients who had a history of treatment for bare-metal coronary stent restenosis.

The association between active MMPs-3 and -9, and TIMP-1 with a history of ISR was independent of clinical and demographic variables, and optimal cut off points were independently associated with large effect sizes for ISR (odds ratios ranging from 2.8 – 4.2).

Active MMPs -3 and -9 showed a dose response relationship with the number of ISR lesions. Levels of both active MMP-3 and -9 were elevated in those with multiple ISR lesions compared to those with a single ISR lesion, as well as higher in those with a single ISR lesion compared to those with no ISR lesions.

This study investigated a number of candidate active MMPs as well as TIMP-1 in patients with a history of ISR, in anticipation of validation in a prospective study being recruited during this thesis. The present discussion will focus on the biology of MMPs and the potential roles they have in vascular disease and ISR, and how these may explain our observed results.

The association of demographic and clinical factors with ISR in the retrospective study is discussed in Appendix 11.5.

3.3.1. Association of active matrix metalloproteinases with a history of in-stent restenosis

While MMPs have been extensively investigated in vascular physiology and pathology, the majority of reports have examined the pro-forms, and the active forms by zymography. As sample preparation for zymographic analysis removes bound TIMPs, the result may not directly reflect that of the endogenous activity (see 1.6.4; 2.8.3). A complex regulatory network controls the action of MMPs through three main steps: transcription, activation and inhibition. Thus measuring only the pro-form, or negating the influence of the TIMPs, could limit understanding of the biological involvement of MMPs in vascular disease. Interestingly, while active MMP-9 was elevated in patients with a history of ISR, pro-MMP-9
was decreased. This highlights that direct measurement of MMP activity may be important for understanding disease processes. Our findings indicate that patients with a history of ISR have elevations specifically of the active form of MMPs in the systemic circulation long after presentation with ISR.

The Vascular Research Group previously demonstrated a strong, independent association between active MMP-9 and a history of ISR. The current analysis upheld this finding in an expanded dataset. Statistical adjustment for known risk variables suggested that each of active MMPs-3 and -9, and TIMP-1 were independently associated with ISR. The analysis included those variables that were significant on univariate analysis, as well as those previously identified in the literatures, specifically: age, sex, waist circumference, HDL-cholesterol, renal impairment, high sensitivity C-reactive protein, coronary artery disease severity, lesion complexity (as quantified by modified ACC/AHA score), the length, diameter and number of stents placed, as well as a history of diabetes mellitus. Both body mass index and waist circumference were associated with ISR, but as they had a strong correlation, waist circumference was preferentially included because it had the strongest association with ISR. A history of diabetes mellitus was included in the model, despite its lack of statistical significance on univariate analysis, previous studies have shown that diabetes is a strong risk factor for ISR. Finally, while the ISR group were more likely to be treated with long-acting nitrates and Ca$^{2+}$ antagonists, medication information was not included in the multivariate model. It is possible that both medications were prescribed to provide better control coronary risk factors and incipient symptoms in the ISR group, hence making medication a surrogate for ISR symptoms rather than a predictor or pathogenic factor.

Some medications are known to alter MMP levels, and this is a potential source of bias, but it appears that medications tend to decrease MMP levels. This may led to association of MMPs with the more highly medicated ISR patients being under-estimated. When long-acting nitrates and Ca$^{2+}$ antagonists were included in the multivariate model, associations between active MMPs-3 and -9, and TIMP-1 remained significant.
3.3.2. Potential roles of individual matrix metalloproteinases

3.3.2.1. Matrix metalloproteinase-9 and ISR

Individual MMPs appear to have specific roles in the response to vascular injury. MMP-9 has been extensively linked to vascular injury and intima hyperplasia, including several observational studies on human PCI and the development of ISR. Targeted MMP-9 knock-out leads to a dramatic, albeit incomplete, reduction in both VSMC migration, and development of intimal thickening in murine models. (For review of the cardiovascular effects of MMP knock-out in murine models see Janssens and Lijnen). Furthermore, MMP-9, but not MMP-2, appears to be necessary for compaction of collagen, which could indicate a role for MMP-9 in the late remodelling and regression of ISR. Overexpression of MMP-9 enhanced VSMC migration, but there was no difference in intimal area at 14 days.

With experimental injury, levels of pro-MMP-9 and zymographic activity rise in the human saphenous vein, in a variety of animal models. Elevated pro-MMP-9 has been seen after PCI, in the coronary and peripheral circulation, although one study suggests this response may be absent with the use of drug-eluting stents. Elevations of pro-MMP-9 have also been associated with the development of intimal hyperplasia. Serum post-procedural pro-MMP-9 and -2 were shown to be predictive of angiographic restenosis by Ge et al. Furthermore, the pre-interventional levels of MMP-9 available for activation were elevated in those who would later undergo further revascularizations following initial PCI or CABG. Significantly, while we saw a borderline association of pro-MMP-9 in those with a history of ISR, in contrast to the findings above, we saw a decreased level. This may be explained by the timing difference in relation to ISR. Where Ye et al. investigated pre-procedural levels and Ge et al. post-procedural levels, we analysed levels long after presentation. Nikkari et al. saw decreased levels of MMPs in restenotic tissue after carotid endarterectomy, and this may represent a later phase of ISR biology, with decreased collagen breakdown, and net accumulation.

While it is understood that activation is one of the key regulatory steps in MMP biology, most genetic knock-out studies have focussed on the expression of the non-activate proteins. However, auto-activating MMPs-3 and -9 have been created by site-directed mutagenesis. While the MMP-3 model has not been utilized in the study of vascular disease, auto-activating MMP-9, G100L, has been used to investigate the role of the active
isoform in plaque rupture. Gough et al.\textsuperscript{426} found that, while expression of transfected normal \textit{MMP9} gene linked to the macrophage-LDL receptor CD68 resulted in a ten-fold increase of MMP-9 protein expression, it did not induce the rupture of atherosclerotic lesions. The increase in MMP-9 expression appeared to be in the pro-form when analysed by zymography. When the modified G100L \textit{MMP9} gene was transfected, all mice studied had evidence of plaque rupture, which was associated with greatly increased elastin degradation, but they also had similar total levels of MMP-9 protein compared to the mice transfected with normal MMP-9. Hence, it appears that the active form is crucial for at least some of the biological functions of MMP-9, and this is likely to be true for other MMPs as well.

Hence, MMP-9 has been extensively linked to intimal hyperplasia and ISR. The present study upholds the previous observation by our group,\textsuperscript{521} in an expanded dataset, that the endogenous active form of MMP-9 is strongly elevated in patients with a history of ISR.

### 3.3.2.2. Matrix metalloproteinase-3 and ISR

The endogenous active level of MMP-3 also had a strong, positive association with ISR, which remained significant on multivariate analysis, and levels were progressively higher in patients who had multiple ISR, compared to those with a single restenotic lesion.

Tissue expression of MMP-3 has been reported to gradually increase in the adventitial layers over 7 days after arterial injury in a mouse model,\textsuperscript{131} partly co-localised with VSMC. Furthermore, while Southgate \textit{et al.}\textsuperscript{368} found that MMP-3 was unable to be detected in the culture medium of migrating VSMC, James \textit{et al.}\textsuperscript{386} detected MMP-3 gene transcripts mechanical injury to VSMC.\textsuperscript{386} This suggests that the role of MMP-3 in vascular injury may not be directly related to VSMC migration. MMP-3 has a broad substrate specificity,\textsuperscript{527} and is able to activate a number of other MMP isoforms, including MMPs-1 and -9,\textsuperscript{302} although MMP-2 and -9 activation in experimental vascular injury can occur independently of MMP-3.\textsuperscript{308} Additionally, MMP-3 has been reported to cleave the cell-cell attachment protein epithelial-cadherin.\textsuperscript{349,528} While expression of epithelial-cadherin is limited to epithelial tissues, a number of cadherins are expressed in vascular tissue, including vascular endothelial-cadherin, which is essential for normal vascular development.\textsuperscript{529} Increased expression of T-cadherin has been linked to VSMC proliferation and migration.\textsuperscript{529} Thus, MMP-3 may have a role in the development of intimal hyperplasia by initiating migration through interactions with the cadherin family of proteins, or by activating other MMPs. However, MMP-3 has not
been directly linked to the development of intimal hyperplasia, either in animal models or in clinical studies. One small study found no association between the level of total MMP-3 available for activation before initial revascularization by PCI or CABG, and the development of adverse cardiac events. Interestingly, while mice with targeted knock-out of the MMP-3 gene have not been investigated for the response to arterial injury, MMP-3 knock-out is associated with similar changes in plaque characteristics to the MMP-9 gene, which has been extensively linked to the development of intimal hyperplasia. Specifically, MMP3 -/- mice fed an atherogenic diet, show evidence of an unstable plaque phenotype with increased plaque area, buried fibrous layers and decreased VSMC content compared to wild-type mice for the MMP genotype. In contrast to MMP-9, the lesion macrophage content was unaltered in the MMP-3 knockout. While it is impossible to infer the contribution of MMP-3 to the development of intimal hyperplasia from this observation, it does suggest that there is a similar or complementary role in the overall tissue functioning of MMPs-3 and -9.

Hence, this is a novel result, and while there does not appear to be a precedent in terms of a functional association between MMP-3 and ISR in the basic science literature, it appears that circulating active MMP-3 levels are elevated in individuals who have a history of ISR.

3.3.2.3. Tissue inhibitor of matrix metalloproteinases-1 and ISR

We observed a significant increase in the circulating plasma level of TIMP-1 in patients with a history of ISR, again upholding the previous finding in this larger dataset. TIMP-1 is one of four endogenous inhibitors of the TIMP class. There is evidence that the four TIMPs have subtly distinct biological roles, for example, TIMP-1 appears to bind more loosely to the ECM than TIMP-2, whereas TIMP-3 strongly binds the ECM. However, all TIMPs can bind most MMPs. TIMP-1 is the most abundant TIMP, and as well as having an inhibitory role towards MMPs, TIMP-1 also acts as a growth factor. A large number of factors appear to regulate both MMP and TIMP expression. Fibrogenic cytokines, including PDGF and TGF-β, stimulate both MMPs and TIMPs, whereas it has been suggested that mechanical disruption and inflammation tend to stimulate MMPs more than TIMPs. TIMP-1 is constitutively expressed in the vascular wall, but is raised with injury, co-localising to intimal VSMC. Likewise, TIMP-2, -3 and -4 are also elevated, but more subtly, and with different topography. TIMP-2 is associated with neointimal and medial cells, whereas TIMP-3 is expressed throughout the arterial wall,
-4 in the adventitia only.\textsuperscript{534} Up-regulation of TIMP inhibition is capable of reducing cell migration \textit{in vitro}, including TIMP-1,\textsuperscript{366,367,535 -2,365,535 and -4.\textsuperscript{534}} Additionally, the overexpression of TIMPs, including TIMP-1,\textsuperscript{366,367 -2,365 and -3,373} attenuate the development of intimal hyperplasia after experimental injury.

Hence, the expression of TIMPs appears to be responsive to vascular injury. However, as the function appears to be one of attenuating migration and the development of intimal hyperplasia, it appears that the elevation of TIMPs is a natural braking mechanism. Corroborating this, TIMP-1 knock-out leads to accelerated VSMC migration and neointimal accumulation.\textsuperscript{364} Rising levels of TIMP-1 have been observed in the coronary circulation after PCI.\textsuperscript{344} However, this elevation was not different between ISR cases and controls in a small study.\textsuperscript{345} Like MMP-1, TIMP-1 is lower in restenotic carotid endarterectomy specimens compared to normal artery walls.\textsuperscript{394} This may reflect reduced remodelling in the neointima of restenotic lesions. All four members of the TIMP family are expressed in the vascular wall during arterial injury.\textsuperscript{387,534} However, TIMP-1 is the most strongly elevated during neointimal formation,\textsuperscript{387} therefore it may be the most likely to be detected in the circulation with ISR, and we only measured TIMP-1 in this study.

\textbf{3.3.2.4. Matrix metalloproteinase-2 and ISR}

Levels of active MMP-2 had an inverse association with ISR, which was statistically significant on univariate association but was not independent of clinical and demographic variables. Both the \textit{MMP2} gene and expressed protein have been extensively linked to ISR. Like MMP-9, mice with the \textit{MMP2} gene knocked out have been shown to have reduced VSMC invasion and attenuation of intimal hyperplasia after experimental injury.\textsuperscript{377,536} Unlike most other MMPs, MMP-2 is constitutively expressed at a low level by a number of cell types,\textsuperscript{276,368} although it is still responsive to various stimuli. MMP-2, including both pro-level and activity, as measured by zymography, rises concurrently with MMP-9 in experimental human vein injury,\textsuperscript{384} and a variety of animal models of arterial injury.\textsuperscript{347,389} MMP-2 expression has also been found to be altered in clinical studies, and has been associated with ISR. A study by Hojo \textit{et al.}\textsuperscript{344} showed that both pro-MMP-2 and the endogenous active form of MMP-2 (by activity ELISA) were elevated in the coronary sinus after PTCA and PCI, compared to immediately before. Furthermore, while pre-interventional levels of pro-MMP-2 in the coronary circulation were not different between patients who
would subsequently develop ISR and those who would not, samples drawn from the coronary sinus were predictive of the percentage angiographic restenosis at six months. The relationship between the active endogenous form of MMP-2, as quantified by activity ELISA, and late loss was not reported. These results were corroborated by Ge et al.,\textsuperscript{345} who showed post-procedural elevations of pro-MMP-2 amongst those who went on to develop clinical ISR, and Ye et al.,\textsuperscript{395} who saw no difference in the pre-procedural total level of MMP-2 available for activation.

However, one factor in determining the associations in the literature may be analysis bias. If one type of test is easier to perform, or more readily available, then the results generated by this test may be more prevalent in the literature. MMP-2 and -9 form the gelatinase class of MMPs, as gelatin is a major substrate of these isoforms, and both can be easily assayed in this medium by substrate zymography.\textsuperscript{330} The ease of assaying both MMPs-2 and -9 may contribute to the large number of associations these enzymes have with vascular healing, and the relative paucity of published associations of other MMPs, i.e. MMP-3 (a Google Scholar search yields 114 000 results for “MMP-9”; “MMP-2” yields 91 000 but “MMP-3” gives only 30 000). Thus, the lack of published associations does not necessarily reflect the importance of each MMP isoform in the development of intimal hyperplasia.

### 3.3.2.5. Matrix metalloproteinase-1 and ISR

A trend between the plasma level of active MMP-1 and a history of ISR was found, but did not reach statistical significance. However, in the study in this thesis, the circulating plasma levels of active MMP-1 were significantly higher in the group with multiple ISR when compared with either patients who had developed a single ISR, or those who had no complications after PCI. This apparent elevation of active MMP-1 with coronary restenosis appears to conflict with the observations of Nikkari et al.\textsuperscript{394} who found little MMP-1 expression in histological sections of carotid restenosis after endarterectomy. The chronic systemic elevation we observed in the present study may reflect a different aspect of neointimal biology than the tissue-level observations by Nikkari et al.\textsuperscript{394} Mechanical disruption of VSMC stimulates increased production of MMP-1 mRNA,\textsuperscript{386} and surgical preparation of the veins increased pro-MMP-1 expression by two-fold, and greatly increased the active fraction when tested using zymography.\textsuperscript{385} In contrast, Lijnen et al.\textsuperscript{131} did not
detect MMP-1 mRNA after balloon injury to rat carotid. However, pro-MMP-1 concentration in peripheral blood rose immediately after carotid stenting. Apart from this, MMP-1 has not been linked to restenosis. MMP-1 has been found in the shoulder of unstable plaques, and in the coronary sinus of patients with unstable coronary disease. Mice as a species do not have MMP-1, so murine models are limited in their usefulness with respect to the functional role of MMP-1. Lemaître et al. utilized an atherosclerotic mouse model, transfecting the MMP1 gene under the control of a cell receptor sensitive to oxidised LDL. This led to expression of MMP-1 in macrophages and foam cells. They found that the MMP1 mice had smaller atherosclerotic lesions than wild-type mice under the same conditions, with MMP1 mice having a stable plaque phenotype with less cellular infiltrate. While it might be expected that dysregulated MMP-1 production, stimulated by the presence of oxidised LDL, would lead to destruction of the fibrous cap and precipitate plaque rupture, it has been suggested that this did not occur because lesions produced by the mouse model are immature. The reasons for the attenuated development of atherosclerotic lesions are not clear, however this may be due, at least in part, of the local environment being altered through the release of sequestered messengers, cleavage of receptors or direct action on non-ECM protein. For example, MMP-1 (as well as other MMPs) has a role in cleaving the inflammatory cytokine pro-TNF-α and on the inhibitory growth factor binding protein insulin-like growth factor-binding protein-3. (For review of the role of Insulin-Like Growth Factor in atherosclerosis and intimal hyperplasia see Bayes-Genis et al.) The study by Lemaître et al. may in fact provide insight into the functional role of MMP-1 outside of the highly regulated expression in response to cellular phenotype and cytokine messaging. Hence, the role that MMP-1 has in the development of intimal hyperplasia is unclear. MMP-1 has a possible role in VSMC migration. Decreased tissue levels have been linked to mature restenotic lesions, and the present study showed an elevation of the active form in the subgroup with multiple ISR. However, only a small amount of work has been done on the role of MMP-1 in intimal hyperplasia and post-PCI restenosis.

3.3.2.6. Possible sources of MMP production in the studies in this thesis

Taken in the context of the literature discussed above, our results may reflect the disparate roles of MMPs in both normal physiology and in disease. The studies in this thesis adds to this body of data by confirming that MMPs are associated with ISR, particularly in the
active form. However, the studies in this thesis correlated MMP levels with a history of ISR, and association of peri-procedural active MMPs was outside the scope of the present study. The source of production for the circulating enzymes is unclear, however a number of possibilities are present. MMPs are known to be expressed in the vascular wall in stable and unstable atherosclerotic plaques and sites of remodelling. Furthermore, it appears that coronary events are associated with inflammation throughout the heart, rather than being limited to the culprit lesion. Circulating inflammatory cells are a potential source as pro-MMP-9 is known to be released from activated neutrophils, which appear to be elevated in the coronary circulation of patients with unstable angina. The myocardium expresses MMP-1, -2, -3 and -9 under normal conditions, and altered expression has been linked with the development of heart failure.

Lastly, various MMP polymorphisms have been linked to both the plasma levels of enzyme, including MMP-1, -2, -3 and -9, and vascular disease, although there has been much conflicting evidence. It is not immediately clear why systemic elevations of active MMPs-3 and -9, and TIMP-1 should be associated with a history of ISR. However, there are a number of plausible mechanisms.

(1) Genetic polymorphisms of MMPs are associated with altered plasma concentrations, and this may be associated with a number of vascular diseases. Increased production of MMPs may alter the local environment by liberating additional paracrine factors from the ECM. A number of lines of evidence suggest that other, as yet unknown, variables influence the development of ISR. Multivariate analysis identified the co-presence of an ISR lesion is a significant risk predictor for the development of ISR, meaning that those who developed ISR were more likely to develop additional ISR at the same time. Angiographic follow up has shown that late loss occurs in a bimodal distribution, implying there may be a distinct sub-population at risk of ISR. Finally, different strains of mice have been reported as having various susceptibilities to the development of ISR, with some strains having concordant vulnerability to atherosclerosis, and others having opposite risk for ISR and atherosclerosis. All of these factors indicate that unknown intra-patient variables probably contribute to ISR, which could potentially be genetic variables. Although there are not yet any conclusive links between MMP polymorphisms and ISR, this does not rule out the possibility of an association.
(2) There is some evidence that concurrent inflammation increases the hyperplastic response of the intima. Coronary artery disease is associated with activation of neutrophils throughout the coronary circulation, and this appears to be associated with a moderately increased response to injury. This is also characterised by a proportional increase of MMP-9 production. Furthermore, rabbits undergoing experimental injury have an increased rate of ISR when systemic inflammation is induced by co-administration of lipopolysaccharide. Hence, MMPs may be markers of chronic inflammation that predisposes to a greater neointimal response.

(3) A number of MMP isoforms, specifically MMP-7 and -12, appear to be associated with the development of complex lesions, as represented by the association of lipid-rich plaque, which are known to be a risk factor for ISR. There is also evidence from pathological studies that the contact between stent struts and necrotic core exacerbates the inflammatory response and intimal hyperplasia. Additionally, co-activation amongst MMP isoforms is common. Hence the level of specific isoforms may reflect the activity of MMPs involved in the development of lesions that are high risk for ISR.

Each of these theories are problematic and speculative. Further testing of these MMP markers in the prospective study may support the above ideas, or suggest new ones. For instance, testing whether measures of ISR lesion complexity are related to MMP variables, or if the profile of MMPs changes with the time course of neointimal development. Echocardiographic data will be collected, which will allow investigation into whether these changes correlate with functional myocardial dysfunction.

3.3.3. Activation state of matrix metalloproteinases

MMPs are regulated at several steps, including activation from the zymogen-precursor state and endogenous inhibition by TIMPs. It is probably the uninhibited active form of these enzymes that propagates the biological effects. TIMP-1 inhibits both MMP-3 and MMP-9 binding in 1:1 stoichiometry. Complexes with multiple MMPs may form, although the implications of this are not clear. For example, the dimers of MMP-9 are associated with similar activity and rates of TIMP-binding, although the monomer is more easily activated by MMP-3.

The substrate-lysis based assay for active MMPs determined the level of active isoforms, and theoretically there is no cross-reactivity with TIMP-bound enzyme. While the
activity assays were specific for the stated MMP isoform, the company reported that low levels of cross reactivity with the TIMP-complexed enzyme can occur (see 2.8.2). As an altered ratio of TIMP to MMP could result in altered binding, we also compared the association between the ratios of the specific active MMP/TIMP-1, but there was no change to the observed associations between MMPs and a history of ISR. The ratio between the active and pro-isoform of MMP-9 was significantly increased with a history of ISR, but this did not appear to give extra information regarding the association with ISR beyond that obtained from the level of active MMP-9 alone. This may indicate that the regulatory mechanisms involved in activating MMP-9 may be more important than those involved in stimulating the production of MMP-9 protein. While a number of physiological activators of MMPs have been identified (Chapter 4, Table 4.11), the plasminogen/urokinase activating system appears to be important in regulating MMPs in response to vascular injury. The healing response in plasminogen -/- mice was impaired after experimental injury of femoral arteries.\textsuperscript{311} This was associated with delayed repopulation of the medial layer by VSMC, with both migration and proliferation of VSMC being impaired. Active MMP-9 was highly expressed in wild-type mice with vascular injury, but undetectable in plasminogen -/- mice, whereas active MMP-2 was not altered\textsuperscript{312}. These results are consistent with plasminogen being the major activating enzyme of MMP-9 for arterial injury.

The present study has linked altered endogenous activity of a number of MMPs with those who previously developed ISR after being treated with coronary stents. While the mechanisms of this activity are unclear, it appears that there is a chronic shift in the zymogen balance in a number of MMP isoforms, which is not countered by TIMP inhibition. Furthermore, the independence of these enzymes indicates that multiple regulatory systems are in play. This indicates that the development of ISR may be associated with a chronic proteolytic state. However, these findings raise a number of questions relating to the pathobiology of ISR: What are the activating systems regulating the different MMP isoforms? How do the levels of active MMPs vary within individuals? These questions were beyond the scope of the present study, and may be addressed in future research.
3.3.4. Circulating matrix metalloproteinases in atherosclerotic disease

As MMPs have been understood to be a factor in the development of atherosclerosis, there has been a surge of interest in the possible role of plasma markers for various aspects of atherosclerotic disease. Much of this effort has been directed at the plasma profiles in acute coronary syndromes, which will be discussed in Chapter 5. The literature regarding the association of MMP markers with the presence and severity of atherosclerosis will be discussed here.

In their study in this thesis TIMP-1 was significantly associated with the severity of CAD. In other reports, levels of circulating pro-MMP-9\textsuperscript{428,564,565} and TIMP-1\textsuperscript{428,565} are higher in CAD patients than controls. While it is clear that circulating MMPs are elevated in CAD, the association with disease severity is less well established. In a study by Samnegard \textit{et al.},\textsuperscript{566} on patients with acute myocardial infarction, there was no correlation between pro-MMP-1, -3 and -9 and the severity of coronary artery disease.

Kalela \textit{et al}\textsuperscript{564} found an association between circulating MMP concentration and the number of diseased vessels, with pro-MMP-9 elevated in those with triple vessel disease, compared to one- and two-vessel disease. Mahajan \textit{et al.}\textsuperscript{565} showed correlations between pro-MMP-9, TIMP-1 and the Gensini score,\textsuperscript{567} (a composite of number, location and degree of stenosis) in 140 patients with coronary artery disease. We did not calculate the Gensini score, but it may be more sensitive than the number of vessels diseased.\textsuperscript{568}

The association between MMPs and atherosclerosis is not limited to the coronary bed. Pro-MMP-9 was positively correlated with both femoral intimal-medial thickness and particularly the presence of echo-lucent plaques, indicating an unstable phenotype.\textsuperscript{436} Both pro-MMP-9 and TIMP-1, but not TIMP-2, were correlated with the severity of angiographically proven peripheral arterial disease.\textsuperscript{430}

In the study in this thesis no MMPs were associated with modified ACC/AHA score. However, in a consecutive study of 27 patients, Tiong \textit{et al.}\textsuperscript{525} saw correlations of both lesion length and lesion complexity with pre-interventional samples of pro-MMP-9. They included patients with unstable coronary disease in their study, and it is known that unstable disease is associated with elevations of pro-MMP-9.\textsuperscript{433,434,569} This may amplify the relationship between production of plaque and systemic expression.
In summary, a number of associations have been made between the presence of atherosclerosis and circulating MMPs, but the association between circulating MMPs and the severity of coronary disease is less well established. One of the reasons for this may be that the angiographic quantification of numbers of diseased vessels is probably a relatively insensitive measure of total burden of atherosclerotic disease. There could be a smaller difference between the burden indicated by the number of vessels than there is between those with coronary disease and unaffected controls.

While it is plausible that circulating markers could be used to aid the detection of stable coronary disease, it is not clear whether the markers described above would contribute to a risk stratification model, such as the Diamond-Forrester model, used to stratify patients for investigation for coronary artery disease. Another interesting question is what elevated circulating markers might mean for the development of future events, or during the acute phase of plaque rupture. Both of these topics have been addressed in the literature, and will be discussed in Chapter 1.

### 3.3.5. Correlation of matrix metalloproteinases with coronary risk factors

This study found higher levels of TIMP-1 in patients with self-reported hypertension, self-reported diabetes and renal impairment, independently of sex, age, history of ISR and medication variables.

TIMP-1 also appeared to be elevated with increasing total cholesterol, waist circumference, and high sensitivity C-reactive protein, and lower with increasing HDL-cholesterol, which were all independent after adjusting for age, sex and history of ISR. Active MMPs-2 and -3 were associated with HDL-cholesterol; active MMP-2 rose with increasing HDL-cholesterol and active MMP-3 fell. These findings were independent of age, sex, history of ISR as well as treatment with statins. Active MMP-9 appeared to be higher in patients prescribed Ca\(^{2+}\) channel antagonists, independently of age, sex, and history of ISR. Lastly, active MMPs-1 and -3 appeared to be lower in people not taking statins, but this may represent uncontrolled confounding, as it is at odds with the literature, and we did not collect information on why patients were not taking statins.

Garvin et al. showed that pro-MMP-9 measurements were higher with a range of risk factors in a normal population, including high blood pressure, diabetes, obesity and smoking. However, for many of these variables they did not present data for each risk factor.
individually, but rather combined as a "risk factor burden." Weiss et al., who specifically correlated pro-MMP-9 with lipid variables, found no association between these variables. In the retrospective study, TIMP-1 was correlated with high-density lipoprotein level. In a large study (n = 891) by Hansson et al., there were small correlations between pro-MMP-9 and serum total cholesterol, low-density lipoprotein and high-density lipoprotein \((r^2 = -0.13, -0.10\) and \(-0.11\) respectively) but not triglycerides; whereas TIMP-1 correlated with high-density lipoprotein and triglycerides \((r^2 = -0.21, 0.11)\), but not low-density lipoprotein or total cholesterol. They did not see an association between circulating levels of either pro-MMP-9 or TIMP-1 with antihypertensive treatment, blood pressure or statins. In a pair of papers describing associations between TIMP-1, and total MMP-9 in the Framingham study population, Sundstrom et al. found higher levels of total MMP-9 with a history of smoking, diabetes mellitus, antihypertensive treatment (though not blood pressure) and heart rate. Increased TIMP-1 was associated with increasing age, total cholesterol, antihypertensive treatment, body mass index, smoking and diabetes, was also lower in women. The current study probably could not detect changes of this size because of its smaller power than the studies by Hansson et al. and Sundstrom et al. Another study measured pro-MMP-9 and TIMP-1 before treatment for hypertension, finding firstly that pro-MMP-9 was higher and TIMP-1 lower in individuals with untreated hypertension, compared to healthy controls, and secondly, that these levels normalised with antihypertensive treatment. Many of the patients in the present study were on antihypertensive treatment at the time of index measurement, which may have obscured the findings of this study.

Taking these findings together, it appears likely that MMP markers correlate with different measures of cardiovascular risk, and that these associations may be attenuated with treatment.

It has been suggested that MMPs can be considered inflammatory markers. A number of MMPs genes contain cytokine-responsive elements, for example MMP-3 has a stromelysin-interleukin responsive element in the promoter region, and MMP-1 and -9 have NF-κβ responsive elements. Furthermore, it has been demonstrated that these MMPs, as well as MMP-2, are released from VSMC by cytokines, and that MMP-9 is released from neutrophil granules upon activation.

Consistent with these observations TIMP-1 had significant correlations with high-sensitivity C-reactive protein measurements, indicating that there is an inflammatory
component to TIMP-1 regulation. This is in agreement with other reports where circulating TIMP-1\textsuperscript{578-580} has been correlated with inflammatory markers, including high sensitivity C-reactive protein, interleukins-6 and -18, tumour necrosis factor-\(\alpha\) and monocyte chemotactic protein-1. Other studies have shown relationships with the pro-forms of MMP-9\textsuperscript{398,579,581} which we could not confirm.

However, in they study in this thesis the active isoforms appeared to have no association with inflammatory markers. Whether the active forms of MMPs are up-regulated by inflammation, like the pro-forms are, is less clear. This raises questions about their regulation \textit{in vivo}. We have detected a significant elevation of the active forms of MMP-3 and -9, and if this activation were through a local inflammatory mechanism, this would be reflected by a significant correlation with systemic inflammatory markers, such as C-reactive protein. Much less work has been done on the active forms of MMPs, and while the activity of MMPs-2, -9 and -13 has been shown to be responsive to the cytokines IL-\(\beta\) and TNF-\(\alpha\)\textsuperscript{582} it does not necessarily follow that all MMP activity has an inflammatory genesis.

3.3.5.1. \textbf{Tissue inhibitor of matrix metalloproteinases-1 and renal impairment}

TIMP-1 was raised in participants with renal impairment, and this was independent of age, sex, history of ISR or treatment with renin/angiotensin system antagonists. TIMP-1 is expressed in the normal glomeruli of both mice\textsuperscript{583} and humans.\textsuperscript{584} Most patients with renal impairment in this study probably had chronic kidney disease, and despite varying aetiologies, fibrosis of the glomeruli and tubulointerstitium is a common pathway in progressive disease.\textsuperscript{585} TIMP-1, along with TIMPs-2 and -3 were elevated with interstitial fibrosis in a murine model of obstructive nephropathy.\textsuperscript{586} Similarly, a rat model of glomerular hyperfiltration – subtotal nephrectomy – demonstrated four-fold higher TIMP-1 expression in association with glomerulosclerosis and interstitial fibrosis. However, the authors were unable to localise TIMP-1 expression. MMP-3 was reduced and TIMP-2 increased compared to normal rats in the tubules, and MMP-1 was elevated in the glomeruli.\textsuperscript{587}

Experimental knock-in of TIMP-1, linked to a cytomegalovirus promoter, led to increased TIMP-1 expression.\textsuperscript{588} As these mice aged, they developed larger kidneys, with decreased MMP-2 and -9 expression, and increased age-related fibrosis, compared to wild-type mice. Consistent with TIMP-1 being involved in the development of fibrosis, TIMP-1 knock-out did not alleviate interstitial fibrosis in a murine model of obstructive nephropathy.
However, this same model had a compensatory increase of TIMP-3 and plasminogen activator-inhibitor-1. These results suggest that elevated TIMP-1 may be sufficient, although not necessary, for the development of interstitial fibrosis.

TIMP-1 expression has also been noted in glomerulosclerosis in humans, with TIMP-1 elevated up to four-fold, and a three-fold increase of TIMP-2, compared to normal glomerular tissue.

Changes in TIMP-1 have also been noted in both serum and urine with chronic renal failure with a number of aetiologies. There was a strong correlation between circulating and urine levels of TIMP-1 \((r = 0.55, p < 0.001)\), as well as with falling creatinine clearance. Although strong correlations with creatinine clearance probably indicate that TIMP-1 levels would have prognostic value, it is not clear if measurements of TIMP-1 would be additive over that of creatinine clearance alone. One study found that TIMP-1 levels were not correlated with the future development of microalbuminuria in patients with type 1 diabetes mellitus.

There are a number of potential sources of TIMP-1 in renal disease, including the diseased parenchyma, accumulation as renal clearance decreases, and systemic contributory variables (e.g. diabetes mellitus and hypertension [reviewed elsewhere in this chapter]). Soylemezoglu et al. measured systemic and urine markers of protein degradation in patients with biopsy-proven renal fibrosis. They found that both circulating and urine collagen fragments correlated strongly with disease activity on renal biopsy. Furthermore, TIMP-1 has a direct correlation with creatinine clearance, rather than with the serum creatinine concentration. Both of these findings suggest that the renal disease process was the source, rather than accumulation with declining renal function.

### 3.3.5.2. Tissue inhibitor of matrix metalloproteinases-1 and diabetes mellitus

Participants with self-reported diabetes mellitus had higher levels of TIMP-1, independently of age, sex and history of ISR. Additionally, this did not appear to be explained by treatment with renin/angiotensin system antagonists.

Elevated plasma TIMP-1 has previously been linked with diabetes mellitus. Diabetes mellitus is associated with a number of ECM changes, particularly basement membrane thickening. Extreme hyperglycaemia is known to have a direct effect on the expression of MMPs and TIMPs. Death et al. cultured human endothelial cells and
macrophages in glucose concentrations of 25 mmol/L for 24 hours, comparing these to normoglycaemic conditions (5 mmol/L) and a mixture of mannitol and glucose (20 mmol/L mannitol, 5 mmol/L glucose). Hyperglycaemia induced mRNA expression of MMPs-1 and -2, and inhibited MMP-3 from endothelial cells, and total MMP-9 protein and activity from macrophages. TIMP-1 expression was not altered by hyperglycaemia. There was no difference in terms of MMP or TIMP expression between the mannitol/glucose mixture and the normoglycaemic control for any comparison. When endothelial cells were subject to even higher levels of glucose (33 mmol/L), the levels of TIMP-2 progressively decreased, and levels of active MMP-2 increased over 48 hours. There was no difference in levels of TIMP-1, total MMPs-2 or -9, or active MMP-9. Incubation with ascorbic acid as well as high levels of glucose, prevented changes in active MMP-2 and TIMP-2, indicating that the mechanism driving the changes in these proteins was the generation of reactive oxygen species.

Another study showed that eight weeks of elevated blood glucose (~15 mmol/L) in mice with streptozotocin-induced type 1 diabetes was not associated with alterations in MMP-9 mRNA or ECM changes. The authors up-regulated the TGFβ1 gene by attaching it to a renin promoter. This was associated with increased MMPs-2 and -9, and TIMP-1, along with increased collagen deposition in the renal tubulointerstitium. When the normoglycaemic/TGF-β1 knock-in mouse was compared to the hyperglycaemic/TGF-β1 knock-in, there appeared to be an interaction between TGF-β1 overproduction and hyperglycaemia, with higher levels of both collagen deposition and expression of MMP-2 and TIMP-1, although comparable levels of MMP-9. These results suggest that extreme hyperglycaemia is able to alter the expression of multiple MMPs and TIMPs, and lower levels of hyperglycaemia may also interact with growth factor pathways to produce altered levels of MMPs and TIMPs.

In clinical studies, TIMP-1 has consistently been elevated in patients with diabetes, although one study in patients with type 1 diabetes did not show an association. In patients with type 2 diabetes, pro-MMPs-2 and -9, total MMP-2 and TIMP-1 and TIMP-2 were higher than age and sex matched controls.

In patients with type 1 diabetes, one study by Maxwell et al showed increased TIMP-1, a non-significant increase in pro-MMP-2, and had a greater proportion of patients with detectable pro-MMP-9. However, Thrailkill et al. saw no difference in TIMPs-1 or -2,
but that the levels of total and active MMP-2 were higher in both the plasma and the urine of patients with diabetes. Different durations of disease in the patients may explain the differences between the two studies. The average duration was not reported by Thrailkill et al.,\textsuperscript{601} although one-quarter had diabetes for less than three years. The median duration of diabetes in the study by Maxwell et al. was 10.5 years. However, the mean ages were 19 years and 27 years in the studies by Thrailkill\textsuperscript{601} and Maxwell,\textsuperscript{596} respectively. Thrailkill et al. reported that urine total MMP-2/creatinine ratio was increased with those having diabetes longer than three years, but they did not report associations with either TIMP. Maxwell et al. did not see an association between any MMP or TIMP and duration of disease. The mean glycated haemoglobin measurements were similar between the two studies.

Thrailkill et al.\textsuperscript{601} examined both the circulating and urinary expression of MMPs in type 1 diabetes, compared to controls. They found that both the total MMP-2 concentration, and total MMP-2/creatinine ratio increased in the urine of patients with diabetes. That the ratio of total MMP-2 to creatinine was elevated suggests that the mechanism of elevation is not due simply to altered urine composition between those with diabetes and controls, or decreasing glomerular filtration rate.

Studies in patients with type 2 diabetes appeared to have shorter disease durations (less than six months,\textsuperscript{594} mean 3.2 years\textsuperscript{593} and not reported\textsuperscript{595}) yet all had strong associations between TIMP-1 and diabetes, whereas only one out of two studies of patients with type 1 diabetes showed an association between TIMP-1 and diabetes. The lack of association in the one study may represent a false negative.

Most papers did not describe associations with MMP or TIMP variables with glycated haemoglobin. In patients with type 1 diabetes plasma total MMP-2 and urinary total MMP-2/creatinine ratio were positively associated with HbA1c,\textsuperscript{601} but not pro-MMPs-2,\textsuperscript{596} -9,\textsuperscript{596} active MMP-2\textsuperscript{601} or TIMPs-1\textsuperscript{596,601} or -2.\textsuperscript{601} TIMP-1 was weakly correlated with HbA1c in one study on patients with type 2 diabetes.\textsuperscript{595} TIMP-1 did not correlate with VCAM-1 or ICAM-1, markers of endothelial activation.\textsuperscript{593}

One study followed a cohort of 65 patients with type 2 diabetes beginning intensive cardiac risk factor management.\textsuperscript{595} The intervention included lifestyle, dietary and pharmacological therapy. After one year, the group had decreased serum total cholesterol, and HbA1c dropped from 7.7 to 7.1%. The use of aspirin, ACE inhibitors and other anti-hypertensive medications increased. There was no change in body mass index or blood
Association of MMP markers with a history of ISR

pressure. TIMP-1 levels also fell modestly but there was no change in pro-MMP-9 or TIMP-2.

Another study investigated MMP expression in internal mammary artery grafts in patients undergoing coronary artery bypass grafting, comparing patients, with either type 1 or type 2 diabetes, to patients without diabetes.\textsuperscript{602} There was decreased expression of a range of MMP variables in the homogenised arterial grafts of patients with diabetes, specifically pro-MMPs-1, -2, and -9, active MMP-2 and TIMP-1. In fact no active MMP-9 was detected. Associated with the decreased MMP and TIMP-1 expression, there was a three-fold increase in type 1 collagen in the arterial grafts from patients with diabetes. These findings suggest that in patients with diabetes, non-atherosclerotic arteries are associated with decreased MMP and TIMP expression, and therefore widespread vascular changes are not the source of altered MMP and TIMP expression in diabetes.

Reports in the literature of associations between TIMPs and MMPs with diabetic nephropathy are discussed elsewhere in this chapter. However, we did not include blood glucose measures, HbA1c, time since diagnosis, or other measures such as presence of microvascular disease in the studies in this thesis.

3.3.5.3. Effect of prescribed medications on matrix metalloproteinases

We observed decreased levels of active MMPs-1 and -3 in the small group not receiving statin therapy at recruitment. This was independent of age, sex and a history of ISR. There was no interaction between the association of active MMPs-1 and -3 with statin therapy and a patient-reported history of dyslipidaemia. This finding is at odds with the literature. Experimental administration of statins \textit{in vitro} to VSMC, endothelial cells and macrophages has been shown to decrease levels of MMPs-1,\textsuperscript{603} -2,\textsuperscript{603,604} -3,\textsuperscript{603} and -9,\textsuperscript{603-606} increase MMP-12,\textsuperscript{607} but not effect TIMPs-1 or -2.\textsuperscript{603} Statin therapy also decreased the level of MMP-2 and -9 activity.\textsuperscript{604} Similar results were seen for a broad range of statins.\textsuperscript{604} This effect does not appear to be tissue-specific, with statins decreasing the expression of MMPs-1, -3 and -9 from lung fibroblasts,\textsuperscript{608} and MMPs-1, -2, -3, -9 and -13 from chondrocytes.\textsuperscript{609} In order to perform these experiments, the investigators have utilized a number of different types of stimulating factors, including interleukin-1,\textsuperscript{603,606,608,609} PDGF,\textsuperscript{603,606} tumour necrosis factor-\(\alpha\),\textsuperscript{605,608} and oncostatin M\textsuperscript{609} as well as phorbol myristate acetate,\textsuperscript{605} or added nothing (unstimulated) to the
culture media. However, results appeared to be consistent despite different stimulating mechanisms.

The effects of statins on MMPs can also be detected within diseased human tissue, with lower levels of MMPs-3 and -9 and lower activity of MMP-9 in the excised aortae of patients undergoing aneurysm repair. Abisi et al. saw a trend towards decreasing in the levels of MMP-3 activity, but this did not achieve statistical significance. Wilson et al. saw no change in the concentrations of MMPs-1, -2, -8 or -13. Of note, neither of the studies by Abisi or Wilson had randomized patients to statins, but rather the statin exposure was determined by clinical indications. Potentially the observed changes could be due to associations with coronary artery disease or dyslipidaemia. A similarly designed study measured MMP-9 in carotid plaques after endarterectomy, finding that MMP-9 expression was much lower in the group treated with statins. However, one randomized control trial confirmed that fluvastatin decreased circulating MMP-9 activity along with measures of inflammation in patients with dyslipidaemia. As statins appear to decrease levels of MMPs-1 and -3 from isolated cells, as well as MMP-3 in diseased human tissue, it is possible that the association of statin therapy with increased levels of MMP-1 and -3 activity we observed is due to uncontrolled confounding. While this effect appeared to be independent of age, sex and histories of ISR and dyslipidaemia, the group not on statins may differ in other important ways not captured by these variables.

Active MMP-9 was higher in those prescribed Ca²⁺ channel blockers, independently of age, sex and history of ISR. There was no interaction between prescription of Ca²⁺ channel blockers and patient-reported history of hypertension, and the level of active MMP-9. Ca²⁺ channel blockers have been shown to have divergent effects on MMP levels. Multiple dihydropyridine-based Ca²⁺ channel blockers have been linked to changes in the activity of MMPs-2 and -9 but not TIMP-1. Non-dihydropyridine-based Ca²⁺ channel blockers do not appear to affect the activity of MMPs-2 and -9 in both in vitro and clinical studies. However, one study showed that a range of dihydropyridine and non-dihydropyridine Ca²⁺ channel blockers could increase the expression of MMP-2 activity from VSMC. Both amlodipine, a dihydropyridine, and diltiazem, a non-dihydropyridine Ca²⁺ channel blocker, also appeared to inhibit VSMC proliferation and suppress MMP-1 expression after stimulation with PDGF. These findings may indicate cell-line specific responses to Ca²⁺ channel blockade.
The different members of the dihydropyridine-based Ca\textsuperscript{2+} channel blockers act differently upon MMP levels. Nifedipine\textsuperscript{616} and felodipine\textsuperscript{614} increase MMP-2 activity; amlodipine decreases MMPs-1\textsuperscript{622} and -2\textsuperscript{616} and increases MMP-9\textsuperscript{615,616,618} activity; and azelnidipine\textsuperscript{619,623} and lercanidipine\textsuperscript{617} decrease active MMP-9, but do not alter TIMP-1.

The different members of the dihydropyridines appear to mediate their effects through different mechanisms. For example, nifedipine increases MMP-2 activity by donating nitric oxide, and amlodipine works through activating tyrosine kinase.\textsuperscript{616}

In clinical populations, cohort studies have indicated that the activity of circulating MMPs-2\textsuperscript{614} and -9\textsuperscript{615} are altered by dihydropyridine Ca\textsuperscript{2+} channel blockers, and one crossover trial has confirmed that lercanidipine decreases active MMP-9.\textsuperscript{617}

However, we did not collect important variables that could potentially be confounding these associations. Specifically, we did not collect information on the type of Ca\textsuperscript{2+} channel blocker prescribed, and we did not directly measure blood pressure. Non-dihydropyridine Ca\textsuperscript{2+} channel blockers account for 70 to 90\% of prescribed Ca\textsuperscript{2+} channel blockers in patients undergoing PCI,\textsuperscript{624,625} which would suggest that any drug-related effect on MMP-9 activity would only be seen in a minority of patients. Hence, like the association of active MMPs-1 and -3 with statin therapy, the association of increased active MMP-9 with Ca\textsuperscript{2+} channel blockers may be due to uncontrolled confounding. The association of active MMP-9 and Ca\textsuperscript{2+} channel blockers was independent of a patient-reported history of hypertension and the number of anti-hypertensive medications prescribed, suggesting that this association was not due to underlying hypertension. The divergent effects of the different Ca\textsuperscript{2+} channel blockers may have precluded the detection of the changes in MMP activity previously reported in association with these medications.

In this dataset active MMP-2 was positively associated with the plasma concentration of HDL-cholesterol. The largest study looking at the impact of lipid markers on circulating MMPs found that pro-MMP-9 was significantly correlated positively with LDL-cholesterol and negatively with HDL-cholesterol,\textsuperscript{573} which was also seen by Tayebjee et al.,\textsuperscript{576} and TIMP-1 negatively correlated with oxidised LDL.\textsuperscript{626} Furthermore, there appeared to be a synergistic effect between hypertriglyceridaemia and pro-MMP-9 concentration, with elevations of both associated with a strong risk of developing coronary events.\textsuperscript{572} However, the association between MMPs and lipid markers has been disputed by a number of smaller studies.
Cicero et al.\textsuperscript{627} found no association between pro-MMP-2 and lipid markers in patients with untreated familial combined hyperlipidaemia compared to healthy controls, however both pro-MMP-9 and TIMP-1 levels were elevated in familial combined hyperlipidaemia.\textsuperscript{627} There was no correlation between either the pro-MMPs or TIMP-1, and any specific circulating lipid concentration in obese patients,\textsuperscript{455} familial combined hyperlipidaemia,\textsuperscript{627} or diabetes.\textsuperscript{594} These studies investigated the association of circulating pro-MMPs with known risk groups for vascular disease. While there was no definite association between MMP markers and a specific circulating lipid marker, the authors noted that pro-MMPs -2 and -9 and TIMP-1 were elevated in the patients with dyslipidaemia compared to non-dyslipidaemic controls. While these studies are small, the association of MMP markers with dyslipidaemia, but not specific markers, suggests that alterations in a number of lipid markers influence MMP markers. To date no studies have addressed the correlation between active MMPs and lipid markers.

### 3.3.6. Confounders of matrix metalloproteinases in clinical studies

It is well known that many variables alter the plasma levels of MMPs, including atherosclerosis,\textsuperscript{428,429} peripheral arterial occlusive disease,\textsuperscript{430} acute coronary syndrome,\textsuperscript{429,433} abdominal aortic aneurysm,\textsuperscript{460-463} ethnicity,\textsuperscript{428} sex,\textsuperscript{428} age,\textsuperscript{428,451} medications\textsuperscript{452-454} and obesity.\textsuperscript{455} Data on many of these variables was collected in order to adjust for bias in the association of MMPs with ISR. A number of relatively common conditions without known or putative associations with ISR, for example cancer,\textsuperscript{449,450} level of physical fitness,\textsuperscript{428} asthma,\textsuperscript{457} liver fibrosis,\textsuperscript{458} surgery,\textsuperscript{456} and rheumatoid arthritis\textsuperscript{459} were not controlled for, and may be a source of bias. Additionally, a number of drugs are known to alter MMP levels,\textsuperscript{452,616,628,629} but there was no evidence for an association between treatment with medication and any MMP.

The degree to which these confounded the study is difficult to estimate, but these conditions are unlikely to have a preponderance in either arm of the study. Hence they are unlikely to cause a significant bias.

The majority of participants in this study were of New Zealand European ethnicity, and age and medications were adjusted for. We noted significant differences with the use of statins and calcium channel inhibitors between the case and control groups, but a probable
explanation for this is that patients in the case group have had further symptom-driven follow up, and so were treated with medications as part of the clinical response to the ISR symptoms.

The inclusion criteria attempted to exclude people who had active cardiac disease. The majority of ISR cases present within one year,\textsuperscript{49,520} and most cases of recurrent ISR develop by six months.\textsuperscript{630,631} Both of these time frames may reflect patterns of clinical follow up as well as the development of symptoms, but the majority of ISR symptoms develop within this time frame. Therefore, the cut off points of one year and six months would exclude most cases currently developing symptomatic ISR, which would be a confounding factor, if it entailed elevated MMP levels.

3.4. Limitations of this study

There were a number of limitations to this study. The major limitation was the retrospective design. While the results suggest that the development of ISR is associated with a chronic proteolytic state, it is not clear if active MMPs-3 and -9, and TIMP-1 are elevated prior to PCI, which would be necessary if the levels of these enzymes were to be used as risk markers. Many different variables alter MMP expression and activation, especially the type of presentation before PCI.\textsuperscript{429,433,632} Therefore, it is important to confirm this finding in a prospective study, and draw blood samples before intervention. Hence, at present the findings of this study lack direct clinical utility.

A further limitation is that demographic and clinical variables collected at recruitment, at least one year after the index PCI. This may have led to confounding through recall bias, further treatment in high-risk subgroups and survival bias.

During this time many would have been receiving dietary, exercise, and pharmacological therapy for secondary prevention of cardiovascular events. A number of drugs alter MMP levels,\textsuperscript{452,616,628,629} and while cardiovascular drugs were controlled for, it is not clear what impact these have had on our results. While ISR often presents as ACS,\textsuperscript{49,633} in one large study there was no difference in mortality at 6 months.\textsuperscript{633} However, it appears that ISR may be associated with an overall, long-term risk of mortality (6.6% vs. 4.0% of those without restenosis in a cohort of 2,272 patients followed for four years), with an increasing rate of mortality for those with a more severe restenotic lesion.\textsuperscript{634} Hence, it could be that the ISR population in the present study is skewed by the death of those with more severe ISR, perhaps leading to an underestimation of the associations of different variables with ISR. On the basis of these points, prospective validation is necessary, and will also allow a
determination as to whether pre-interventional levels could guide the choice of revascularisation therapy.

In this study, the definition of ISR was an angiographic diameter stenosis of >50% of the vessel reference diameter in ≥1 projections, by visual assessment by a single experienced cardiologist. However, after the original PCI procedure, no further angiography was planned, rather, the re-assessment was clinically driven (i.e. either a recurrence of symptoms suggestive of ischaemia, or an unstable presentation). The corollary of this is that the control group received no angiographic follow up, and measures such as luminal late loss and diameter stenosis were impossible to calculate. While there has been some debate over the validity of these measures as substitute end points in stent trials, they are widely used in the cardiovascular literature, and correlate strongly with the need for revascularisation.

The area of intimal hyperplasia is present in varying degrees in the stented population. Therefore, it is possible to correlate various measures with the intimal area, reflecting a dose-response effect. Furthermore, one-half to one-third of patients with angiographic restenosis have no clinical symptoms. However, we did not include follow up angiography in this study, and quantitative angiographic endpoints were not included. Therefore some of the control group will have had significant intimal hyperplasia, which would have qualified them as having angiographic restenosis, but in the absence of symptoms, were not re-studied. Two reasons for this were (1) the retrospective nature of this study precluded capturing data at specified intervals from the original PCI as many patients were recruited long after the original PCI, and additionally, this was not contemporary clinical practice during the period which these patients underwent intervention. (2) While coronary angiography is a relatively safe procedure, it is still associated with risks to the individual. The rate of complications appears to be under 2%, and includes a wide range of adverse events, from bleeding and pseudoaneurysm formation at the puncture site, renal failure due to contrast medium administration, vessel perforations, embolic events and risk from radiation exposure. Mortality is rare, but cases have been reported. Another limitation is that a single cardiologist interpreted all angiograms. While they were blinded to MMP measurements, this could lead to a systematic error and obscure relationships between disease and MMP measurements.

It would have been interesting to correlate the plasma levels of active MMPs to angiographic measures of intimal hyperplasia, however, this was outside the scope of the
present research.

Because not all angiographic restenosis, defined by diameter stenosis of >50% of the vessel detected by routine angiography, is symptomatic,\textsuperscript{520,637} it could be argued that cases included in this definition do not need revascularisation and therefore do not have clinically important disease. Thus, our results suggest that MMP markers are associated with a history of development of symptomatic disease, but this association may be blunted by not taking into account the fact that the area of intimal hyperplasia is a continuous variable.\textsuperscript{65}

Active MMPs-1 and -3 appeared to be lower in people not taking statins, but this may represent uncontrolled confounding. These associations did not appear to be explained by sex, age, history of ISR event or patient-reported dyslipidaemia. However, we did not collect the indication for statin therapy. Additionally, the number of patients not on statins was small and may represent a chance finding.

\textbf{3.5. Conclusions}

The main findings of this study are that levels of active MMPs-3 and -9, and TIMP-1 were significantly elevated in patients who have a history of ISR compared with patients who received coronary stents without a return of symptoms at one year. These associations were independent of established clinical and demographic risk factors.

The adjusted odds ratios associated with active MMPs-3 and -9, and TIMP-1 were as large, if not larger, than many variables currently established in the literature,\textsuperscript{46,518,519} indicating that there may be a role for the use of active MMP markers in risk prediction for ISR. Additionally, there appeared to be a dose-response with increasing levels of active MMPs-3 and -9, but not TIMP-1, in patients with multiple ISR lesions. These findings need to be further assessed in a prospective study, particularly in order to test the clinical utility of these associations.

A number of exploratory comparisons were made between MMPs and inflammatory, cardiovascular and medication variables. TIMP-1 was higher in patients with more severe coronary artery disease, self-reported hypertension, self-reported diabetes and renal impairment, all independent of sex, age, history of ISR and medication variables. TIMP-1 also appeared to be elevated with increasing total cholesterol, waist circumference, high sensitivity C-reactive protein and lower with increasing HDL-cholesterol, after adjusting for age, sex and history of ISR.
Active MMPs-1 and -3 appeared to be lower in people not taking statins and active MMP-9 higher in those prescribed Ca\(^{2+}\) channel antagonists. These associations were independent of potential confounding variables that we did collect, but these associations may represent uncontrolled confounding.
4. Performance of MMP markers in ISR predictive risk models

4.1. Introduction

In Chapter 3 it was shown that the active circulating forms of MMPs-3 and -9, and TIMP-1 were elevated in those with a history of ISR. A case was made linking this finding to the biology of arterial repair with migration and proliferation of VSMCs and the deposition of a proteoglycan-rich neointimal lesion, concluding that elevated levels of the active form of these enzymes could represent individual susceptibility to mounting a hyperplastic response. A further goal of this project is to investigate whether these enzymes could be useful in a clinical situation. Active MMP and TIMP measurements could possibly add to clinical and demographic risk factors in guiding selection of drug-eluting stents or coronary artery bypass grafting over bare-metal stenting, or appropriately direct more intensive early follow up.

With the advent of molecular biology, biomarker studies have proliferated in many areas of medicine. Some biomarkers have been successfully integrated into medical practice, for example troponin testing has been integrated into the universal definition of myocardial infarction. However, for many more biomarkers, their roles are less well delineated. For a biomarker to be clinically useful it must be easy to measure; acceptable to the patient; have a strong; independent association with the disease; be able to alter clinical management and finally; must provide further information in addition to that provided by more easily obtainable variables.

Recently, guidelines for the development of biomarkers have been produced, recommending several stages of biomarker assessment. These include reporting how the putative biomarker contributes to the prediction of risk already provided by established and utilized risk factors.

There is extensive literature on clinical and angiographic predictors of ISR with both bare-metal and drug-eluting stents. However, while a number of risk prediction scores for ISR have been developed, none have penetrated into contemporary clinical practice.

While it is recognised that the association of increased active MMPs-3 and -9, and
TIMP-1 with ISR needs to be confirmed prospectively, this chapter will investigate how active MMPs-3 and -9, and TIMP-1 add to established risk factors in the prediction of ISR. The aims of this chapter are to (1) assess the contribution active MMPs-3, -9 and TIMP-1 collectively make to ISR risk prediction, (2) assess the contribution of active MMPs-3, -9 and TIMP-1 to ISR risk prediction in the context of clinical and demographic factors in the retrospective study and (3) assess the contribution of active MMPs-3 and -9, and TIMP-1 to ISR risk prediction in the context of previously developed risk models.
4.2. Results

4.2.1. Independence of associations between MMPs and ISR

The correlations between all MMPs and TIMP-1 are displayed in Table 4.1. Active MMP-1 had positive, moderately strong correlations with both active MMP-3 and MMP-9. TIMP-1 was moderately, negatively correlated with active MMP-2, and positively with active MMPs-3 and -9. Finally, active MMP-3 and -9 were moderately correlated with each other. Hence, there may be important confounding relationships between each of the MMP variables most strongly associated with a history of ISR, active MMPs-3 and -9, and TIMP-1. This indicates that they may be surrogates for each other, and potentially share underlying regulating mechanisms. Therefore, it is important to determine whether these markers are independent of each other in their association with ISR.

The associations between MMP markers and a history of ISR, adjusted for clinical and demographic risk factors, as well as other MMP markers, are displayed in Table 4.2. Univariate and multivariate adjustment for clinical and demographic risk factors alone (from Table 3.6, Chapter 3) are given for comparison. Active MMPs-3 and -9, and TIMP-1 remained strongly associated with ISR, indicating that the association each of these markers had with ISR was independent of each other. However, active MMPs-1, -2 and pro-MMP-9 were not associated with ISR independently of clinical, demographic and other MMP markers. When the optimal cut off points were associated in the multivariate model, active MMPs-3 and -9, and TIMP-1 remained associated with ISR. Elevated active MMP-3 and TIMP-1 both indicated an approximate doubling of risk, and active MMP-9 indicated an approximate quadrupling of risk. When other MMPs were included in the multivariate model, the effect size of the associations of active MMPs-3 and -9, and TIMP-1 with ISR remained similar.
Performance of MMP markers in ISR predictive risk models

Table 4.1 Correlations between MMP markers in the retrospective study

<table>
<thead>
<tr>
<th></th>
<th>Active MMP-1</th>
<th>Active MMP-2</th>
<th>Active MMP-3</th>
<th>Active MMP-9</th>
<th>Pro-MMP-9</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Active MMP-2</td>
<td>-0.031</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Active MMP-3</td>
<td>0.47†</td>
<td>-0.16</td>
<td>0.20*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>0.32†</td>
<td>0.05</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pro-MMP-9</td>
<td>0.06</td>
<td>0.06</td>
<td>0.09</td>
<td>-0.08</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.06</td>
<td>-0.18*</td>
<td>0.22*</td>
<td>0.21*</td>
<td>-0.08</td>
<td>–</td>
</tr>
</tbody>
</table>

Results are Spearman’s rho.

*p < 0.05; †p < 0.0001. Blue colouring indicates a correlation coefficient with an absolute value between 0.15 and 0.3; red colouring indicates a correlation coefficient with an absolute value between 0.3 and 0.5.
Performance of MMP markers in ISR predictive risk models

Table 4.2 Multivariate association of MMPs with ISR, including adjustment for MMP markers

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
<th>Adjusted OR + MMPs (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1, ng/mL</td>
<td>1.32 (1.03 – 1.68)*</td>
<td>1.32 (0.96 – 1.80)</td>
<td>0.84 (0.57 – 1.25)</td>
</tr>
<tr>
<td>Active MMP-2, ng/mL</td>
<td>0.80 (0.64 – 1.02)</td>
<td>0.81 (0.60 – 1.09)</td>
<td>0.83 (0.59 – 1.17)</td>
</tr>
<tr>
<td>Active MMP-3, ng/mL</td>
<td>1.56 (1.22 – 2.00)‡</td>
<td>1.71 (1.23 – 2.37)†</td>
<td>1.54 (1.02 – 2.31)*</td>
</tr>
<tr>
<td>Active MMP-9, ng/mL</td>
<td>2.29 (1.71 – 3.08)§</td>
<td>2.38 (1.65 – 3.45)§</td>
<td>2.27 (1.47 – 3.50)‡</td>
</tr>
<tr>
<td>Pro-MMP-9, ng/mL</td>
<td>0.87 (0.69 – 1.10)</td>
<td>0.78 (0.58 – 1.06)</td>
<td>1.02 (0.73 – 1.43)</td>
</tr>
<tr>
<td>TIMP-1, ng/mL</td>
<td>1.50 (1.18 – 1.92)‡</td>
<td>1.69 (1.19 – 2.42)†</td>
<td>1.62 (1.05 – 2.78)*</td>
</tr>
<tr>
<td>Active MMP-1, ≥ 2 ng/mL</td>
<td>1.41 (0.89 – 2.28)</td>
<td>1.45 (0.79 – 2.64)</td>
<td>0.82 (0.41 – 1.64)</td>
</tr>
<tr>
<td>Active MMP-2, ≥ 15 ng/mL</td>
<td>0.55 (0.35 – 0.89)*</td>
<td>0.62 (0.34 – 1.14)</td>
<td>0.58 (0.29 – 1.15)</td>
</tr>
<tr>
<td>Active MMP-3, ≥ 3 ng/mL</td>
<td>2.57 (1.57 – 4.14)§</td>
<td>3.09 (1.65 – 5.77)‡</td>
<td>2.30 (1.14 – 4.65)*</td>
</tr>
<tr>
<td>Active MMP-9, ≥ 2 ng/mL</td>
<td>4.63 (2.83 – 7.61)§</td>
<td>4.17 (2.21 – 7.88)§</td>
<td>4.26 (2.11 – 8.61)§</td>
</tr>
<tr>
<td>Pro-MMP-9, ≥ 35 ng/mL</td>
<td>0.91 (0.55 – 1.48)</td>
<td>0.88 (0.42 – 1.65)</td>
<td>1.39 (0.68 – 2.84)</td>
</tr>
<tr>
<td>TIMP-1, ≥ 220 ng/mL</td>
<td>2.16 (1.35 – 3.46)†</td>
<td>2.82 (1.44 – 5.55)†</td>
<td>2.32 (1.10 – 4.91)*</td>
</tr>
</tbody>
</table>

Adjusted model includes age, sex, diabetes, waist circumference, hs-CRP, CAD severity, ACC/AHA score, stent diameter and length, number of sites stented, and impaired renal function. “Adjusted OR” model includes all of the above variables. “Adjusted OR + MMPs” model includes all of the above variables as well as all other MMP variables. All associations with continuous MMP variables are first transformed by logarithm, and each association is for a one standard deviation increase. Unadjusted and multivariate (“Adjusted OR”) associations from Table 3.6, Chapter 3 are provided for comparison.

*p < 0.05, †p < 0.01, ‡p < 0.001, §p < 0.0001 all for ISR vs. stent.
There was no evidence for poor model fit.
Table 4.3 displays the proportion of cases and controls with combinations of elevated markers along with the univariate and multivariate odds ratios. A history of ISR was associated with a significant increase in the number of elevated markers. Around one quarter of controls had no elevated markers, whereas the ISR group had half this number. While around half of the controls group had at least one elevated marker, nearly half of the ISR group had two elevated markers. Finally, nearly one quarter of the ISR group had all three markers elevated, whereas only a small number of the control group had all markers elevated. Increasing prevalence of elevated markers was associated with higher odds ratios, and having all three markers elevated was associated with an adjusted odds ratio of over 10. When combinations of elevated markers were compared, all combinations were associated with increasing effect sizes. While both cases and controls generally had at least one elevated marker, the number of cases with each possible pair of markers elevated was 2.3 to 3.5 fold higher than the number of controls with the same pair of markers. Over two-thirds of ISR cases had at least two elevated markers, compared to only one third of controls. Having all three markers elevated was also strongly associated with ISR, identifying around a quarter of patients with ISR, compared to just over six percent of controls. Thus there appears to be additive benefit for prediction of ISR by including all of active MMPs -3 and -9, and TIMP-1.

It also appeared that there was a biological gradient associated with the number of ISR lesions (Figure 4.1). Whereas three quarters of controls had one or fewer elevated marker, nearly 80% of those with multiple ISR had two or more markers elevated. There was also a significant increase in the rate of multiple ISR when all three markers were elevated.
Table 4.3 Association of combinations of elevated MMPs with ISR

<table>
<thead>
<tr>
<th>Number of cut offs</th>
<th>CAD with stent, n = 151</th>
<th>ISR, n = 152</th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>37 (24.5%)</td>
<td>19 (12.5%)</td>
<td>‡</td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>75 (49.7%)</td>
<td>35 (23.0%)</td>
<td>0.91 (0.47 – 1.73)</td>
<td>1.00 (0.41 – 2.42)</td>
</tr>
<tr>
<td>Two</td>
<td>29 (19.2%)</td>
<td>62 (40.8%)</td>
<td>4.25 (2.17 – 8.32)‡</td>
<td>6.83 (2.65 – 17.61)‡</td>
</tr>
<tr>
<td>Three</td>
<td>10 (6.6%)</td>
<td>36 (23.7%)</td>
<td>7.09 (3.03 – 16.59)‡</td>
<td>10.16 (3.36 – 30.74)‡</td>
</tr>
<tr>
<td>At least 1 cut off</td>
<td>114 (75.5%)</td>
<td>133 (87.5%)*</td>
<td>2.28 (1.28 – 4.06)*</td>
<td>3.07 (1.34 – 7.04)*</td>
</tr>
<tr>
<td>Both active MMPs-3 and -9</td>
<td>14 (9.3%)</td>
<td>50 (32.9%)†‡</td>
<td>4.60 (2.50 – 8.45)‡</td>
<td>4.07 (1.91 – 8.68)‡</td>
</tr>
<tr>
<td>Both active MMP-3 and TIMP-1</td>
<td>26 (17.2%)</td>
<td>59 (38.8%)‡</td>
<td>3.23 (1.94 – 5.39)‡</td>
<td>4.91 (2.02 – 7.56)‡</td>
</tr>
<tr>
<td>Both active MMP-9 and TIMP-1</td>
<td>19 (12.6%)</td>
<td>61 (40.1%)‡</td>
<td>4.64 (2.67 – 8.06)‡</td>
<td>5.88 (2.90 – 11.89)‡</td>
</tr>
<tr>
<td>At least 2 cut offs</td>
<td>50 (33.1%)</td>
<td>102 (67.1%)‡</td>
<td>5.45 (3.34 – 8.96)‡</td>
<td>6.76 (3.48 – 13.13)‡</td>
</tr>
<tr>
<td>All active MMPs-3, -9 and TIMP-1</td>
<td>10 (6.6%)</td>
<td>36 (23.7%)‡</td>
<td>4.41 (2.17 – 8.96)‡</td>
<td>5.00 (2.15 – 11.61)†</td>
</tr>
</tbody>
</table>

*p < 0.05; †p < 0.001; ‡p < 0.0001, all for ISR vs. stent.

Adjusted model includes age, sex, diabetes, waist circumference, hs-CRP, CAD severity, ACC/AHA score, stent diameter and length, number of sites stented, and impaired renal function.

There was no evidence for poor model fit. Column percentages for analyses of the number of individuals multiple markers being elevated do not sum to 100% as those with greater than two markers elevated are included in each comparison.
Performance of MMP markers in ISR predictive risk models

Figure 4.1 Association of MMP cut off points with number of ISR lesions
The number of elevated biomarkers (active MMP-3 $\geq$ 3 ng/mL; active MMP-9 $\geq$ 2 ng/mL; TIMP-1 $\geq$ 220 ng/mL) compared to the number of ISR lesions for each given patient.
Percentages for no ISR (white bars) were 23.0, 50.0, 19.6, 7.4; single ISR lesion (pink bars) were 13.7, 19.1, 40.9, 26.4; and multiple ISR lesions (red bars) were 12.5, 8.3, 37.5, 41.7. *$p < 0.0001$ versus no ISR, †$p < 0.008$ versus single ISR lesion.
4.2.2. Contribution of MMP markers to clinical and demographic factors from the retrospective study

The associated area under the curve of individual MMP markers, and MMP markers in combination with clinical and demographic risk markers are given in Table 4.4. The discriminatory ability of individual MMPs was moderately good, but not as high as the combination of clinical and demographic risk factors. When MMP cut off points were included along with the clinical and demographic risk factors, each combination was associated with improved discrimination over the clinical and demographic risk factors alone. When all three MMP markers were included, the highest rate of discrimination was achieved, an absolute increase of 8% discrimination between cases and controls. This increase was statistically significant.

The classification of cases and controls by the logistic regression model after the inclusion of the same variables, with progressive inclusion of MMP variables, is given in Table 4.5. The addition of MMP variables was associated with a progressive increase in the proportion of overall correct predictions (i.e. ISR cases classified as cases, controls classified as controls), and improving model fit, as indicated by the decreasing Bayesian Information Criteria (BIC). Active MMP-9 appeared to contribute the most of any single marker, with active MMP-3 and TIMP-1 making similar contribution to classification and BIC. When active MMP-3 and TIMP-1 were both included, the overall number of correct predictions was similar to that of active MMP-9 alone. However the combination of active MMP-3 and TIMP-1 appeared to be more specific, with more controls correctly classified, and active MMP-9 more sensitive, with more controls correctly classified. When either active MMP-3 or TIMP-1 was included with active MMP-9, incremental improvement in the classification of both cases and controls was provided over the inclusion of active MMP-9 alone. The inclusion of all three markers gave the best overall model, as well as the most sensitive model. With the addition of MMP markers, an absolute increase of nearly 10% of participants were correctly classified over the clinical and demographic factors alone.
Performance of MMP markers in ISR predictive risk models

Table 4.4 Discriminatory capacity of adding elevated MMP markers to clinical and demographic variables

<table>
<thead>
<tr>
<th>MMP marker</th>
<th>AUC</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1, ng/mL</td>
<td>0.63</td>
<td>0.56 – 0.70</td>
</tr>
<tr>
<td>Active MMP-3, ng/mL</td>
<td>0.63</td>
<td>0.56 – 0.69</td>
</tr>
<tr>
<td>Active MMP-9, ng/mL</td>
<td>0.67</td>
<td>0.60 – 0.73</td>
</tr>
<tr>
<td>Risk group</td>
<td>0.74</td>
<td>0.67 – 0.79</td>
</tr>
<tr>
<td>Risk group + TIMP-1</td>
<td>0.76</td>
<td>0.70 – 0.81</td>
</tr>
<tr>
<td>Risk group + active MMP-3</td>
<td>0.76</td>
<td>0.70 – 0.82</td>
</tr>
<tr>
<td>Risk group + active MMP-9</td>
<td>0.79</td>
<td>0.73 – 0.84</td>
</tr>
<tr>
<td>Risk group + active MMP-3 and -9</td>
<td>0.81*</td>
<td>0.75 – 0.86</td>
</tr>
<tr>
<td>Risk group + active MMP-3 and TIMP-1</td>
<td>0.78</td>
<td>0.72 – 0.83</td>
</tr>
<tr>
<td>Risk group + active MMP-9 and TIMP-1</td>
<td>0.80*</td>
<td>0.74 – 0.85</td>
</tr>
<tr>
<td>Risk group + active MMP-3, -9 and TIMP-1</td>
<td>0.82†</td>
<td>0.75 – 0.86</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. risk group. †p < 0.01 vs. risk group. Risk set includes age, sex, diabetes, waist circumference, hs-CRP, CAD severity, ACC/AHA score, stent diameter and length, number of sites stented, and impaired renal function. AUC = area under the curve. When combined with the risk group, all MMP markers are included as cut off points.
**Table 4.5 Classification of ISR cases and controls with different sets**

<table>
<thead>
<tr>
<th></th>
<th>Predicted controls correct</th>
<th>Predicted cases correct</th>
<th>Overall predictions correct</th>
<th>Change overall from risk set</th>
<th>BIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk set</td>
<td>74.0%</td>
<td>59.8%</td>
<td>67.0%</td>
<td>-</td>
<td>357</td>
</tr>
<tr>
<td>Risk set + TIMP-1</td>
<td>72.4%</td>
<td>63.9%</td>
<td>68.3%</td>
<td>1.3%</td>
<td>347</td>
</tr>
<tr>
<td>Risk set + active MMP-3</td>
<td>73.2%</td>
<td>63.1%</td>
<td>68.3%</td>
<td>1.3%</td>
<td>350</td>
</tr>
<tr>
<td>Risk set + active MMP-9</td>
<td>74.8%</td>
<td>67.2%</td>
<td>71.1%</td>
<td>4.1%</td>
<td>338</td>
</tr>
<tr>
<td>Risk set + active MMP-3, TIMP-1</td>
<td>77.2%</td>
<td>63.9%</td>
<td>71.1%</td>
<td>4.1%</td>
<td>343</td>
</tr>
<tr>
<td>Risk set + active MMP-9, TIMP-1</td>
<td>78.0%</td>
<td>72.1%</td>
<td>75.1%</td>
<td>8.1%</td>
<td>334</td>
</tr>
<tr>
<td>Risk set + active MMP-3, -9</td>
<td>79.5%</td>
<td>73.0%</td>
<td>76.3%</td>
<td>9.3%</td>
<td>335</td>
</tr>
<tr>
<td>Risk set + all three markers†</td>
<td>76.4%</td>
<td>77.1%</td>
<td>76.7%</td>
<td>9.7%</td>
<td>322</td>
</tr>
</tbody>
</table>

*Bayesian information criteria, lower numbers indicate better overall set fit. Risk set includes age, sex, diabetes, waist circumference, hs-CRP, CAD severity, ACC/AHA score, stent diameter and length, number of sites stented, and impaired renal function. Results given as percent. † All three markers indicates the number of MMP cut-offs (active MMP-3, MMP-9 and TIMP-1) present.
4.2.3. Performance of previously derived risk models in the retrospective study

The adjusted odds ratios from the PRESTO and ITVR scores in the original publications, and those derived from our New Zealand study population, are provided in Table 4.6 and Table 4.7. The direction of associations and effect sizes between the original studies and the present study were generally similar but differed in the following associations: between ISR and female sex; non-smoking; and hypertension; with all three appearing to be risk characteristics in the original studies, but protective in this New Zealand study population. The reason for this is unclear, but the confidence intervals for all three associations were wide and included the odds ratio for the original study in each case.

Also, in our New Zealand retrospective ISR study the impact of vessel size was much larger than in the original PRESTO study. This is likely due to a small sample size in the reference category (vessel diameter > 4.0mm), as there were only 25 individuals in this study in this category. Both the PRESTO and the ITVR score were moderately discriminative when applied to this study population, with areas under the curve of 0.64 (95% CI 0.58 to 0.69) and 0.63 (95% CI 0.57 to 0.68), respectively. There was no evidence of poor model fit, as indicated by the Hosmer-Lemeshow test giving a non-significant p value.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Original PRESTO publication</th>
<th>NZ retrospective study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC/AHA type C</td>
<td>1.8 (1.3 – 2.6, ( p = 0.001 ))</td>
<td>3.1 (1.7 – 5.8, ( p = 0.0003 ))</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.4 (1.0 – 1.9, ( p = 0.034 ))</td>
<td>2.0 (1.0 – 3.8, ( p = 0.049 ))</td>
</tr>
<tr>
<td>Female sex</td>
<td>1.2 (0.9 – 1.5, ( p = 0.32 ))</td>
<td>0.9 (0.5 – 1.5, ( p = 0.57 ))</td>
</tr>
<tr>
<td>Stent length &gt;20mm*</td>
<td>2.1 (1.3 – 3.2, ( p = 0.001 ))</td>
<td>1.5 (0.9 – 2.5, ( p = 0.16 ))</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>1.4 (1.1 – 1.8, ( p = 0.022 ))</td>
<td>0.9 (0.5 – 1.5, ( p = 0.64 ))</td>
</tr>
<tr>
<td>Vessel size*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 4.0 mm (reference)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5 – 4.0 mm</td>
<td>1.2 (0.6 – 2.5, ( p = 0.68 ))</td>
<td>5.3 (1.6 – 17.4, ( p = 0.006 ))</td>
</tr>
<tr>
<td>3.0 – 3.5 mm</td>
<td>1.4 (0.7 – 2.9, ( p = 0.32 ))</td>
<td>7.2 (2.4 – 21.4, ( p = 0.0004 ))</td>
</tr>
<tr>
<td>&lt; 3.0 mm</td>
<td>1.8 (0.9 – 1.5, ( p = 0.15 ))</td>
<td>7.6 (2.5 – 23.1, ( p = 0.0003 ))</td>
</tr>
</tbody>
</table>

Not included: previous PCI (score 2), unstable angina (score 1).
Modified from Singh et al.518
Sample size original PRESTO study ISR n = 601, controls n = 711; for retrospective study ISR n = 152, controls n = 151.
*Lesion length and diameter in original paper. ACC/AHA = modified American College of Cardiology/American Heart Association score.
Table 4.7 Comparison of ITVR risk score variables and ISR in the original publication and retrospective study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Original publication</th>
<th>Retrospective study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per year</td>
<td>0.99 (0.98 – 0.99, ( p = &lt; 0.0001 ))</td>
<td>0.98 (0.96 – 1.01, ( p = 0.11 ))</td>
</tr>
<tr>
<td>Diabetes mellitus*</td>
<td>1.53 (1.22 – 1.92, ( p = 0.0002 ))</td>
<td>1.80 (0.93 – 3.47, ( p = 0.08 ))</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.23 (1.07 – 1.41, ( p = 0.003 ))</td>
<td>0.83 (0.50 – 1.40, ( p = 0.45 ))</td>
</tr>
<tr>
<td>Lesion length &gt;20mm</td>
<td>1.47 (1.23 – 1.75, ( p = &lt; 0.0001 ))</td>
<td>1.76 (1.00 – 3.10, ( p = 0.049 ))</td>
</tr>
<tr>
<td>Smoker</td>
<td>0.72 (0.61 – 0.85, ( p = 0.0001 ))</td>
<td>1.10 (0.66 – 1.83, ( p = 0.72 ))</td>
</tr>
<tr>
<td>Single lesion treated</td>
<td>0.58 (0.51 – 0.66, ( p = &lt; 0.0001 ))</td>
<td>0.46 (0.24 – 0.85, ( p = 0.014 ))</td>
</tr>
</tbody>
</table>

Modified from Singh et al.\textsuperscript{519}

Sample size original study: target lesion revascularisation n = 1 609, controls n = 9 874; for retrospective study: ISR n = 152, controls n = 151.

*Defined as insulin and/or oral treatment by Singh et al.\textsuperscript{519}
Contribution of MMP markers to previously derived risk models in the retrospective study

The odds ratios for optimum cut off points for active MMPs-3, -9 and TIMP-1 after adjustment for components of the PRESTO and ITVR scores are displayed in Table 4.8. In both of these models the optimum cut off points for active MMPs-3, -9 and TIMP-1 retained robust effect sizes and statistical significance. In the derivation of both the PRESTO and the ITVR scores, the number of points for each variable was assigned from doubling the adjusted odds ratio, and rounding the resulting number. This was also applied to cut off points for active MMPs-3, -9 and TIMP-1.

The resulting discrimination, model fit, and correct classifications after including MMP variables in each model are displayed in Table 4.9. The addition of MMP variables improved model fit, discrimination and classification. It also contributed an absolute increase of correct classification of 8.6% to the PRESTO score and 9.0% to the ITVR score, and improved discrimination by up to an absolute increase of 6% and 7%, respectively. However, it appeared that different combinations of MMP variables gave the best performance in terms of different metrics, and the best combinations were different in each model. In terms of model fit (R² and BIC) the best scores for both the PRESTO and the ITVR scores included all of active MMPs-3 and -9, and TIMP-1. However, in both models the scores that included only active MMP-9, or that which included both active MMPs-3 and -9 gave marginally better discrimination. This did not equate with better prediction of case or control status, the best classification with the PRESTO score included all three markers, and with the ITVR score only active MMPs-3 and -9. Nevertheless, these findings demonstrate that the MMP markers appear to contribute incrementally to the predictive power of previously derived risk models.
### Table 4.8 Contribution of MMP markers to predictive risk models

<table>
<thead>
<tr>
<th></th>
<th>Adjusted OR (95% CI)</th>
<th>Assigned score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRESTO score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active MMP-3, ≥ 3 ng/mL</td>
<td>1.9 (1.1 – 3.4, ( p = 0.03 ))</td>
<td>4</td>
</tr>
<tr>
<td>Active MMP-9, ≥ 2 ng/mL</td>
<td>3.8 (2.1 – 7.0, ( p &lt; 0.0001 ))</td>
<td>8</td>
</tr>
<tr>
<td>TIMP-1, ≥ 220 ng/mL</td>
<td>2.4 (1.3 – 4.3, ( p = 0.005 ))</td>
<td>5</td>
</tr>
<tr>
<td><strong>ITVR score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active MMP-3, ≥ 3 ng/mL</td>
<td>1.87 (1.05 – 3.35, ( p = 0.04 ))</td>
<td>4</td>
</tr>
<tr>
<td>Active MMP-9, ≥ 2 ng/mL</td>
<td>3.72 (2.06 – 6.73, ( p &lt; 0.0001 ))</td>
<td>8</td>
</tr>
<tr>
<td>TIMP-1, ≥ 220 ng/mL</td>
<td>2.15 (1.17 – 3.95, ( p = 0.014 ))</td>
<td>4</td>
</tr>
</tbody>
</table>

Assigned score for the PRESTO and ITVR scores were calculated by multiplying the \( \beta \) coefficients from the multivariate models by two for each of the relevant scores, when active MMPs-3 and -9, and TIMP-1 were included.
### Table 4.9 Contributions of MMP markers to risk models in terms of discrimination, model fit and classification

<table>
<thead>
<tr>
<th>Model</th>
<th>AUC</th>
<th>$R^2$</th>
<th>BIC*</th>
<th>Overall predictions correct</th>
<th>Overall change from original model</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRESTO</td>
<td>0.64 (0.58 – 0.69)</td>
<td>0.10</td>
<td>469</td>
<td>63.2%</td>
<td>–</td>
</tr>
<tr>
<td>PRESTO + TIMP-1</td>
<td>0.64 (0.58 – 0.69)</td>
<td>0.14</td>
<td>409</td>
<td>66.8%</td>
<td>3.6%</td>
</tr>
<tr>
<td>PRESTO + active MMP-3</td>
<td>0.65 (0.59 – 0.70)</td>
<td>0.14</td>
<td>382</td>
<td>69.2%</td>
<td>6.0%</td>
</tr>
<tr>
<td>PRESTO + active MMP-9</td>
<td>0.70 (0.64 – 0.75)†</td>
<td>0.17</td>
<td>407</td>
<td>70.2%</td>
<td>7.0%</td>
</tr>
<tr>
<td>PRESTO + active MMP-3, TIMP-1</td>
<td>0.65 (0.59 – 0.70)</td>
<td>0.15</td>
<td>372</td>
<td>67.2%</td>
<td>4.0%</td>
</tr>
<tr>
<td>PRESTO + active MMP-9, TIMP-1</td>
<td>0.69 (0.64 – 0.74)*</td>
<td>0.21</td>
<td>387</td>
<td>71.2%</td>
<td>8.0%</td>
</tr>
<tr>
<td>PRESTO + active MMP-3, -9</td>
<td>0.70 (0.65 – 0.75)†</td>
<td>0.19</td>
<td>362</td>
<td>70.3%</td>
<td>7.1%</td>
</tr>
<tr>
<td>PRESTO + active MMP-3, -9 and TIMP-1</td>
<td>0.69 (0.64 – 0.74)*</td>
<td>0.21</td>
<td>350</td>
<td>71.8%</td>
<td>8.6%</td>
</tr>
<tr>
<td>ITVR</td>
<td>0.63 (0.57 – 0.68)</td>
<td>0.06</td>
<td>431</td>
<td>61.8%</td>
<td>–</td>
</tr>
<tr>
<td>ITVR + TIMP-1</td>
<td>0.63 (0.57 – 0.69)</td>
<td>0.10</td>
<td>403</td>
<td>62.2%</td>
<td>0.4%</td>
</tr>
<tr>
<td>ITVR + active MMP-3</td>
<td>0.65 (0.59 – 0.70)</td>
<td>0.13</td>
<td>369</td>
<td>64.4%</td>
<td>2.6%</td>
</tr>
<tr>
<td>ITVR + active MMP-9</td>
<td>0.70 (0.65 – 0.75)‡</td>
<td>0.14</td>
<td>399</td>
<td>68.2%</td>
<td>6.4%</td>
</tr>
<tr>
<td>ITVR + active MMP-3, TIMP-1</td>
<td>0.64 (0.59 – 0.70)</td>
<td>0.12</td>
<td>353</td>
<td>64.0%</td>
<td>2.2%</td>
</tr>
<tr>
<td>ITVR + active MMP-9, TIMP-1</td>
<td>0.69 (0.63 – 0.74)†</td>
<td>0.17</td>
<td>381</td>
<td>69.9%</td>
<td>8.1%</td>
</tr>
<tr>
<td>ITVR + active MMP-3, -9</td>
<td>0.70 (0.65 – 0.75)‡</td>
<td>0.17</td>
<td>345</td>
<td>70.8%</td>
<td>9.0%</td>
</tr>
<tr>
<td>ITVR + active MMP-3, -9 and TIMP-1</td>
<td>0.69 (0.64 – 0.74)†</td>
<td>0.18</td>
<td>339</td>
<td>69.2%</td>
<td>7.4%</td>
</tr>
</tbody>
</table>

*$p < 0.05$ compared to basic risk models. †$p < 0.001$ compared to basic risk models. ‡$p < 0.0001$ compared to basic risk model.
4.2.5. Ability of MMP markers to reclassify individuals across score quartiles in previously derived risk models

The ability of addition of MMP variables to reclassify individuals across quartiles of the PRESTO and ITVR scores is displayed in Table 4.10. When MMP markers were added, a greater proportion of controls and a smaller proportion of cases were classified in the lowest quartile of risk score. Additionally, a greater proportion of cases and a smaller proportion of controls were classified in the highest quartile of risk score. Similar effects were seen when MMP markers were included in both the PRESTO and ITVR models. The data did not deviate significantly from the models, as indicated by the non-significant p values with the Hosmer-Lemeshow goodness-of-fit test. When MMP variables were added to each model, around 20% of study participants were correctly classified to a higher quartile, and 20% of study participants were correctly classified to a lower quartile (i.e. those with the disease classified into a higher risk category and those without the disease classified into a lower risk category). However, between 13 to 18% were also inappropriately classified up or down at least one quartile of risk. The net reclassification index (the net proportion of cases and controls with a more appropriate classification after addition of variables to the model) for addition of MMP variables was 10.0% for the PRESTO score and 9.9% for the ITVR score. When only reclassification into or out of quartiles one and four (i.e. those at the highest and lowest calculated risk) was considered, inclusion of MMP variables resulted in a net reclassification index of 7.3% and 8.6%, for the PRESTO and ITVR scores, respectively. This indicates that the inclusion of MMP variables in risk prediction models has potential for reclassifying patients into clinically useful categories (i.e. including MMP variables reclassifies a substantial proportion of patients into very high and very low risk groups). Finally, this ability to reclassify individuals across quartiles of risk score is extrapolated to a hypothetical clinical population in Figure 4.2.
## Table 4.10 Contribution of MMP variables to reclassification across quartiles of ISR risk scores

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>% correctly reclassified*</th>
<th>% incorrectly reclassified*</th>
<th>Net reclassification index %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRESTO score alone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>32.6%</td>
<td>31.5%</td>
<td>21.9%</td>
<td>14.0%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ISR</td>
<td>18.0%</td>
<td>19.1%</td>
<td>27.8%</td>
<td>35.1%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>PRESTO + active MMPs-3, -9 and TIMP-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>34.3%</td>
<td>30.9%</td>
<td>25.3%</td>
<td>9.6%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ISR</td>
<td>16.5%</td>
<td>19.6%</td>
<td>24.7%</td>
<td>39.2%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Reclassification with MMP markers and PRESTO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>up = 20.4%</td>
<td>down = 20.6%</td>
<td>10.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>down = 18.1%</td>
<td>up = 12.9%</td>
<td></td>
</tr>
<tr>
<td>Reclassification with MMP markers and PRESTO to and from quartiles 1&amp; 4 only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>up = 15.5%</td>
<td>down = 13.6%</td>
<td>7.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>down = 8.3%</td>
<td>down = 13.5%</td>
<td></td>
</tr>
<tr>
<td><strong>ITVR score alone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>32.0%</td>
<td>29.8%</td>
<td>24.2%</td>
<td>14.1%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ISR</td>
<td>18.6%</td>
<td>20.6%</td>
<td>25.8%</td>
<td>35.1%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>ITVR + active MMPs-3, -9 and TIMP-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>34.8%</td>
<td>29.2%</td>
<td>27.0%</td>
<td>9.0%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ISR</td>
<td>16.0%</td>
<td>21.1%</td>
<td>23.2%</td>
<td>39.7%</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Reclassification with MMP markers and ITVR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>up = 20.4%</td>
<td>down = 19.9%</td>
<td>9.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>down = 17.5%</td>
<td>up = 12.9%</td>
<td></td>
</tr>
<tr>
<td>Reclassification with MMP markers and ITVR to and from quartiles 1&amp; 4 only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>up = 16.8%</td>
<td>down = 12.3%</td>
<td>8.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>down = 7.6%</td>
<td>down = 12.9%</td>
<td></td>
</tr>
</tbody>
</table>

*The percentage reclassified include the whole study population as the denominator.
Figure 4.2 Reclassification by active MMPs-3 and -9, and TIMP-1 with increasing quartiles of risk model score with estimated ISR risk in a hypothetical population.

Blue = Modified PRESTO score. Red = ITVR score. Light colour indicate risk model alone. Dark colours indicate risk model with addition of cut off points of active MMPs-3 and -9, and TIMP-1 to the model.

Estimate of ISR risk derived from observed proportion of ISR cases in each quartile and an assumed rate of clinical restenosis of 10%.
This figure was generated using the data from Table 4.10, with retrospective study data adjusted to derive a hypothetical population with an average clinical restenosis rate of 10%. For more information on the extrapolation of the study data, see Appendix 11.6. Before MMP variables were added, each quartile of risk score was associated with an increasing risk for ISR. Quartile one was associated with a risk of around 5%; quartile two 7%; quartile three 10% and quartile four 23%. The estimated risk was similar for both the PRESTO and the ITVR scores.

There appear to be two main effects of including MMP variables into these models (Figure 4.2). The first is that the estimated risk of ISR in the lowest quartile of score dropped from ~5% to 2.5%. Secondly, the estimated risk for ISR in the highest quartile rose from ~20% to 33 – 38%. In other words, it appears that the addition of MMP variables to the risk prediction was associated with the separation of the top and bottom quarter of patients with a difference in ISR risk of an order of magnitude.

4.3. Discussion

The main findings of this chapter was that although active MMPs-3, -9 and TIMP-1 were correlated with each other, they were associated with a history of ISR independently of each other, as well as independently of other active MMPs (-1 and -2) and pro-MMP-9. It also appeared that there was incremental value in including elevated levels of active MMPs-3, -9 and TIMP-1, where having greater numbers of different elevated MMP markers gave stronger associations with ISR, and a greater likelihood of multiple ISR lesions.

Although this study was retrospective, and thus MMP measurements were performed in samples from after the time point when the ISR lesion had developed clinically, analysis was performed to test whether MMP variables would improve prediction of ISR in the context of clinical and demographic risk factors, assuming similar alterations in MMP variables could be detected prior to intervention. In the absence of an appropriate, well-validated ISR prediction model, two approaches were utilized to investigate whether MMP variables might contribute to improved ISR risk prediction in the context of previously established clinical and demographic risk factors. The first approach looked at whether the addition of MMP variables to the factors significantly associated with ISR in the retrospective study improved model fitness, discrimination and classification, without using a formal prediction model.
The second approach utilized two formal prediction models, the PRESTO score, which had been previously derived, and the ITVR score, which was derived from effect sizes from a large study. MMP variables appeared to contribute to improved prediction both in the context of risk factors from the retrospective study and when added to the PRESTO and ITVR scores. The addition of active MMPs-3, -9 and TIMP-1 to the two formal risk scores appeared to identify individuals at very low risk and very high risk, with half of the study population having approximated ISR risk either ≤ 2.5% or ≥ 30%.

4.3.1. Complementary roles of active MMPs-3 and -9, and TIMP-1 in the prediction of ISR

Active MMPs-3 and -9, and TIMP-1 were all correlated with each other, but the associations of these variables with ISR all remained significant when all three variables were included in a multiple regression model, without evidence for poor model fit. Thus, despite being correlated, these MMP variables also appeared to be independently associated with ISR. It is likely that MMP variables may be under multiple different regulatory mechanisms, both through biochemical relationships with inflammatory and proteolytic stimuli, and also through mechanistic roles in the development of ISR. Because the activity assays used in this study relied on endogenous activity to participate in a colour-forming reaction, the observed correlations with active MMPs-3, -9 and TIMP-1 was not explained by MMP/TIMP complexes.

Instead, both the observed correlations and the independence of associations between active MMPs-3, and -9 and TIMP-1 with ISR are likely due to differences in roles of these proteins in ISR, and different regulatory and activation mechanisms. The observed results are consistent with multiple inter-related regulatory mechanisms.

Putative roles for each of MMPs-3, -9 and TIMP-1 are discussed in Chapter 3.3.2. Briefly, MMP-9 may be important for VSMC migration and later compaction of the ECM during the maturation of the neointima. MMP-3 is up-regulated in the adventitial layer of injured arteries, but is not associated with migrating VSMC. MMP-3 may have a role in activating other MMPs and cleaving cell-cell attachment proteins, perhaps allowing the initiation of VSMC migration. TIMP-1 appears to have a moderating effect,
with TIMP-1 knock-out accelerating VSMC migration and exacerbating intimal hyperplasia.\textsuperscript{364} Experimental over-expression has the opposite effect, producing the suppression of intimal hyperplasia.\textsuperscript{366,367}

The results of experimental addition of growth factors and enzymes in stimulating the production and activation of MMPs-3, -9 and TIMP-1 are displayed in Table 4.11. While it has been demonstrated that the activation of MMP-9 may occur independently of MMP-3,\textsuperscript{308} MMP-3 is known to activate both MMP-9 and MMP-1.\textsuperscript{283} It appears that MMP-1, -3, and -9 are activated by plasmin, whereas MMP-2 is not.\textsuperscript{283,312} Activation by plasmin may account for some of the increase in active MMPs-1, -3 and -9, which would explain the correlations between the plasma levels of MMP-1 and the levels of -3 and -9. Furthermore, while there are a large number of factors known to both regulate and activate MMPs, plasminogen appears to be important in regulating MMPs in the response to vascular injury (as discussed Chapter 3.3.3) with plasminogen gene knock-out mice overall having reduced intimal hyperplasia.\textsuperscript{305,311,312} While there is some overlap between stimulating factors of MMPs and TIMP-1,\textsuperscript{642} a number of signalling molecules have been shown to have differing effects, such as chemokine (C-C motif) ligand 5 and chemokine (C-X-C motif) ligand 12, which stimulate the production of MMP-9 mRNA and protein levels, but have no effect on TIMP-1.\textsuperscript{643}

Furthermore, some signalling molecules appear to directly stimulate TIMP-1 production while simultaneously down-regulating MMP production. Administration of the anti-inflammatory cytokine interleukin-10 to cell cultures containing tissue macrophages resulted in increased TIMP-1 release and decreased release of MMP-9.\textsuperscript{644} The direct action of MMP activity may also result in increased TIMP production. For example, MMP-9 can activate transforming growth factor-\(\beta\),\textsuperscript{357} which then stimulates TIMP-1 production.\textsuperscript{358} In this way, MMP-mediated TIMP release may act as a physiological braking system, preventing ECM breakdown outside of the control of upstream signalling. One further possibility is that TIMP-1 may be elevated to keep other MMPs, not measured in this study, in check. This could explain how TIMP-1 was correlated with active MMPs-3 and 9 but still independently associated with ISR. The list in Table 4.11 is almost certainly incomplete, but it serves to illustrate that MMPs and TIMPs are regulated by a number of mechanisms, which appear to have both overlapping and mutually exclusive elements, and helps to explain the observed results. One of the consequences of these independent relationships with ISR is that
Table 4.11 Known regulating factors for active MMPs-3 and -9, and TIMP-1

<table>
<thead>
<tr>
<th>MMP-3</th>
<th>Production stimulated</th>
<th>Activated by</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>vascular endothelial growth factor</strong></td>
<td><strong>645</strong></td>
<td>kallikrein&lt;sup&gt;631&lt;/sup&gt;</td>
</tr>
<tr>
<td>interleukin-1α&lt;sup&gt;293&lt;/sup&gt;</td>
<td></td>
<td>plasmin&lt;sup&gt;651&lt;/sup&gt;</td>
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<tr>
<td>interleukin-6&lt;sup&gt;646&lt;/sup&gt;</td>
<td></td>
<td>chymotrypsin&lt;sup&gt;651&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β&lt;sup&gt;647&lt;/sup&gt;</td>
<td></td>
<td>neutrophil elastase&lt;sup&gt;651&lt;/sup&gt;</td>
</tr>
<tr>
<td>PDGF&lt;sup&gt;352&lt;/sup&gt;</td>
<td></td>
<td>APMA&lt;sup&gt;651&lt;/sup&gt;</td>
</tr>
<tr>
<td>prostaglandin F&lt;sub&gt;2&lt;/sub&gt;α&lt;sup&gt;648&lt;/sup&gt;</td>
<td></td>
<td>rat mast cell proteinase&lt;sup&gt;652&lt;/sup&gt;</td>
</tr>
<tr>
<td>histamine&lt;sup&gt;649&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>interleukin-17&lt;sup&gt;650&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td>vascular endothelial growth factor&lt;sup&gt;645&lt;/sup&gt;</td>
<td>MMP-3&lt;sup&gt;656&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β&lt;sup&gt;647&lt;/sup&gt;</td>
<td></td>
<td>trypsin&lt;sup&gt;656&lt;/sup&gt;</td>
</tr>
<tr>
<td>thrombin&lt;sup&gt;294&lt;/sup&gt;</td>
<td></td>
<td>MMP-26&lt;sup&gt;657&lt;/sup&gt;</td>
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<td>APMA&lt;sup&gt;656&lt;/sup&gt;</td>
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<td>nitric oxide&lt;sup&gt;658&lt;/sup&gt;</td>
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<tr>
<td>interleukin-8&lt;sup&gt;290&lt;/sup&gt;</td>
<td></td>
<td>plasmin&lt;sup&gt;659&lt;/sup&gt;</td>
</tr>
<tr>
<td>interleukin-6&lt;sup&gt;646,653&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF&lt;sup&gt;352&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF&lt;sup&gt;296&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour growth factor-α&lt;sup&gt;296&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 40&lt;sup&gt;654&lt;/sup&gt;</td>
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<td>CCL5&lt;sup&gt;643&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>CXCL12&lt;sup&gt;643&lt;/sup&gt;</td>
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</tr>
<tr>
<td>nitric oxide&lt;sup&gt;655&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
<td>TGF-β&lt;sup&gt;358&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td>interleukin-1β&lt;sup&gt;296&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF&lt;sup&gt;296&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour necrosis factor-α&lt;sup&gt;296&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>interleukin-10&lt;sup&gt;644&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>leptin&lt;sup&gt;660&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

CCL5 = chemokine (C-C motif) ligand 5; CD 40 = cluster of differentiation 40; CXCL12 = chemokine (C-X-C motif) ligand 12; GM-CSF = granulocyte-macrophage colony-stimulating factor.
the inclusion of multiple markers was associated with stronger relationships with both the presence of ISR, and the number of ISR lesions. Active MMPs-3 and -9, and TIMP-1 predicted ISR independently of clinical and demographic variables. Neither PRESTO or ITVR include biochemical variables, and perhaps the independent contribution that active MMPs-3 and -9, and TIMP-1 make to ISR prediction derive from these proteins giving an indication of the individual biological healing response. Elevated high sensitivity C-reactive protein has been linked with a modestly increased risk for ISR. While neither the PRESTO nor the ITVR scores included high sensitivity C-reactive protein as a variable, our results indicate that active MMPs-3 and -9, and TIMP-1 are associated with ISR independently of this marker.

### 4.3.2. Inclusion of active MMPs-3 and -9, and TIMP-1 in risk prediction models

The contribution of active MMPs-3 and -9, and TIMP-1 to prediction of ISR in the context of clinical and demographic variables was made in two ways. Firstly, MMP variables were sequentially added to clinical and demographic risk factors that had significant associations with ISR from Chapter 3.2.1. This resulted in improved model fitness and discrimination. Secondly, the MMP variables were considered in the context of previously reported risk classification models for ISR. The inclusion of active MMPs-3 and -9, and TIMP-1 in the PRESTO and ITVR score improved model fitness, discrimination and reclassification.

The overall discrimination as assessed by area under the curve has previously been recommended as a good measure of analysing whether a prediction model is useful. However, reclassification based measures have been promoted as a more sensitive indicator of improved predictive ability, by assessing the ability of an included term to stratify cases and controls over risk categories. For example, in the Women’s Health Study, a prospective cohort of 26,901 initially healthy females followed for ten years, the inclusion of a term for systolic blood pressure added an absolute increase of 1.1% to the area under the curve for a conventional risk model for incident cardiovascular disease. However, the same addition of systolic blood pressure led to more participants being classified into a more appropriate
category, with a net reclassification index of nearly 10%. This was largely due to females who later developed incident cardiovascular disease being classified into higher risk categories.

One modelling study showed that clinically significant improved reclassification was unlikely to occur if there was no increase in discrimination, but that greatly improved net reclassification (i.e. up to 10.0% in the prediction of developing type 2 diabetes) could occur in the context of giving a small (~3%) improvement in area under the curve. Our results suggest that the inclusion of active MMPs-3 and -9, and TIMP-1 could correctly reclassify ~10% of patients with ISR across quartiles of risk score, assuming that similar results could be detected before intervention.

It appears that no studies have yet reported the net reclassification index associated with any biomarker for ISR, although a number of studies have used this index to evaluate both traditional and novel risk factors for the incidence of coronary artery disease. Reclassification measures have been used in PCI-related scores, but only for cardiac mortality with left main PCI and mortality at one year after STEMI. On the other hand, there are many studies utilizing these methods in the primary prediction of cardiovascular disease, which is the role for which these methods were first developed. Our finding that ~10% of patients could be correctly reclassified for ISR was not as good as including age, HbA1c (for people with diabetes only) or lipid markers in the prediction of incident cardiovascular disease (net reclassification index 19.5%, 11.0%, and 13.9% respectively) but were similar to some other conventional risk markers, including systolic blood pressure (8.5%) and HDL (9.8%), and compared favourably to other novel markers such as measurements of circulating progenitor cells (1.5%), high sensitivity C-reactive protein (-3.4 to 12.0%) and aortic pulse wave velocity (5.5%). A family history of premature coronary artery disease led to worse prediction of incident coronary disease, despite a significant and independent association (-2.2%).

However, a number of plaque imaging modalities have recently been reported to give substantial reclassification. Intimal-medial thickness and measures of carotid plaque were identified by ultrasound in the Atherosclerosis Risk In Communities study, a prospective study of 15,792 participants with 15 years of follow up and 1,812 events. The presence of both carotid plaque and intimal thickness in the top quartile was associated with nearly 10
times the risk for incident coronary artery disease (24.7/1,000 vs. 2.9/1,000 person years). When included in a model of conventional risk factors, the addition of intimal medial thickness and measures of carotid plaque were associated with a net reclassification index of 9.9%.^{673}

Recently, coronary calcium scoring was found to improve reclassification for incident coronary disease in the Multi Ethnic Study of Atherosclerosis. This is a prospective study of 6,814 patients without cardiovascular disease, and 209 events during 5.8 years of follow up. The inclusion of coronary artery calcium score improved discrimination by an absolute increase of 5%, and gave a net reclassification index of 25%. The number of participants went on to develop events in the highest risk category nearly doubled when coronary artery calcium was added to the base risk score.^{674}

When ISR risk was estimated, the contribution of including MMP markers appeared to help identify both very low risk, and very high-risk individuals. Inclusion of MMP variables may allow one-quarter of patients to be classified with an ISR risk below 2.5%, and a further one-quarter above at least 30%. While no formal risk cut offs for decision making have been defined, it seems reasonable that patients with an ISR risk of less than 2.5% may represent safe candidates for bare-metal stenting. Patients with a higher risk, particularly if rates of ISR were to be 35% or greater, stand to gain more incremental benefit from drug-eluting stenting or coronary artery bypass grafting.

The ability of high sensitivity C-reactive protein to reclassify people for the primary prevention of cardiovascular disease has been criticized for simply shuffling patients between low risk categories, with little, if any, clinical benefit for the expense.^{218} This did not appear to be the case with active MMPs-3 and -9, and TIMP-1, as their inclusion in the models for ISR contributed to the identification of both very high risk and very low risk groups.

4.3.3. Justification of risk model comparison

The intent of investigating the contribution of MMP variables to conventional risk scores was to determine if these markers could offer any predictive information over and above that readily obtainable by history taking, physical examination and the PCI procedure. However, if these models were indeed not the best available, or correctly applied, then the comparison is a “straw man” and the conclusions may be erroneous.^{193} Furthermore, if the
external model is poorly fitted to the data from this study, then the comparisons could over- or underestimate the contribution of additional markers.\textsuperscript{675} Each of the previously developed models has limitations, both within the model itself and in application to this dataset. The MAHI score requires assessment with a proprietary angina questionnaire and excludes patients with acute myocardial infarction;\textsuperscript{640} the EVENT score was derived for drug-eluting stents.\textsuperscript{641}

However, one score, the PRESTO score was likely the most applicable. The PRESTO trial was a large randomized control trial, which included over 11,000 patients undergoing PCI with coronary stenting between 1999 and 2000. The aim of the PRESTO trial was to test whether the drug tranilast, an anti-inflammatory and VSMC proliferation and migration inhibitor, would reduce death, myocardial infarction, or ischaemia-driven target vessel revascularization. There was no effect of tranilast on this outcome, or any of the secondary outcomes including late loss, as assessed by intravascular ultrasound.\textsuperscript{162} It is likely, that as the PRESTO trial was carried out during a similar time period to the recruitment of this study, clinical practices and PCI technology were similar. However, the PRESTO score was derived for a single lesion only with protocol angiographic restenosis as the outcome and includes scores for diagnosis of unstable angina and previous PCI, which were not included in the data collection for the retrospective study. For this chapter, lesion length and reference vessel diameter were replaced with stent length and diameter in the PRESTO score. There are differences between reference vessel/lesion measurements and stent dimensions. Stent length systematically overestimates lesion length and oversizing with high balloon pressures/stent size may overestimate reference vessel size. However, in the absence of lesion measurements in the retrospective study, stent characteristics were used. This is a reasonable assumption, as the measurements are likely to correlate with each other. Additionally, the original publication of the PRESTO score found that the discrimination of the score for the validation cohort was modest, associated with an area under the curve of only 0.63.\textsuperscript{518}

There were problems with generalizing the PRESTO score, because it was derived from single-lesion PCI only and had poor predictive value. A second score, designated the ITVR score, was utilized. This score was derived from the same study as the PRESTO score,\textsuperscript{162} but instead used the multivariate $\beta$-coefficients for ischaemia-driven target vessel revascularisation, which were reported seperately.\textsuperscript{519} It has previously been shown that ISR
is the predominant driving factor for target vessel revascularisation. In the original publication, the variables only predicted target vessel revascularisation with an area under the curve of 0.66, only slightly better than the PRESTO score. We were unable to include all of the variables that were significant on multivariate analysis, including prior coronary artery bypass grafting, presentation with unstable angina, ostial lesion, left-anterior descending artery procedure and use of atherectomy. While there were a number of differences between the study in this thesis and the study population from which the PRESTO and ITVR scores were derived, the overall discrimination between cases and controls was similar to that of the original studies, with area under the curve of 0.64 and 0.63, respectively. There was also no evidence for poor model fit, indicated by non-significant $p$ values by the Hosmer-Lemeshow statistic. Hence, it appears that despite their limitations, the scores used to compare the inclusion of MMP variables were robust. Of note, the discriminatory power of the scores used in this chapter (both in the original PRESTO and ITVR studies and our own New Zealand study) were similar to that of two other studies, a recently described score for predicting target lesion revascularisation after drug-eluting stenting, which gave an area under the curve of 0.63; and a older score predicting restenosis after balloon angioplasty, which had an area under the curve of 0.62.

Reclassification, and its derivatives, demonstrate the clinical benefit of a novel marker by showing whether its addition alters the risk classification of patients such that it changes clinical management. As there are no formal risk levels for clinical decision making in coronary revascularisation, analysis was performed using quartiles of the calculated risk scores.

The prediction of ISR from variables that were significantly associated with ISR in the retrospective study led to better discrimination than either the ITVR or PRESTO scores (area under the curve 0.74 vs. 0.66 and 0.63, respectively), but this is probably due to overfitting. In larger studies, this may be avoided by utilizing a discovery and a validation cohort, although in practice, investigators often do not attempt this. The method of splitting the cohort was used in the derivation of the PRESTO score, but not the ITVR. Hence, the reported discrimination of that score (area under the curve 0.63) may be an overestimate. Nevertheless, the improvement in discrimination from including MMP variables was comparable in the three models (our own retrospective study 8%, PRESTO 5% and ITVR
6%).

Therefore, despite limitations in the application of the conventional risk scores to the retrospective study, the overall results of discrimination with and without MMP variables were similar, and this comparison is likely to be a good benchmark to test the inclusion of MMP variables.

### 4.3.4. Considerations in biomarker assessment

The aim of the present chapter was to understand whether MMP-related markers may contribute to risk prediction in the context of currently established risk markers.

Thus far it appears that active MMPs-3 and -9, and TIMP-1 may satisfactorily fulfil proposed criteria for the prediction of ISR.\textsuperscript{191,194} Firstly, a number of animal studies have identified MMPs as important pathogenic factors in the development of restenosis after arterial injury (reviewed in Chapter 1.6.7).\textsuperscript{194} In Chapter 3.2.2 it was demonstrated that active MMPs-3 and -9, and TIMP-1 could be detected in the circulation of people who had previously developed ISR, with a strong relationship, independent of clinical and demographic risk factors. In this chapter it was shown that these proteins appear to be associated with ISR independently of each other, and they appear to add information beyond that provided by currently established risk predictors. Importantly, they appear to identify a substantial proportion of the population at very low risk or very high risk of ISR. The measurement of these putative markers is probably acceptable to the general population as it is performed by a process that is currently used in a hospital laboratory environment (\textit{i.e.} enzyme-linked immunosorbent assay). Furthermore, it is possible that biomarkers that can improve prediction of ISR assist in the management of patients. If it were better known who would develop ISR, therapies more resistant to ISR such as drug-eluting stenting and coronary artery bypass grafting could be preferentially chosen.\textsuperscript{678,679}

### 4.4. Limitations

There were a number of limitations to this study. Firstly, this study was retrospective, meaning that the exposure (\textit{i.e.} MMP levels) were measured after the outcome had developed.
Thus it was assumed that MMP levels could be extrapolated to before the PCI procedure. This assumption is tested prospectively in the next chapter.

Active MMPs-3 and -9, and TIMP-1 were correlated with each other. While this may be problematic for regression analysis, there was no evidence for poor model fit. As a result of the relative small size of this study, the data set was not split into training and validation groups. Thus, there was no independence of training and testing for the contribution of MMP markers. This may have led to an overestimate of the benefit of including MMP markers.

The analysis of the contribution of MMP markers to conventional risk factors was limited by the absence of a validated risk model for clinical restenosis. Therefore, MMP markers were tested in the context of a number of risk models. However, not all of the variables included in these models were recorded in the current study and the outcome measures were not identical. Nevertheless, the discrimination for the outcomes in the original publications were similar to that obtained in this study.

Finally, the contribution to ISR risk was extrapolated by multiplying the size of the control group. This was used to give an indication of the risk of ISR in each quartile of risk score. However, any biases that were present in the recruitment of the control group will remain.

4.5. Conclusions

Active MMPs-3 and -9, and TIMP-1 appear to be associated with a history of ISR independently of each other. Furthermore, they appear to have an additive relationship when used to predict the development of ISR, and multiple elevated markers is also associated with multiple ISR lesions. The addition of active MMPs-3 and -9, and TIMP-1 to a number of risk models improved model fitness, discrimination and classification. When active MMPs-3 and -9, and TIMP-1 were included in two previously developed risk models, they appeared to help identify a low and a high risk group, with nearly half the study population having estimated risk of ISR ≤ 2.5% or ≥ 30%.

Addition of MMP markers to clinical and demographic variables may be able to help predict the risk of ISR and could potentially help to appropriately guide revascularisation decisions or direct early follow up.
5. Impact of index clinical presentation on MMPs

5.1. Introduction

MMPs have been linked to vascular disease in a number of divergent ways. Others have previously shown that pro-MMPs are altered with coronary artery disease severity, acute coronary syndrome, myocardial infarction, heart failure and ejection fraction.\textsuperscript{30,437} Hence, there may be multiple variables that alter MMP level at clinical presentation.

This reflects the multiple roles that MMPs have in the pathophysiology of heart disease. ECM remodelling is an important part of atherosclerosis and plaque rupture, with thrombosis leading to acute coronary syndrome and acute myocardial infarction.\textsuperscript{680} The thickness of the fibrous cap covering intra-mural lipid pools is probably the key determinant in the susceptibility of the lesion to rupturing.\textsuperscript{681} MMPs have been demonstrated in the vulnerable “shoulder region” of human plaques\textsuperscript{363} and mouse models with MMP knock in/outs demonstrate divergent roles of MMP species.\textsuperscript{379} This effect of the presence and absence of specific MMPs can have a striking effect on the atherosclerotic phenotype, for example the absence of MMPs-3 and -9 lead to a large increase of collagen layers in the fibrous plaque,\textsuperscript{420} indicating that these MMPs facilitate the degradation of this protective barrier. On the other hand, the absence of MMP-12 leads to smaller lesions but with thinner caps and decreased macrophage content, suggesting that perhaps MMP-12 has an anti-inflammatory role and may assist the development of unstable atherosclerosis.\textsuperscript{420} However, while the function of these MMPs at the tissue level appear to be important for atherogenesis, a number of clinical studies have also demonstrated correlations between measurements of circulating MMPs and various indices of atherosclerotic burden, and clinical presentation with coronary disease.\textsuperscript{429,433,569,682}

Another important confounder may be myocardial dysfunction. There are a number of different patterns of myocardial dysfunction, with different aetiologies.\textsuperscript{437} Acute coronary syndrome and acute myocardial infarction are associated with both reversible\textsuperscript{683} and permanent myocardial dysfunction,\textsuperscript{684} due to both transient ischaemia and necrotic loss of cardiomyocytes. These processes appear to have effects on the myocardium both at the site of
Impact of index clinical presentation on MMPs

ischaemia and also may have effects in distant areas of the myocardium. The main structural consequences of myocardial dysfunction and infarction are cardiomyocyte hypertrophy, increase in other cell types, and increased production of ECM.

After ischaemia, large increases of collagen types I and III are produced by supporting cells both within the infarcted region, as well as in remote areas. Multiple MMPs appear to be involved in remodelling after myocardial infarction.

Important to notice from the perspective of biomarker studies is that as well as changes in tissue levels, circulating concentrations of both collagen degradation products and MMPs have been shown to be altered in permanent myocardial dysfunction and myocardial stunning, and in both systolic and diastolic dysfunction. Some MMPs have been noted to be correlated with various echocardiographic measures.

This chapter examines the relationship between potential confounders, in the realms of cardiovascular disease presentation before PCI, and the levels of active MMP-9 and TIMP-1.

The aims of this chapter were to examine the association of index clinical presentation and circulating active MMP-9 before PCI, and at three- and six-months.

5.2. Results

5.2.1. Description of the prospective study

Of approached potential participants approached, 402 out of 494 were included in the study (81.4%; Figure 5.1). Fifteen declined consent, 64 did not meet eligibility criteria, one sample was lost due to laboratory error and one person died during the PCI procedure. A further 11 patients had PCI attempted but the procedure was unsuccessful and was abandoned. Seven patients were excluded for inflammatory conditions, specifically rheumatoid arthritis, polymyalgia rheumatica, systemic lupus erythmatosus and inflammatory bowel disease. During recruitment of this study, a parallel study randomizing patients undergoing PCI to an anti-inflammatory drug was being undertaken. Inadvertently, a number of patients (n = 16) were included in both studies. When the study was unblinded, those receiving the active drug were excluded (n = 8), but those receiving placebo were retained in this study. At one year follow up four participants were lost to follow up (1.0%), and six withdrew consent (1.5%).
Impact of index clinical presentation on MMPs

Figure 5.1 Study flow diagram for the prospective study
ISR = in-stent restenosis; MMP = matrix metalloproteinases; non-ISR events = non-in-stent restenosis events.
Thirty-five participants developed clinically-symptomatic, angiographically-proven ISR (8.7%) which will be discussed in Chapter 7. Additionally, 30 (7.5%) participants suffered other cardiovascular events; these outcomes will be discussed in Chapter 8. A further 16 participants were not considered eligible for selection as controls due to other events during the follow up year (surgery, n = 8; bony fracture, n = 1; incident heart failure, n = 2; non-cardiac death, n = 5). This left 311 patients who were asymptomatic at one year, who were considered eligible for selection as controls. Ninety-eight patients were randomly selected from this group, stratified by index clinical presentation to both the ISR and the non-ISR cardiac events group.

5.2.2. Description of study population by index clinical presentation

At the time of PCI there were significant differences in the rates of patient-reported hypertension and medications (Table 5.1). Most of this difference was in the stable angina group, with higher rates of hypertension, medication with aspirin, long-acting nitrates, beta-blockers, Ca\(^{2+}\) channel inhibitors, and combination anti-hypertensive therapy when compared to either those with acute coronary syndrome or acute myocardial infarction. The unstable angina group were more likely to be on aspirin therapy than the acute myocardial infarction group but groups were otherwise similar.

Lipid results, and high sensitivity C-reactive protein are displayed in Table 5.2. While there was no difference in reporting of dyslipidaemia, there was a higher rate of prescription of statins in the stable angina group compared to either acute coronary syndrome or those with acute myocardial infarction. There was no difference in lipid markers. There was a trend towards higher levels of high sensitivity C-reactive protein with more acute myocardial infarction, but this was not statistically significant.

Clinical and angiographic factors are displayed in Table 5.3. Because the stable angina group underwent elective procedures, there was a shorter time from hospital admission to PCI in the stable angina group, with most people undergoing revascularisation on the day of admission.
Table 5.1 Demographic variables by index presentation

<table>
<thead>
<tr>
<th></th>
<th>Stable angina n = 39</th>
<th>Unstable angina n = 23</th>
<th>Non-STEMI n = 64</th>
<th>STEMI n = 37</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>64.2 ± 11.2</td>
<td>63.3 ± 9.2</td>
<td>61.6 ± 11.7</td>
<td>62.9 ± 9.7</td>
<td>0.71</td>
</tr>
<tr>
<td>Sex, male</td>
<td>33 (84.6%)</td>
<td>17 (73.9%)</td>
<td>52 (81.3%)</td>
<td>27 (72.0%)</td>
<td>0.55</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>101.2 ± 11.3</td>
<td>102.4 ± 11.3</td>
<td>102.7 ± 12.6</td>
<td>96.7 ± 12.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Ethnicity, NZ European</td>
<td>33 (84.6%)</td>
<td>20 (87.0%)</td>
<td>53 (83.3%)</td>
<td>29 (78.4%)</td>
<td>0.87</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>33 (84.6%)</td>
<td>15 (65.2%)</td>
<td>47 (74.6%)</td>
<td>20 (54.1%)</td>
<td>0.023</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5 (12.8%)</td>
<td>6 (26.1%)</td>
<td>5 (7.9%)</td>
<td>3 (8.1%)</td>
<td>0.11</td>
</tr>
<tr>
<td>eCrCl, &lt; 60mL/min</td>
<td>4 (10.5%)</td>
<td>6 (28.6%)</td>
<td>7 (11.5%)</td>
<td>9 (28.1%)</td>
<td>0.066</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>51.9% ± 17.6%</td>
<td>49.8% ± 10.8%</td>
<td>51.5% ± 13.3%</td>
<td>46.1% ± 9.9%</td>
<td>0.065</td>
</tr>
<tr>
<td>ACE-I/ARB</td>
<td>14 (35.9%)</td>
<td>18 (45.5%)</td>
<td>20 (31.3%)</td>
<td>13 (35.1%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>30 (76.9%)</td>
<td>12 (54.6%)</td>
<td>29 (45.3%)</td>
<td>20 (54.1%)</td>
<td>0.018</td>
</tr>
<tr>
<td>Ca^{2+} antagonist</td>
<td>13 (33.3%)</td>
<td>5 (22.7%)</td>
<td>7 (10.9%)</td>
<td>4 (10.8%)</td>
<td>0.019</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td>19 (48.7%)</td>
<td>7 (31.8%)</td>
<td>13 (20.3%)</td>
<td>4 (10.8%)</td>
<td>0.0011</td>
</tr>
<tr>
<td>Aspirin</td>
<td>37 (94.9%)</td>
<td>19 (86.4%)</td>
<td>33 (51.6%)</td>
<td>21 (56.8%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Number of antihypertensives</td>
<td>3 (7.7%)</td>
<td>5 (21.7%)</td>
<td>28 (43.8%)</td>
<td>15 (40.5%)</td>
<td>0.0092</td>
</tr>
</tbody>
</table>

Results are mean ± standard deviation or number (column percentage). Medications are those prescribed at time of hospital admission. eCrCl = Estimated creatinine clearance, Cockroft-Gault; non-STEMI = non-ST-segment elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction. * p < 0.05 vs. acute coronary syndrome. † p < 0.05 vs. acute myocardial infarction. ‡ p < 0.01 vs. acute coronary syndrome. § p < 0.01 vs. acute myocardial infarction.
<table>
<thead>
<tr>
<th></th>
<th>Stable angina n = 39</th>
<th>Unstable angina n = 23</th>
<th>Non-STEMI n = 64</th>
<th>STEMI n = 37</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of dyslipidaemia</td>
<td>25 (65.8%)</td>
<td>17 (73.9%)</td>
<td>34 (54.0%)</td>
<td>20 (54.1%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Statin</td>
<td>35 (89.7%)*</td>
<td>14 (60.9%)</td>
<td>26 (41.3%)</td>
<td>20 (54.1%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.46 ± 1.16</td>
<td>4.44 ± 0.96</td>
<td>4.78 ± 0.93</td>
<td>4.57 ± 1.19</td>
<td>0.42</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.60 ± 1.13</td>
<td>2.60 ± 0.98</td>
<td>2.89 ± 0.82</td>
<td>2.78 ± 1.05</td>
<td>0.45</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.11 ± 0.35</td>
<td>1.04 ± 0.28</td>
<td>1.05 ± 0.29</td>
<td>1.05 ± 0.32</td>
<td>0.76</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.54 ± 1.01</td>
<td>1.75 ± 0.92</td>
<td>1.83 ± 1.11</td>
<td>1.61 ± 0.67</td>
<td>0.68</td>
</tr>
<tr>
<td>hs-CRP, mg/L†</td>
<td>2.4 (1.0 – 3.7)</td>
<td>2.0 (1.2 – 2.7)</td>
<td>3.8 (1.9 – 5.2)</td>
<td>2.5 (1.4 – 5.9)</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Results are mean ± standard deviation, number (column percentage) or median (interquartile range). Index presentation indicates the state at percutaneous coronary intervention. Medications are those prescribed at time of hospital admission.

* p < 0.0001 compared to either acute coronary syndrome or acute myocardial infarction.

† High sensitivity C-reactive protein levels of greater than 10mg/L excluded (n = 37) due to presumed acute inflammation.

HDL = high density lipoprotein; hs-CRP = high sensitivity C-reactive protein; LDL = low density lipoprotein.
### Table 5.3 Clinical and angiographic variables by index clinical presentation

<table>
<thead>
<tr>
<th></th>
<th>Stable angina (n = 39)</th>
<th>Unstable angina (n = 23)</th>
<th>Non-STEMI (n = 64)</th>
<th>STEMI (n = 37)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAD severity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1VD</td>
<td>16 (41.0%)</td>
<td>6 (26.1%)</td>
<td>25 (39.1%)</td>
<td>25 (39.1%)</td>
<td>0.92</td>
</tr>
<tr>
<td>2VD</td>
<td>13 (33.3%)</td>
<td>11 (47.8%)</td>
<td>24 (37.5%)</td>
<td>24 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>3VD</td>
<td>10 (25.7%)</td>
<td>6 (26.1%)</td>
<td>15 (23.4%)</td>
<td>15 (23.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Time to PCI, days</strong></td>
<td>0 (0 – 0)</td>
<td>2 (1 – 3)</td>
<td>2 (1 – 3)</td>
<td>2 (1 – 4)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Index ACC/AHA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>A</td>
<td>1 (2.5%)</td>
<td>3 (13.0%)</td>
<td>7 (11.1%)</td>
<td>3 (8.1%)</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>11 (28.2%)</td>
<td>4 (17.3%)</td>
<td>17 (27.0%)</td>
<td>9 (24.3%)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>12 (30.8%)</td>
<td>11 (47.8%)</td>
<td>22 (33.3%)</td>
<td>8 (21.6%)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>15 (38.4%)</td>
<td>5 (21.7%)</td>
<td>18 (28.6%)</td>
<td>17 (46.0%)</td>
<td></td>
</tr>
<tr>
<td>Reference diameter, mm</td>
<td>3.1 ± 0.6</td>
<td>3.1 ± 0.6</td>
<td>2.9 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>0.025</td>
</tr>
<tr>
<td>MLD, mm</td>
<td>0.9 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>0.7 ± 0.4</td>
<td>0.7 ± 0.5</td>
<td>0.0028</td>
</tr>
<tr>
<td>Lesion length, mm</td>
<td>22.0 (14.2 – 29.2)</td>
<td>14.7 (11.6 – 24.9)</td>
<td>16.3 (10.0 – 25.9)</td>
<td>18.0 (12.0 – 23.8)</td>
<td>0.14</td>
</tr>
<tr>
<td>Stent diameter, mm</td>
<td>3.0 ± 0.4</td>
<td>3.2 ± 0.5</td>
<td>3.0 ± 0.4</td>
<td>3.1 ± 0.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Stent length, mm</td>
<td>34.0 (18.5 – 48.0)</td>
<td>24 (16.0 – 40.5)</td>
<td>24 (15.0 – 38.0)</td>
<td>24 (17.3 – 37.0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Number of stents</td>
<td>1.7 ± 0.9</td>
<td>1.8 ± 1.5</td>
<td>1.4 ± 0.6</td>
<td>1.6 ± 0.9</td>
<td>0.21</td>
</tr>
<tr>
<td>Diameter stenosis, %</td>
<td>75% (70 – 80%)</td>
<td>65% (60 – 71%)</td>
<td>76% (67 – 85%)</td>
<td>76% (60 – 92%)</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>Event</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>Control</td>
<td>23 (59.0%)</td>
<td>15 (65.2%)</td>
<td>36 (56.3%)</td>
<td>24 (64.9%)</td>
<td></td>
</tr>
<tr>
<td>ISR</td>
<td>9 (23.1%)</td>
<td>5 (21.7%)</td>
<td>16 (25.0%)</td>
<td>5 (13.5%)</td>
<td></td>
</tr>
<tr>
<td>Non-ISR event</td>
<td>7 (17.9%)</td>
<td>3 (13.0%)</td>
<td>12 (18.8%)</td>
<td>8 (21.6%)</td>
<td></td>
</tr>
</tbody>
</table>

Results displayed as mean (± standard deviation), percent (number) and median (interquartile range).

CAD = coronary artery disease; ISR = in-stent restenosis; MLD = minimum luminal diameter; VD = vessels diseased.
While the median time from admission to PCI for ST-elevation myocardial infarction might be expected to be shorter than two days, the study hospital was a tertiary referral center, and 25/37 of the STEMI cases were initially treated with thrombolysis and subsequently referred for definitive revascularisation.

There were significant alterations in angiographic characteristics by clinical presentation. Patients with acute myocardial infarction had smaller minimum luminal diameters. Those with unstable angina had less diameter stenosis and the reference diameter in the NSTEMI group was smaller than other presentations. While diameter stenosis might be expected to be higher in the STEMI group, the majority of patients had undergone thrombolysis at initial presentation. Of those with primary PCI 8/11 had total occlusions.

5.2.3. Association of plasma MMP variables and clinical presentation.

There was no association between the clinical presentation and the index level of either active MMP-9 or TIMP-1. Amongst the STEMI group, active MMP-9 levels were significantly lower amongst those undergoing primary PCI than those undergoing planned, deferred PCI (median 0.24 [IQR 0.02 – 0.46] vs. 0.55 [IQR 0.31 – 0.87] ng/mL, p = 0.013). This remained significant when adjusting for the development of events, age, sex, diastolic dysfunction and waist circumference. One person underwent rescue PCI and had an index active MMP-9 level of 0.92 ng/mL. The primary PCI group had a shorter hospital admission-to-PCI time, with a median (IQR) of intervention on the day of admission (0 – 1 days) compared to the adjunctive PCI group, with a median of three days (2 – 4 days), p = 0.0002. Increasing time from initial hospital admission in the STEMI group was associated with higher active MMP-9, but this is potentially a surrogate measure of having initial thrombolysis then adjunctive PCI. There was no change in TIMP-1 levels by type of revascularization. Nor was there any association between time from hospital admission and levels of active MMP-9 or TIMP-1 with any other clinical presentation.

All clinical presentation groups had an increase of active MMP-9 at three months, but this increase was numerically larger in the STEMI group compared to all other groups (Table 5.4). When the association between index clinical presentation and changes in active MMP-9 was performed in the control group alone to isolate the effects of the development of clinical events, there was a significant increase of active MMP-9 in the STEMI group compared to both stable angina and non-STEMI groups (Table 5.5, Figure 5.2). Changes in
Table 5.4 MMP variables by index presentation with the whole cohort included

<table>
<thead>
<tr>
<th></th>
<th>Stable angina</th>
<th>Unstable angina</th>
<th>Non-STEMI</th>
<th>STEMI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Index active MMP-9, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 39</td>
<td>0.51 (0.14 – 0.88)</td>
<td>0.62 (0.35 – 0.85)</td>
<td>0.53 (0.26 – 0.80)</td>
<td>0.55 (0.27 – 0.83)</td>
<td>0.99</td>
</tr>
<tr>
<td>3-month active MMP-9, ng/mL</td>
<td>0.83 (0.27 – 1.4)</td>
<td>0.93 (0.34 – 1.52)</td>
<td>0.79 (0.37 – 1.21)</td>
<td>0.75 (0.37 – 1.13)</td>
<td>0.73</td>
</tr>
<tr>
<td>6-month active MMP-9, ng/mL</td>
<td>0.59 (0.22 – 0.97)</td>
<td>1.12 (0.66 – 1.58)</td>
<td>0.87 (0.6 – 1.14)</td>
<td>0.79 (0.43 – 1.15)</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Index TIMP-1, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 39</td>
<td>106.0 ± 19.7</td>
<td>104.4 ± 26.6</td>
<td>107.6 ± 27.1</td>
<td>104.2 (± 26.2)</td>
<td>0.83</td>
</tr>
<tr>
<td>3-month TIMP-1, ng/mL</td>
<td>109.4 ± 33.5</td>
<td>100.0 ± 27.4</td>
<td>105.7 ± 27.5</td>
<td>95.9 (± 20.3)</td>
<td>0.26</td>
</tr>
<tr>
<td>6-month TIMP-1, ng/mL</td>
<td>108.1 ± 25.4</td>
<td>105.2 ± 21.7</td>
<td>109.5 ± 30.0</td>
<td>96.7 (± 18.1)</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>3-month active MMP-9 Δ, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.13 (0 – 0.48)</td>
<td>0.28 (0 – 0.69)</td>
<td>0.13 (0 – 0.47)</td>
<td>0.26 (0 – 0.58)</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td><strong>6-month active MMP-9 Δ, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.06 (0 – 0.35)</td>
<td>0.25 (0 – 0.68)</td>
<td>0.12 (0 – 0.41)</td>
<td>0.26 (0 – 0.59)*</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td><strong>3-month TIMP-1 Δ, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4 ± 26.1</td>
<td>-2.1 ± 13.8</td>
<td>-3.1 ± 34.2</td>
<td>-8.7 (± 24.5)</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td><strong>6-month TIMP-1 Δ, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.42 ± 22.8</td>
<td>1.2 ± 19.4</td>
<td>0.92 ± 28.3</td>
<td>-6.8 (± 27.4)</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

Results displayed as mean ± standard deviation, percent (number) and median (interquartile range). Results by Kruskall Wallis and ANOVA. Active MMP-9 = active matrix metalloproteinase-9. TIMP-1 = tissue inhibitor of matrix metalloproteinase-1. Δ = delta, calculated as the index value subtracted from the specified time point.
Figure 5.2 Change in active MMP-9 at three and six months in control group only
With ISR and non-ISR event groups excluded. Error bars indicate 95% confidence interval.
Table 5.5 MMP variables by index presentation for controls only

<table>
<thead>
<tr>
<th></th>
<th>Stable angina (n = 23)</th>
<th>Unstable angina (n = 15)</th>
<th>Non-STEMI (n = 36)</th>
<th>STEMI (n = 24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index active MMP-9, ng/mL</td>
<td>0.62 (0.07 – 0.79)</td>
<td>0.62 (0.50 – 0.87)</td>
<td>0.51 (0.30 – 0.84)</td>
<td>0.53 (0.26 – 0.79)</td>
<td>0.67</td>
</tr>
<tr>
<td>3-month active MMP-9, ng/mL</td>
<td>0.50 (0.52 – 1.19)</td>
<td>0.99 (0.84 – 1.78)</td>
<td>0.67 (0.33 – 1.03)</td>
<td>0.87 (0.67 – 1.59)</td>
<td>0.086</td>
</tr>
<tr>
<td>6-month active MMP-9, ng/mL</td>
<td>0.59 (0.31 – 1.02)</td>
<td>1.20 (0.97 – 1.33)</td>
<td>0.87 (0.54 – 1.11)</td>
<td>0.97 (0.50 – 1.23)</td>
<td>0.22</td>
</tr>
<tr>
<td>Index TIMP-1, ng/mL</td>
<td>108.1 ± 22.4</td>
<td>96.5 ± 42.4</td>
<td>106.3 ± 26.6</td>
<td>102.7 ± 18.4</td>
<td>0.62</td>
</tr>
<tr>
<td>3-month TIMP-1, ng/mL</td>
<td>108.9 ± 30.0</td>
<td>104.4 ± 34.0</td>
<td>106.2 ± 27.2</td>
<td>93.6 ± 21.2</td>
<td>0.25</td>
</tr>
<tr>
<td>6-month TIMP-1, ng/mL</td>
<td>108.5 ± 26.8</td>
<td>107.4 ± 22.3</td>
<td>106.5 ± 25.9</td>
<td>95.4 ± 15.1</td>
<td>0.28</td>
</tr>
<tr>
<td>3-month active MMP-9 Δ, ng/mL</td>
<td>0.08 ± 0.47</td>
<td>0.20 ± 1.39</td>
<td>0.04 ± 0.67</td>
<td>0.69 ± 0.88</td>
<td>0.022</td>
</tr>
<tr>
<td>6-month active MMP-9 Δ, ng/mL</td>
<td>-0.05 ± 0.82</td>
<td>0.44 ± 0.42</td>
<td>0.07 ± 0.73</td>
<td>0.57 ± 1.02</td>
<td>0.049</td>
</tr>
<tr>
<td>3-month TIMP-1 Δ, ng/mL</td>
<td>0.4 ± 23.5</td>
<td>-0.85 ± 15.3</td>
<td>-0.55 ± 31.3</td>
<td>-10.0 ± 20.1</td>
<td>0.47</td>
</tr>
<tr>
<td>6-month TIMP-1 Δ, ng/mL</td>
<td>0.41 ± 25.1</td>
<td>3.2 ± 14.3</td>
<td>-1.9 ± 26.0</td>
<td>-5.9 ± 13.9</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Results displayed as mean ± standard deviation, percent (number) and median (interquartile range). Results by Kruskall Wallis and ANOVA. Active MMP-9 = active matrix metalloproteinase-9. TIMP-1 = tissue inhibitor of matrix metalloproteinase-1. Δ = delta, calculated as the index value subtracted from the specified time point. Only those without ISR nor non-ISer events at one year were included (i.e. controls only).
active MMP-9 with STEMI were independent of left ventricular ejection fraction measured at index. When adjusting for age, sex and waist circumference, the relationship between index STEMI and change in active MMP-9 at three months remained statistically significant. The relationship between STEMI and change in active MMP-9 at three months was independent of left ventricular ejection fraction and diastolic dysfunction (see Chapter 8.2.4 for analysis of active MMP-9 and TIMP-1 with diastolic dysfunction and left ventricular ejection fraction). There was no change in TIMP-1 by index clinical presentation (Table 5.4, Table 5.5).

5.3. Discussion

5.3.1. Impact of index clinical presentation on MMPs

At the time of PCI, levels of active MMP-9 and TIMP-1 were not significantly altered according to clinical presentation, but there was an increase in active MMP-9 over three and six months amongst those who had initially presented with STEMI. Increased levels of active MMP-9 were observed in those initially suffering STEMI who were subsequently referred for definitive revascularisation after initial thrombolysis compared to those who had primary PCI. Specifically designed studies have examined the relationship between MMP-related markers and clinical presentation of coronary artery disease, many of which notably measured MMP levels at specific time points after the onset of symptoms, finding increased levels of some pro-MMPs-2,318,452,433,569,581,682 and TIMP-2.580 The lack of an initial change with presentation may be due to the varying time from symptom onset to measurement because blood samples in this study were taken immediately before PCI. While an increase of active MMP-9 with time from hospital admission (and subsequently symptom onset) was observed in those with STEMI, there was no correlation between time from hospital admission and active MMP-9 for people with any other presentation. Consequently, it may be more likely that the observed increase of active MMP-9 is an effect of thrombolysis itself, possibly due to direct activation by plasmin.310 However, due to the design of this study, a vascular or myocardial origin cannot be definitively ruled out.

There was a sustained alteration of active MMP-9 at three and six months in the STEMI group. This was independent of the ejection fraction at index. Elevated levels of circulating MMPs -2 and -9 have been noted to be elevated in the days after MI,682 and along with pro-MMP-3443 are predictive of the occurrence of heart failure.443 MMP-9 protein was found to be nearly two-fold higher in the myocardium distant to the infarcted region months after
experimental ligation of sheep arteries. Clinical studies suggest that around 10% of the myocardium may be infarcted after STEMI even with contemporary treatment. Acute myocardial infarction has been associated with increasing levels of pro- and active MMP-2 over three weeks. Increased coronary sinus levels of pro-MMPs-2 and -9 which were correlated with brain natriuretic peptide, confirming both the cardiac origin and suggesting regulation by altered myocardial physiology. Inokubo et al. could not confirm that pro-MMP-9 was initially higher in acute myocardial infarction than in controls, but levels of TIMP-1 displayed increasing levels in the myocardial infarction group over three weeks, reaching a peak of approximately double baseline at 10 days. In two studies, levels of pro-MMP-9 trended downwards from the initial measurements the second day, and then remained constant at the last measurement three weeks later. Because of the lack of control measurements in these two studies, it is not clear if these late measurements are normal, but it appears that levels are initially high with presentation of acute myocardial infarction and then decrease over the first day.

One prospective study has tracked the circulating levels of MMPs for six months in a clinical population after myocardial infarction, noting that pro-MMP-9 was associated with increasing left ventricular dilation over this time period. Serial measurements of pro-MMP-3 and total MMP-3 (including pro-, active- and TIMP-bound fractions) showed that while there were no changes over the first two days, levels were elevated on days three and four and appear to remain elevated at three months. Pro-MMP-3 levels before discharge were not correlated with echocardiographic parameters before discharge, but they were predictive of left ventricular ejection fraction at five months, with index levels of pro-MMP-3 correlating with the degree of worsening of left ventricular functioning. While we did not measure left ventricular function at three and six months, sustained MMP-9 activation with myocardial remodelling is the most likely explanation for the observed increase.

A number of groups have sampled biomarkers in blood from different areas of the vascular anatomy, which has allowed insight into the sources and roles of these biomarkers. The locations studied have been liberated debris, thrombectomy specimens, the event-related coronary artery, the coronary sinus, aorta and peripheral venous circulation. One elegant study was able to demonstrate elevated levels of inflammatory markers throughout the heart with unstable angina: not just in the blood draining the event-related artery. They cannulated the great cardiac vein, a tributary of the coronary sinus that selectively drains the left – but not the right – coronary artery.
Patients with right coronary events had elevated inflammatory markers in the great cardiac vein compared to both within individual peripheral measurements and patients undergoing angiography for stable disease. These findings suggest that the inflammatory response is not solely generated in the culprit artery and related vascular bed, but rather inflammatory molecules are released from the myocardium in non-ischaemic areas with acute coronary syndrome. Of note, this effect did not appear to be present when the total and active forms of MMPs-2 and -9 were measured in this way with elevated MMPs in the venous blood draining the territory of the left coronary or circumflex culprit lesion, but normal levels if the lesion was in the right coronary. By examining the difference between two locations, the anatomical source of the biomarker can logically be estimated. The distal blood in the event-related blood probably gives the best indication of the biological milieu within the culprit lesion. Coronary sinus measurements could do this as well, but may be confounded by ischaemic changes in the myocardium – the pre-capillary location of the distal event-related artery obviates this potential confounder. The peripheral venous measurements likely give an indication of the systemic nature of the disease, or a diluted measurement of the specific lesional change. Some groups have also sampled blood from the ascending aorta. The relevance of aortic compared to peripheral venous measurements is less clear. Presumably this is a convenient location to sample from, as the angiography catheter must traverse this area (whereas venous collection would entail an additional separate venepuncture procedure). However, two groups included both peripheral venous and aortic measurements at the same time points. Funayama et al. found similar levels of IL-6 in the peripheral and aortic circulation, but Robertson et al. found that whereas the concentration of pro-MMP-9 was higher in the aorta post-procedurally, the change in venous pro-MMP-9 was not significant. Three possible explanations for this are that this is a chance finding, made more likely due to multiple comparisons; there could be an attenuated level of pro-MMP-9 due to exposure to some factor in the systemic circulation; or finally there is a real difference related to differential expression of proteins into the heart chambers.

Acute coronary syndrome is associated with increased MMP9 gene expression and increased pro-MMP-9 in liberated debris and in thrombus. Levels are elevated above those of more distal regions (e.g. aortic, peripheral circulation) in the same individual, or from the same sampling locations in patients undergoing PCI for stable disease. There was no change in MMP2 gene or pro-MMP-3 expression. These results demonstrate that acute coronary syndromes are associated with altered pro-MMP-9
genetic and protein expression related to the culprit lesion. However, while the invasive measurements made in the coronary sinus and culprit arteries are anatomically close to the culprit lesion, measurements may be confounded by the presence of thrombus. Thrombus has been linked with altered MMPs, with both through the plasminogen activating system and platelet activation and degranulation being associated with the release and activation of MMPs. Hence, these invasive measurements, as well as measurements from thrombectomy specimens and liberated debris may simply be surrogates of the presence of thrombus and activated platelets, rather than leakage of the underlying proteolytic activity in the plaque into the circulation.

Peripheral measurements suggest that pro-MMPs-2, -3, -9, -12, as well as total MMPs -2 and -9 and TIMPs-1 and -2 are higher in patients with coronary artery disease compared to healthy volunteers. Additionally, pro-MMPs -2, -8, -9, -12, and TIMP-2 are higher in the peripheral circulation of those with acute coronary syndrome or myocardial infarction than stable angina. Interestingly, pro-MMP-9 levels were higher with plaque rupture measured by intravascular ultrasound and there was no association of pro-MMPs-2 and -9 with troponin levels measured in paired samples. This perhaps indicates the levels are not directly derived from the ischaemic myocardium. However, other studies could not confirm a relationship of higher pro-MMPs-1, -2, -3, -9, and TIMP-1 in acute coronary syndrome compared to stable angina. The discrepancy is likely due to the relatively small size of the studies, which did not allow them to confirm the findings. The changes were usually in the same direction as the former studies. Changes in MMPs-2, -3 and -9 with plaque rupture are consistent with the observation that these enzymes are elevated in the vulnerable shoulder regions of unstable plaques. In summary, it has been clearly demonstrated by others that pro-levels of MMP-9 are altered in the coronary sinus and pro-MMPs-2, -8 and -9, as well as TIMP-2 are altered in the peripheral circulation with acute coronary syndromes. Pro-MMPs-1 may not have a role in the development of coronary artery disease and plaque rupture. Additionally, levels of pro-MMPs -3 and -9 are up-regulated in the months after acute myocardial infarction and correlate with left ventricular dysfunction. However, the groups that have looked at the association of MMPs and clinical presentation with coronary disease have largely used the pro-form ELISAs, and it is unknown exactly what happens with the active forms. This study design is different to some other studies, measuring MMPs at the time of angiography rather than a fixed time after admission and this may account for there
being no difference in measured active MMP-9 and TIMP-1 between clinical presentations at index.

Patients with stable angina were more likely to report a diagnosis of hypertension and be receiving anti-hypertensive therapy, aspirin, statins and long-acting nitrates. There are two likely explanations for these observations. The first is that as stable angina is not, by definition, an acute presentation, these patients have had previous contact with specialist cardiologists and risk factor management, and only subsequently have come forward for planned procedures. Or secondly, that a previous diagnosis or at least provisional diagnosis of coronary artery disease had been made so cardiovascular drugs had been initiated. The high rates of statins, beta-blockade and aspirin therapy in this patient group is encouraging and suggests a high rate of optimal medical therapy in this patient group.\textsuperscript{707} Those in the unstable angina group were more likely to be prescribed aspirin than those with acute myocardial infarction. This may be due to the unstable angina group having had previous diagnoses of coronary artery disease. However, aspirin therapy has also been linked to presentation with less severe forms of acute coronary syndrome,\textsuperscript{708} perhaps due to a thrombolytic effect.

Nevertheless, the results of the current study suggest that active MMP-9 may change in the months after presentation of STEMI, with levels rising over 3 - 6 months in this group. Levels of active MMP-9 also appear to rise in the unstable angina group over 3 - 6 months, but were unable to confirmed statistically.

\textbf{5.4. Limitations}

As this study was designed to examine the relationships between active MMP-9 and TIMP-1, and the development of events after PCI, it was not optimally designed to detect changes in these proteins according to clinical presentation of atherosclerotic disease. The initial blood sample was taken immediately before proceeding to PCI, resulting in variable times from onset of symptoms to blood sampling. This may have obscured alterations in active MMP-9 and TIMP-1 with clinical presentation. Furthermore, the majority of those with STEMI had undergone thrombolysis in the previous few days. Therefore we could not separate the contributions of time from symptom onset with STEMI, from those of thrombolysis, on the measured concentrations of active MMP-9 and TIMP-1. Finally, it was noted that active MMP-9 increased at three and six months in those with STEMI, and this was attributed to myocardial remodelling. This was not associated with left ventricular ejection
fraction at baseline, but echocardiographic measurements were not available at later time points to compare with three and six month levels of active MMP-9.

5.5. Conclusions

This study demonstrated that index clinical presentation with STEMI was associated with a rise of active MMP-9 over three and six months compared to other presentations. This was independent of clinical and demographic variables, as well as left ventricular ejection fraction at baseline. The association of changes in active MMP-9 and TIMP-1 with cardiovascular events should be adjusted for index clinical presentation with ST-elevation myocardial infarction.
6. Impact of pre-analytical factors on MMPs

6.1. Introduction

Since planning both this study and the retrospective study, a number of papers on the impact of pre-interventional aspects of MMP sampling have been published. Notably, Rouy et al.\textsuperscript{709} suggest that both the inactive protein and the functional activity of MMP-9 decline significantly with long-term storage. This observation has not been confirmed, and seems to sit at odds with other published studies that appear to have successfully measured MMPs many years after sampling.

Additionally, it is well accepted that there is seasonal variation in cardiovascular events,\textsuperscript{710-713} with a preponderance in the winter months. This is likely due, in part, to changes in risk factors,\textsuperscript{714-717} but a number of circulating biomarkers linked to atherosclerosis have also been implicated.\textsuperscript{718,719} However, it is unknown whether MMPs vary by time of year. Such an effect could obscure associations in clinical studies, for example if the duration of sample collection differed for cases and controls, or the increased variability could obscure the relation between the measurement and the outcome of interest.

The aims of this chapter were to assess (1) the impact of seasonality and (2) elapsed storage time at -80°C on assayed concentration of active MMP-9 and TIMP-1.
6.2. Results

6.2.1. Description of the prospective study

A description of the study population for the prospective study is provided in Chapter 5. The prospective study population (n = 163) had a mean age of 62.8 years (SD 10.7), and 78.7% were male. The retrospective study group were described in Chapter 3. The retrospective study population had a mean age of 63.0 years (SD 9.4) and 72.3% were male included in this chapter (n = 303). All participants had angiographically-proven coronary artery disease.

6.2.2. Sample storage time at -80°C and assayed concentrations of MMPs

There was a broad range of storage times at -80°C, with some samples being stored greater than three years, and others less than three months (Figure 6.1A). For the associations of active MMP-9 and TIMP-1, logistic regression clustered by participant code was used to preserve the independence of observations. There was no association between storage time at -80°C and assayed concentration of active MMP-9. Similar results were obtained when only the index sample was included. There was also no association in the whole cohort between the three month delta of active MMP-9 and time elapsed in storage at -80°C (r² = 0.006, p = 0.75).

In the retrospective study (Chapter 3) the storage times ranged from less than three months to as long as two years, but the exact dates of sample storage time for each sample in the retrospective study were not collected. However, the study code (i.e. the unique participant identifying number) was used as ranking variable for storage time (i.e. the lower study codes indicate earlier recruitment and longer storage time) to approximate the distribution of sample storage with time. When this was done there was no effect of storage time on active MMPs-3, -9 or pro-MMP-9 in either the controls or the ISR group of the retrospective study.

There appeared to be a small association between storage time at -80°C and assayed concentration of TIMP-1 (Figure 6.1B), with higher levels in samples with longer storage time. This was no longer significant after adjustment for age and sex, indicating that this is
Figure 6.1 Correlation between measured concentrations of active MMP-9 and TIMP-1, with storage time
A. Correlation of active MMP-9 with time elapsed in storage at -80°C.
B. Correlation of TIMP-1 with time elapsed in storage at -80°C.
From the prospective study population. Results are by multiple regression, clustering for each individual to preserve the independence of observations, with active MMP-9 level transformed by natural logarithm. Each comparison included the whole cohort from the prospective study, including controls, the ISR group and the non-ISR events group. Index and 3- and 6-month follow up data points were included. For the active MMP-9 comparison, n = 426 samples from 163 individuals; for the TIMP-1 comparison, n = 422 samples from 163 individuals.
6.2.3. Sampling month and assayed concentrations of MMPs

In order to test whether there was a seasonal variation in active MMP-9 and TIMP-1 measurements, the measurements of active MMP-9 and TIMP-1 were correlated with the month of sample collection. Samples were collected over a period of three years from February 2007 – February 2010. The index, three- and six-month measurements for the whole cohort for whom MMP measurements were available (controls [n = 98], ISR events group [n = 35] and non-ISR events group [n = 30]) were included. Every month of the year was represented. Logistic regression was clustered by participant code to preserve the independence of observations.

Assayed levels of active MMP-9 and TIMP-1 were similar irrespective of the months of the year the sample was collected in (Figure 6.2A and B respectively). To compensate for the fact that the index, three- and six-month measurements for each individual are not independent of each other, a further analysis with only the index sample was undertaken. Similar results were obtained when only the index sample was included, and when only controls were included. Furthermore, there was no evidence for an association when adjustment for the development of clinical events was included in the multivariate model, or when the analysis was repeated in the control group alone.
Figure 6.2 Association of active MMP-9 and TIMP-1 with month of sampling
A. Association between active MMP-9 measurement and the month of sampling. Results are median and interquartile ranges.
B. Association between TIMP-1 and the month of sampling. Results are mean and 95% confidence interval. Results are by multiple regression, clustering for each individual to preserve the independence of observations, with active MMP-9 level transformed by natural logarithm.
Each comparison included index and 3- and 6-month follow up data points from the whole cohort from the prospective study (controls, ISR group, non-ISR events group). For the active MMP-9 comparison, n = 426 samples from 163 individuals; for the TIMP-1 comparison, n = 422 samples from 163 individuals.
6.3. **Discussion**

6.3.1. **Impact of sample storage time at -80°C on assayed concentrations of MMPs**

There was no association between elapsed storage time at -80°C and assayed concentration of TIMP-1 or active MMP-9. This observation is inconsistent with the findings of Rouy *et al.*,709 who found that after two years of storage at -80°C, total MMP-9 antigen declined to 65% of that measured at one month in a cohort of patients with acute myocardial infarction. This relationship appeared to be asymptotic, reaching 1% of the original assayed concentration at three and a half years. Further, this relationship appeared to extend to the functional activity of MMP-9 assayed by zymography. The level of TIMP-1 appeared to be stable.

We measured the endogenous active levels of MMP-9, and TIMP-1 in samples stored for a wide range of times. Some samples were stored for less than three months, and some samples stored for three years, yet there was no evidence for a dramatic decline in either the pro- or active- form of MMP-9, as seen by Rouy *et al.*709 The pro-form of MMP-9 also appeared to be stable in the retrospective study.

Souza-Tarla *et al.*338 examined the effect of freeze-thaw cycles and storage temperature on the stability of pro-MMPs-2 and -9, and the zymographic activity of MMP-9, finding that there was no significant difference between MMPs stored at -20°C for one month and those at -80°C. Furthermore, all MMP levels tested remained constant for up to five freeze-thaw cycles, indicating that MMP levels are quite stable. Fainardi *et al.*502 suggest that from the findings of Souza-Tarla *et al.* and Rouy *et al.*, that storage for up to 36 months cannot be considered inappropriate, as MMPs appear to be freeze-thaw and temperature stable, and are apparently stable from three months to at least two years.502 While a number of groups have examined circulating MMPs in various conditions, many over long periods of time, there is no direct confirmation of the findings of Rouy *et al.* Furthermore, there is little corroborating evidence, in fact reports in the literature have been highly variable. A number of reports in which samples had been stored for around two years found that pro-MMP-9 in EDTA plasma read between 20ng/mL and 70ng/mL.571,720,721 These measurements are low compared to the report of Jung *et al.*503 as they attempted to catalogue the response of different MMPs to
Impact of pre-analytical factors on MMPs

anticoagulants. This could been seen as consistent with a decline with storage, but this relies on assuming that Jung et al. did not store their plasma for a significant amount of time. Perhaps the most informative corroborating report of a decrease of pro-MMP-9 were the efforts of a single group, who showed a halving in pro-MMP-9 concentration in samples from patients with breast cancer after around three years of storage compared to healthy controls at around two years of storage, although this is not properly controlled and the observed effect could equally be from the different disease status.

On the other hand, a number of reports suggest that at least some MMPs are stable in storage. Kisand et al. examined the rate of degradation of MMP-7 and TIMP-1 when stored at a range of temperatures. They utilized the Arrhenius equation, which describes the temperature dependence of the rate of chemical reaction, to model the decay of these analytes. While MMP-7 was sensitive to temperature, they found it was remarkably stable when stored at -75°C, predicting that the recovery would remain >90% for tens of thousands of years. However, while TIMP-1 was more stable at room temperature than MMP-7, it would degrade to 90% of the baseline measure by 17 months.

A number of long-term studies have examined the role of MMPs in the prediction of cardiovascular events. While most studies have not precisely reported the time from beginning of blood storage to assay, it appears that readings of various MMPs in serum may remain high, without evidence of degradation. A number of groups have reported high pro-MMP-9 (>500ng/mL) levels in serum for greater than three, and up to 16 years, whereas other groups have found much lower levels with less storage time. However, in one large study reporting the median sample storage time as 4.1 years, the pro-MMP-9 concentration among the control group at this time point was 47.8ng/mL. This indicates that pro-MMP-9 levels do not drop to near zero, as reported by Rouy et al.

Thus it is clear that many variables alter the assayed concentration of MMPs, many of which have not been discovered until the advent of MMP specific ELISA, which allow accurate quantification of levels in biological samples. While it has been reported that pro-MMP levels progressively decline over years at -80°C in citrate samples indicating lability, others have suggested that they are resistant to change with multiple freeze-thaw cycles and storage at -20°C, indicating stability.

There were a number of limitations to the findings of Rouy et al. Their findings were exclusively in citrate samples, which have been associated with a decrease in a number of
MMPs, including pro- and active- MMP-9,\textsuperscript{490} and as discussed previously, citrate chelates Zn\textsuperscript{2+}, which is needed for MMP functioning. Together, these points raise the question as to whether the observed effects are citrate-dependent. Rouy \textit{et al.} used an activity ELISA, but saw no endogenous MMP-9 activity at any time point. The reason for this is unclear, but it may be due to the citrate anticoagulant chelating Zn\textsuperscript{2+} ions essential for MMP activity, or lower sensitivity with an older assay. While the authors conclude that there is no endogenous circulating MMP activity, this is disputed in the literature\textsuperscript{501,615,724} and in our own findings. Rouy \textit{et al.} saw very high levels of pro-MMP-9 at baseline. Such high levels have previously only been reported in serum.\textsuperscript{40,401,721,725} It may be that this relates to a prolonged blood-drawing-to-centrifugation time, or that levels in other studies have always been measured after some degradation has occurred. The results of Fainardi \textit{et al.}\textsuperscript{502} are purportedly generated immediately after blood drawing, and their results in heparin are similar to ours after two years storage.

Of note, in this thesis active MMPs were measured in heparin and pro-MMP-9 and TIMP-1 in EDTA samples, whereas Rouy \textit{et al.} used citrate samples. Additionally, while Rouy \textit{et al.} used an MMP-9 activity ELISA assay (RPN 2630, Amersham Biosciences [Amersham Biosciences was subsequently taken over by GE Healthcare Life Sciences]), which is no longer made, and is different to the product used to measure active MMP-9 in these studies (RPN 2634, GE Healthcare Life Sciences). The specific differences between these two assays are unclear, but notably, Rouy \textit{et al.} were unable to detect endogenous activity in any sample.

Furthermore, it is known that citrate, the anticoagulant used by Rouy \textit{et al.}, has biological effects on MMP concentrations,\textsuperscript{490} therefore degradation of MMP-9 may be restricted to this anticoagulant. Significantly, there is some data to suggest that the endogenous activity levels are stable over time, as evidenced by similar levels immediately after sampling,\textsuperscript{502} and between two-to-five years for serum.\textsuperscript{726}

In summary, it is clear that the reported levels of pro-MMP-9 are stable from around three months until at least two years,\textsuperscript{709} and the fact that high levels have been seen at least ten years after blood sampling in serum\textsuperscript{401} (and moderate levels at least four years after blood sampling in EDTA\textsuperscript{398}) disputes the observation by Rouy \textit{et al.} that levels drop to 1\% after two years. Many studies have shown consistent positive results, indicating that even if there is a decrease in concentrations during long-term storage, they may still reflect \textit{in vivo} biology.
Impact of pre-analytical factors on MMPs

This is explained by Rouy et al.\textsuperscript{709} by the fact that a geometric decrease in MMP levels should be similar for both cases and controls, although they point out that asymmetrically recruited studies may have erroneous results.

There is little corroborating evidence for the findings of Rouy et al., although as discussed, there is some evidence for a decrease in MMP-9 in EDTA samples. It is unclear what effect this may have on the active endogenous fraction of MMPs, or indeed, other pro-MMPs. However, there was no evidence for a systematic decrease of active MMP-9, TIMP-1 or pro-MMP-9 in either the retrospective or the prospective dataset.

6.3.2. Impact of sampling month on assayed concentrations of MMPs

There was no interaction between month of sampling and assayed concentration of either active MMP-9 or TIMP-1 in the prospective dataset. In the southern hemisphere the winter months are June – August, and to be in line with the changes seen in cardiovascular risk variables\textsuperscript{718} it was hypothesised that higher levels of active MMP-9 would be seen through these months. However, there appears to be no effect. The recruitment for the retrospective study was mostly carried out between February – July so this analysis was not performed in the retrospective study.

There is variation in the timing of vascular events by time of year, including stroke,\textsuperscript{712,727-730} cardiovascular disease,\textsuperscript{710,711,727,729,731,732} stent thrombosis\textsuperscript{733} and sudden death\textsuperscript{713} and generally speaking, the risks of vascular events are higher in the winter and lower in the summer.\textsuperscript{710,712,713,727-732} While this is undoubtedly partly due to alterations in blood pressure,\textsuperscript{714} glycaemic control\textsuperscript{715} and lipids,\textsuperscript{716,717} other circulating markers such as inflammatory mediators have also been noted to vary markedly in the same timeframes. These markers include fibrinogen,\textsuperscript{718} D-dimer,\textsuperscript{718} tissue-plasminogen activator,\textsuperscript{718} von-Willebrand factor,\textsuperscript{718} interferon-\textgreek{},\textsuperscript{719} tumour necrosis factor-\textgreek{},\textsuperscript{719} interleukin-1\textgreek{},\textsuperscript{719} interleukin-12,\textsuperscript{719} soluble CD 40 ligand.\textsuperscript{734} In contrast, levels of C-reactive protein appear to be stable.\textsuperscript{718}

The seasonal change in circulating MMPs has not been assessed in other studies, and while it is still possible that part of the seasonal variation in atherosclerotic events is explained by variation in MMP activity, this does not appear to be reflected in the circulating concentration.
6.4. Limitations

The main limitations of this study will be discussed within the context of each study finding. This study aimed to identify whether important degradation of active MMP-9 and TIMP-1 occurred during storage at -80°C. Secondly, this study aimed to determine whether there was seasonal variation in levels of active MMP-9 and TIMP-1.

There were three limitations in the design of the experiment testing the relationship of storage time at -80°C and seasonal variation to assayed concentrations of active MMP-9 and TIMP-1. The first is that optimally this experiment would be performed utilizing the same subjects with repeated blood samples across the range of the variable of interest. However, it is unlikely that individual variation in these makers completely obscured an important relationship.

Secondly, the inclusion of up to three data points (representing six months of time) from each individual in the study compromised the statistical assumption of non-independence. In order to preserve independence, two measures were put in place. Firstly, logistic regression clustering for individual identifying study code was used. Secondly, the analyses were repeated including only the index active MMP-9 and TIMP-1 measurements.

Finally, including the whole cohort in these analyses meant that some individuals developed cardiovascular events, a potential confounding variable. Therefore, the development of events was included in the multivariate model, and the analysis also repeated in the control group alone.

6.5. Conclusions

This study demonstrated that was no evidence of degradation of active MMP-9 and TIMP-1 with up to three years of storage at -80°C. Nor was there any evidence of seasonal variation of active MMP-9 and TIMP-1. Active MMP-9 and TIMP-1 appear to be robust in storage at -80°C for a number of years, so studies of these proteins need not be limited by a shorter storage time. Active MMP-9 and TIMP-1 do not appear to have a seasonal variation, indicating both that the season in which the blood sample is taken is not a confounder of assayed level and that alterations in active MMP-9 and TIMP-1 may not be an important factor in the seasonal differences in cardiovascular disease incidence.
7. Prospective association of MMP markers and the development of ISR

7.1. Introduction

Alteration in the expression of MMPs is one of the key concepts in the development of atherosclerosis and the complications thereof. Central to the development of ISR is smooth muscle cell migration to the intimal layer and ECM formation.

While there has been much interest in the role of MMPs, in vascular disease, it may be the relatively under-investigated active fraction that is particularly meaningful. A sensitive ELISA-based assay for quantifying endogenous MMP activity is available. MMP function is regulated at a number of levels, and conventional ELISA recognises only the pro- and total-forms. Furthermore, zymographic techniques for assessing activity are known to interfere with TIMP binding. Hence, the insight conventional techniques allow into in vivo MMP biology may be limited.

We previously reported that elevations of active MMPs -3 and-9 as well as TIMP-1 were associated with a history of ISR in a clinically stable population. Katsaros et al. reported that pre-interventional active MMP-9 and early post-interventional elevations of active MMPs -2 and -9 were associated with the development of ISR in patients with stable angina undergoing treatment with drug eluting stents. As described in Chapter 5, multiple studies have linked clinical presentation with acute coronary syndromes and myocardial remodelling with changes in MMPs. One of the design features of this study was to include patients with acute coronary syndromes, in order to detect whether this would have an impact on the ability of MMP markers to predict ISR. There were two main aims to the study detailed in this chapter, (1) whether pre-interventional circulating active MMP-9 and TIMP-1 levels were predictive of the development of ISR and (2) whether post-interventional changes in circulating active MMP-9 and TIMP-1 at 3- and 6- months after PCI reflected the development of ISR.
7.2. Results

7.2.1. Description of the control population

The clinical and demographic descriptors comparing the randomly selected control group to the larger eligible cohort are displayed in Table 7.1. There was no significant difference between the two groups in terms of age, sex, ethnicity or index clinical presentation.

<table>
<thead>
<tr>
<th></th>
<th>Not included in analysis n = 213</th>
<th>‘Control sub-group’ n = 98</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63.5 ± 11.8</td>
<td>61.0 ± 10.1</td>
<td>0.75</td>
</tr>
<tr>
<td>Sex, male</td>
<td>159 (74.0%)</td>
<td>76 (77.6%)</td>
<td>0.33</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>101.0 ± 12.4</td>
<td>98.7 ± 12.4</td>
<td>0.73</td>
</tr>
<tr>
<td>Ethnicity*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ European</td>
<td>184 (86.4%)</td>
<td>77 (79.4%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Māori/Pacific Island</td>
<td>7 (3.3%)</td>
<td>5 (5.2%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>22 (10.3%)</td>
<td>15 (15.5%)</td>
<td></td>
</tr>
<tr>
<td>Index clinical presentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable angina</td>
<td>53 (24.8%)</td>
<td>24 (24.2%)</td>
<td>0.55</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>19 (8.9%)</td>
<td>14 (14.1%)</td>
<td></td>
</tr>
<tr>
<td>Non-STEMI</td>
<td>83 (38.8%)</td>
<td>37 (37.4%)</td>
<td></td>
</tr>
<tr>
<td>STEMI</td>
<td>59 (27.6%)</td>
<td>24 (24.2%)</td>
<td></td>
</tr>
</tbody>
</table>

Results displayed as mean (± standard deviation), and number (%). NZ European = New Zealand European; Non-STEMI = Non-ST-segment elevation myocardial infarction; STEMI = ST-segment elevation myocardial infarction.

7.2.2. Description of clinical population

Of those approached, 432 out of 494 were included in the study (87.5%; see Figure 5.1 Study flow diagram for prospective study). Fifteen declined consent, 45 did not meet eligibility criteria, one sample was lost due to laboratory error and one person died during the PCI procedure. At one year follow up, 35 participants developed angiographically-proven ISR (8.1%). A group of 98 non-ISR participants were selected as ‘controls’ for active MMP-9 and TIMP-1 assessment. These were frequency matched by index cardiac presentation. At baseline, patients with ISR were older, had significantly larger waist circumference and BMI (Table 7.2). There were no other significant differences in the clinical and demographic variables. There was no difference in medication between groups. The predominant ethnicity
was New Zealand European, with a minority being Māori, Pacific Island or “other.” There was a trend towards more patients with renal impairment in the ISR group.

Table 7.2 Baseline demographic variables stratified by one-year outcome

<table>
<thead>
<tr>
<th></th>
<th>Control n = 98</th>
<th>ISR n = 35</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>61.0 ± 10.1</td>
<td>66.8 ± 10.3</td>
<td>0.0047</td>
</tr>
<tr>
<td>Sex, male</td>
<td>76 (77.6%)</td>
<td>26 (74.3%)</td>
<td>0.82</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>98.7 ± 12.4</td>
<td>107.9 ± 11.8</td>
<td>0.0002</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.7 ± 4.9</td>
<td>30.1 ± 5.2</td>
<td>0.016</td>
</tr>
<tr>
<td>Ethnicity*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ European</td>
<td>77 (79.4%)</td>
<td>29 (82.9%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Māori/Pacific Island</td>
<td>5 (5.2%)</td>
<td>4 (11.4%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>15 (15.5%)</td>
<td>2 (5.7%)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.69 ± 1.05</td>
<td>4.39 ± 0.99</td>
<td>0.18</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.69 ± 0.73</td>
<td>1.84 ± 1.41</td>
<td>0.45</td>
</tr>
<tr>
<td>LDL</td>
<td>2.85 ± 0.92</td>
<td>2.53 ± 0.78</td>
<td>0.12</td>
</tr>
<tr>
<td>HDL</td>
<td>1.07 ± 0.28</td>
<td>1.02 ± 0.35</td>
<td>0.45</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>3.8 (1.4 – 13.9)</td>
<td>7.5 (2.4 – 16.0)</td>
<td>0.21</td>
</tr>
<tr>
<td>Hypertension</td>
<td>68 (70.1%)</td>
<td>25 (71.4%)</td>
<td>0.88</td>
</tr>
<tr>
<td>Diabetes</td>
<td>14 (14.4%)</td>
<td>4 (11.4%)</td>
<td>0.78</td>
</tr>
<tr>
<td>Pack years</td>
<td>10.4 (0 – 26.0)</td>
<td>10.0 (0 – 34.5)</td>
<td>0.46</td>
</tr>
<tr>
<td>eCrCl &lt; 60mL/min</td>
<td>11 (14.1%)</td>
<td>10 (29.4%)</td>
<td>0.067</td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td>32 (33.0%)</td>
<td>14 (40.0%)</td>
<td>0.54</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>52 (53.6%)</td>
<td>19 (54.3%)</td>
<td>0.94</td>
</tr>
<tr>
<td>Ca²⁺ antagonist</td>
<td>13 (13.4%)</td>
<td>9 (25.7%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Statin</td>
<td>58 (59.8%)</td>
<td>20 (57.1%)</td>
<td>0.79</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td>22 (22.7%)</td>
<td>13 (37.1%)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Results displayed as mean (± standard deviation), number (%) and median (interquartile range).

* Chi-square test for trend. ACE-I = Angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; BMI = body mass index; eCrCl = estimated creatinine clearance, Cockraft-Gault model; HDL = High-density lipoprotein; hs-CRP = high-sensitivity C-reactive protein.

As the groups used for MMP assessments were frequency matched by presentation, there was no difference in clinical presentation (Table 7.3). However, ISR patients had significantly longer lesion length and total stent length implanted.
Table 7.3 Baseline clinical variables stratified by one-year outcome

<table>
<thead>
<tr>
<th></th>
<th>Control n = 98</th>
<th>ISR n = 35</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Index presentation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable Angina</td>
<td>23 (23.5%)</td>
<td>9 (25.7%)</td>
<td>0.61</td>
</tr>
<tr>
<td>Unstable Angina</td>
<td>15 (15.3%)</td>
<td>5 (14.3%)</td>
<td></td>
</tr>
<tr>
<td>NSTEMI</td>
<td>36 (36.7%)</td>
<td>16 (45.7%)</td>
<td></td>
</tr>
<tr>
<td>STEMI</td>
<td>24 (24.5%)</td>
<td>5 (14.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>CAD severity</strong></td>
<td></td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>1VD</td>
<td>45 (45.9%)</td>
<td>10 (28.6%)</td>
<td></td>
</tr>
<tr>
<td>2VD</td>
<td>31 (31.6%)</td>
<td>16 (45.7%)</td>
<td></td>
</tr>
<tr>
<td>3VD</td>
<td>22 (22.5%)</td>
<td>9 (25.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Time to PCI, days†</strong></td>
<td>1 (0 – 3)</td>
<td>1 (0 – 2)</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Index ACC/AHA score</strong></td>
<td></td>
<td></td>
<td>0.069</td>
</tr>
<tr>
<td>A</td>
<td>11 (11.2%)</td>
<td>2 (5.7%)</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>29 (29.6%)</td>
<td>4 (11.5%)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>31 (31.6%)</td>
<td>13 (37.1%)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>27 (27.6%)</td>
<td>16 (45.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Reference diameter, mm</strong></td>
<td>3.06 ± 0.62</td>
<td>2.93 ± 0.41</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>MLD, mm</strong></td>
<td>0.72 ± 0.43</td>
<td>0.83 ± 0.46</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Diameter stenosis, %</strong></td>
<td>76.1 ± 13.4</td>
<td>71.3 ± 15.7</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Lesion length, mm</strong></td>
<td>16.8 (11.1 – 26.6)</td>
<td>21.0 (14.2 – 38.3)</td>
<td>0.043</td>
</tr>
<tr>
<td><strong>Stent diameter, mm</strong></td>
<td>3.07 ± 0.46</td>
<td>2.96 ± 0.35</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Stent length, mm</strong></td>
<td>24.0 (16.0 – 36.0)</td>
<td>36.0 (18.5 – 57.5)</td>
<td>0.0024</td>
</tr>
<tr>
<td><strong>Number of stents</strong></td>
<td>1.52 ± 0.8</td>
<td>1.83 ± 1.04</td>
<td>0.083</td>
</tr>
<tr>
<td><strong>Ejection fraction, %</strong></td>
<td>47.5 ± 10.9</td>
<td>53.8 ± 9.4</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Results displayed as mean (± standard deviation), number (%) and median (interquartile range). * Chi^2 test for trend. † Time to PCI in days from initial hospital admission for the initial symptomatic episode.

ACC/AHA = modified American College of Cardiology/American Heart Association score; ACE-I = angiotensin converting enzyme inhibitor; CAD = coronary artery disease; MLD = minimal luminal diameter; NSTEMI = non-ST elevation myocardial infarction; STEMI = ST elevation myocardial infarction; VD = vessels diseased.
7.2.3. Description of restenosis presentation

The median time to the development of ISR symptoms was 138 days, with 66% of patients presenting with an acute coronary syndrome (Table 7.4). Lesional characteristics are also displayed. There was no difference in Mehran score, time to symptom recurrence or number of ISR lesions for those with unstable ISR presentations.

Table 7.4 Characteristics of ISR presentation for the prospective cohort

<table>
<thead>
<tr>
<th>ISR group</th>
<th>n = 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to symptom recurrence, days</td>
<td>138 (90 – 189)</td>
</tr>
<tr>
<td>Presentation with ISR</td>
<td></td>
</tr>
<tr>
<td>Stable angina</td>
<td>12 (34.3%)</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>7 (20.0%)</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>16 (45.7%)</td>
</tr>
<tr>
<td>STEMI</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mehran score*</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16 (45.7%)</td>
</tr>
<tr>
<td>II</td>
<td>6 (17.1%)</td>
</tr>
<tr>
<td>III</td>
<td>5 (14.3%)</td>
</tr>
<tr>
<td>IV</td>
<td>8 (22.9%)</td>
</tr>
<tr>
<td>Number of ISR lesions</td>
<td>1.42 ± 0.55</td>
</tr>
<tr>
<td>% ISR stenosis</td>
<td>85% (70% – 99%)</td>
</tr>
</tbody>
</table>

Results are mean ± standard deviation, median (interquartile range) or number (percent).
*The Mehran score was derived from the lesion with the greatest percent stenosis. NSTEMI = non-ST elevation myocardial infarction; STEMI = ST elevation myocardial infarction.

7.2.4. Changes in plasma active MMP-9 but not TIMP-1 levels predict the development of ISR

Plasma active MMP-9 and TIMP-1 were not significantly different between the ISR and control groups at any time point (Table 7.5) However, when the change in each group was considered, the increase of active MMP-9 from index to three-month measurement in those who developed ISR was significantly larger than the control group (Figure 7.1A). Similar results were obtained by natural logarithm transformation of the index and three month active MMP-9 variables and inclusion of the index value as a covariate. There was no difference in change of active MMP-9 between controls and the ISR group at six months, and the change at three months was similar between those who would present with ISR before and after the
Table 7.5 Plasma active MMP-9 and TIMP-1 at index, 3- and 6 months by outcome at one year

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISR</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 98*</td>
<td>n = 35*</td>
<td></td>
</tr>
<tr>
<td>Index active MMP-9</td>
<td>0.59 (0.28 – 0.81)</td>
<td>0.56 (0.42 – 0.75)</td>
<td>0.67</td>
</tr>
<tr>
<td>3-month active MMP-9</td>
<td>0.82 (0.39 – 1.27)</td>
<td>1.10 (0.55 – 1.76)</td>
<td>0.14</td>
</tr>
<tr>
<td>6-month active MMP-9</td>
<td>0.87 (0.49 – 1.18)</td>
<td>1.04 (0.28 – 1.45)</td>
<td>0.59</td>
</tr>
<tr>
<td>3-month Δ active MMP-9</td>
<td>0.24 ± 0.83</td>
<td>0.63 ± 0.92</td>
<td>0.03</td>
</tr>
<tr>
<td>6-month Δ active MMP-9</td>
<td>0.20 ± 0.83</td>
<td>0.26 ± 0.63</td>
<td>0.79</td>
</tr>
<tr>
<td>Index TIMP-1</td>
<td>105.7 ± 24.0</td>
<td>122.2 ± 28.3</td>
<td>0.16</td>
</tr>
<tr>
<td>3-month TIMP-1</td>
<td>103.5 ± 27.6</td>
<td>102.6 ± 29.3</td>
<td>0.89</td>
</tr>
<tr>
<td>6-month TIMP-1</td>
<td>104.4 ± 23.8</td>
<td>107.0 ± 32.0</td>
<td>0.70</td>
</tr>
<tr>
<td>3-month Δ TIMP-1</td>
<td>-2.8 ± 2.7</td>
<td>-8.6 ± 5.5</td>
<td>0.30</td>
</tr>
<tr>
<td>6-month Δ TIMP-1</td>
<td>-1.7 ± 2.4</td>
<td>-8.1 ± 10.4</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Results displayed as median (interquartile range) or mean ± standard deviation. All active MMP-9 and TIMP-1 measurements are ng/mL. Results by Mann Whitney U and unpaired t-test. Three- and six-month change were calculated by subtracting each individuals index measurement from the three- and six-month values, respectively. *n displayed is for index measurement. n for the three- (control = 91, ISR = 30) and six- (control = 85, ISR = 17) month measurements decreased due to missing values and censoring due to ISR presentation. Active MMP-9 = active matrix metalloproteinase-9; TIMP-1 = tissue inhibitor of matrix metalloproteinases-1.
Figure 7.1 Alteration of active MMP-9 at three- and six- months after PCI by index presentation and development of ISR
Changes in level of active MMP-9 at three- and six- months were calculated by subtracting the index measurement from the relevant time point. All results by unpaired t-test. Error bars are 95% confidence interval.
(A) Alteration of active MMP-9 at three- and six- months after PCI by ISR outcome with all participants included.
(B) Alteration of active MMP-9 at three- and six- months after PCI by ISR outcome. Those initially presenting with STEMI were excluded.
Prospective association of MMP markers and the development of ISR

six-month sampling point, (0.65 ± 0.94 vs. 0.62 ± 0.93 ng/mL, p = 0.92). Index active MMP-9 and TIMP-1 levels were similar between those who presented with ISR before three-months, and those who presented after. Using multiple regression, the change in active MMP-9 at three months was independently associated with the development of ISR when age, sex, waist circumference, level of TIMP-1, number and average diameter of stents, stent length, as well as clinical presentation with STEMI and time to PCI were included. The adjusted odds ratio was 1.89 (95% CI 1.04 – 3.45, p = 0.037) per ng/mL increase.

As shown in Chapter 5, there was no acute association between the clinical presentation and the index level of either active MMP-9 or TIMP-1. However, those with an initial presentation of STEMI had greater increases at three months than the stable angina and NSTEMI groups (Figure 5.2, Chapter 5). When patients initially presenting with STEMI were excluded from the univariate analysis comparing change in active MMP-9 and ISR, the association remained significant (controls [n = 68] vs. ISR [n = 25], 0.08 ± 0.76 vs. 0.76 ± 0.95 ng/mL, p = 0.0006) (Figure 7.1B). With STEMI removed, the ROC area-under-the-curve was 0.72 (95% CI 0.59 – 0.82), and the optimal cut-off in terms of sensitivity and specificity was an index to 3-month increase of 0.42ng/mL. The sensitivity was 0.52 and the specificity was 0.78, with 18.5% of controls vs. 46.4% of ISR cases having increases of this magnitude. This was associated with an odds ratio of 3.8 (95% CI, 1.5 – 10.1, p = 0.007). Kaplan Meier analysis using this cut-off value is reproduced in Figure 7.2A. A further exploratory subgroup analysis excluding both STEMI and UA was performed, where the change was again significantly greater in the ISR group (controls [n = 57] vs. ISR [n = 21] 0.06 ± 0.59 vs. 0.78 ± 0.95 ng/mL, p = 0.0002). The ROC area-under-the-curve was 0.76 (95% CI 0.64 – 0.86), and the optimal cut-off was an index to 3-month increase of ≥ 0.32 ng/mL. This cut-off point was associated with a sensitivity of 0.67 and specificity of 0.75, with 66.7% of cases and 24.6% of controls having elevations of higher than this level. The odds ratio was 6.1 (95% CI, 2.1 – 18.3, p = 0.0011). Kaplan Meier analysis using this cut-off value is reproduced in Figure 7.2B.
Figure 7.2 Kaplan Meier analysis of presentation-specific increase in active MMP-9 at three months and ISR
Cut-off points were derived for optimum sensitivity and specificity by ROC analysis.
A. Participants with an index clinical presentation of STEMI excluded from analysis.
B. Participants with index clinical presentations of STEMI and UA excluded from analysis.
7.2.5. Association of plasma active MMP-9 and TIMP-1 levels and ISR characteristics

The associations between the circulating levels of active MMP-9 and TIMP-1 with ISR characteristics are displayed in Table 7.6. There were significant associations between MMP levels and a number of ISR characteristics including time to ISR symptoms and ISR percent stenosis. The percent stenosis of the ISR lesion positively correlated with index active MMP-9 levels (Spearman’s rho = 0.56, \( p = 0.006 \)) (Table 7.6, Figure 7.3). Correlations were of similar magnitude at three and six months, but did not reach statistical significance. Furthermore, TIMP-1 had an inverse correlation with percent stenosis of the ISR lesion, but this was only significant at the three-month time point (Spearman’s rho = -0.43, \( p = 0.022 \)).

![Figure 7.3 Correlation of ISR percent stenosis and index active MMP-9 measurement](image)

The level of active MMP-9 at 3 and 6 months appeared to increase by a greater numerical amount amongst those ISR patients who had acute coronary syndrome as an ISR presentation but there was no significant difference on formal analysis. TIMP-1 had a
suggestive inverse association with the number of ISR lesions, but this did not reach significance. Mehran score was not associated with MMP variables.

Table 7.6 Association of active MMP-9 and TIMP-1 with ISR presentation variables

<table>
<thead>
<tr>
<th></th>
<th>Index n = 35</th>
<th>3 months n = 33</th>
<th>6 months n = 18</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>active MMP-9 (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of ISR presentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA, n = 12</td>
<td>0.65 (0.50 – 0.98)</td>
<td>0.69 (0.51 – 1.33)</td>
<td>0.78 (0.49 – 1.17)</td>
</tr>
<tr>
<td>UA, n = 7</td>
<td>0.55 (0.26 – 0.83)</td>
<td>1.12 (0.52 – 3.00)</td>
<td>1.12 (0.72 – 1.15)</td>
</tr>
<tr>
<td>NSTEMI, n = 16</td>
<td>0.56 (0.35 – 0.78)</td>
<td>1.12 (0.72 – 1.15)</td>
<td>1.10 (0.97 – 1.40)</td>
</tr>
<tr>
<td><strong>Mehran score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, n = 16</td>
<td>0.56 (0.35 – 0.85)</td>
<td>0.93 (0.51 – 1.33)</td>
<td>1.66 (1.37 – 2.13)</td>
</tr>
<tr>
<td>II, n = 6</td>
<td>0.61 (0.48 – 0.87)</td>
<td>1.64 (1.13 – 2.74)</td>
<td>0.99 (0.46 – 1.10)</td>
</tr>
<tr>
<td>III, n = 5</td>
<td>0.37 (0.07 – 0.64)</td>
<td>1.11 (0.23 – 2.13)</td>
<td>0.31 (–)‡</td>
</tr>
<tr>
<td>IV, n = 8</td>
<td>0.60 (0.44 – 0.85)</td>
<td>0.66 (0.52 – 1.35)</td>
<td>0.50 (0.21 – 1.02)</td>
</tr>
<tr>
<td><strong>Time to ISR symptoms</strong></td>
<td>ρ = -0.022</td>
<td>ρ = 0.065</td>
<td>ρ = 0.41</td>
</tr>
<tr>
<td><strong>Number of ISR lesions</strong></td>
<td>ρ = 0.19</td>
<td>ρ = 0.19</td>
<td>ρ = 0.12</td>
</tr>
<tr>
<td><strong>% ISR stenosis</strong></td>
<td>ρ = 0.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                          |              |                 |                 |
| **TIMP-1 (ng/mL)**       |              |                 |                 |
| Type of ISR presentation |              |                 |                 |
| SA, n = 12               | 108.2 ± 22.7 | 113.0 ± 15.6    | 114.6 ± 36.1    |
| UA, n = 7                | 105.4 ± 42.5 | 97.5 ± 17.2     | 103.4 ± 25.4    |
| NSTEMI, n = 16           | 101.7 ± 24.7 | 102.1 ± 21.8    | 112.9 ± 2.2     |
| **Mehran score**         |              |                 |                 |
| I, n = 16                | 113.1 ± 36.4 | 96.1 ± 18.8     | 106.1 ± 21.6    |
| II, n = 6                | 114.9 ± 20.5 | 118.0 ± 53.8    | 117.8 ± 32.1    |
| III, n = 5               | 123.2 ± 23.9 | 118.7 ± 21.0    | 95.2 (–)‡       |
| IV, n = 8                | 101.6 ± 13.4 | 95.6 ± 17.4     | 102.8 ± 46.5    |
| **Time to ISR symptoms**| R² = 0.35    | R² = 0.23       | R² = -0.26      |
| **Number of ISR lesions**| ρ = -0.10§   | ρ = -0.15§      | ρ = -0.034      |
| **% ISR stenosis**       | ρ = -0.34§   | ρ = -0.43|| | ρ = -0.22      |

Results are Spearman’s correlation (*), Pearson’s correlation (†), median (interquartile range) and mean ± standard deviation. No participants had a STEMI as a presentation of ISR.‡One observation only, so measures of variability are not applicable. NSTEMI = non-ST-elevation myocardial infarction; UA = unstable angina; SA = stable angina.§Pink indicates a trend (p <0.1); and ||blue indicates statistically significant (p < 0.05).

### 7.3. Discussion

This study evaluated the circulating levels of active MMP-9 and TIMP-1 in patients who had undergone bare-metal coronary stent placement, in an attempt to link alterations of these proteins to clinical events. The main finding of this study was that the development of
clinical ISR is associated with a greater intra-individual rise of active MMP-9 over three months than that of controls in an all-comers population undergoing PCI.

When clinical and demographic confounding factors were included in a multivariate model, the relationship between the three-month delta of active MMP-9 and the development of ISR remained statistically significant. While the ability to adjust for confounding factors was limited by the relatively small number of cases, this indicates that the increase in active MMP-9 from baseline is an independent predictor of the development of ISR. We were unable to confirm the hypothesis generated from the retrospective study, which was to test whether pre-intervention MMP-9 levels might predict development of ISR (Chapter 3). This discussion will cover the findings of the present study in relation to the previous study and the wider literature.

7.3.1. Association of plasma MMPs and ISR.

As previously mentioned, the pathophysiology of ISR involves vascular smooth muscle proliferation and migration into the injured intimal layer, and the subsequent production of proteoglycan-rich ECM leading to a hypocellular neointima which stenoses a stented artery. As discussed in greater detail in Chapter 3.3.2, animal models indicate a strong role for MMPs in this process, with experimental knock-out greatly limiting its development, but also suggesting that MMP expression is up-regulated for the duration of its development (Figure 7.4). A number of studies have examined the relationship between MMPs and ISR in clinical populations, and they can be broadly separated into pre-interventional and post-interventional MMP measurements. One helpful system is that of Niccoli et al. who suggest that pre-interventional measurements are more likely to reflect individual susceptibility and post-interventional measurements likely to reflect the biology of the developing restenotic lesion. As such, the aspects of other literature that deal with pre-interventional measurements have been dealt with in more detail in Chapter 3, and the main focus of the present discussion will be post-interventional levels. A number of studies have now associated alterations of MMPs with coronary interventions and ISR (Table 7.7). Levels of active MMP-9, but not active MMP-2, rise transiently in the coronary circulation immediately after intervention. This is followed by a prolonged increase in pro- and active-
Figure 7.4 Serial changes in intimal composition, MMP-9 and TIMP-1 expression in murine tissue after balloon injury. Adapted from Zou et al.383

A. Change in composition of developing neointima relative to the final area.
B. MMP-9 protein, MMP-9 activity and TIMP-1 expression in the developing neointima. MMP-9 and TIMP-1 expression quantified by Western blotting. MMP-9 activity quantified by zymography. The discrepancy between increased MMP-9 protein relative to TIMP-1 protein despite stable MMP-9 activity from day 14 onwards was explained by a concomitant increase of TIMP-1 “activity” on reverse zymography (i.e TIMP-1 mediated protection from proteolytic degradation by experimentally administered MMPs). This may obscure the detection of activity of MMP-9 protein in the active form. Additionally, human ISR takes longer to develop, around three to six months. If MMP alterations are important for the continuing remodeling of the neointima, it is likely that changes could be detected in human tissues at later time points. Samples from six mice were used to derive each time point. While the precise source of active MMP-9 seen in the studies in this thesis is unclear (and is covered later in this discussion), this provides a framework for discussing the present results.
Prospective association of MMP markers and the development of ISR

Table 7.7 Overview of clinical studies associating MMPs with restenosis

<table>
<thead>
<tr>
<th>Author</th>
<th>Patient group</th>
<th>Sampling location</th>
<th>Intervention</th>
<th>Outcome</th>
<th>MMP</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jones</td>
<td>History of previous PCI, Case control, n = 152 with ISR, n = 151 controls.</td>
<td>Peripheral venous &gt; 6 months post PCI</td>
<td>BMS</td>
<td>ISR (angiographically confirmed)</td>
<td>Pro-MMP-9, -10</td>
<td>Increased active MMPs -3 and -9 in cases. No change in pro- or latent MMP-9, or latent MMP-3.</td>
</tr>
<tr>
<td>Katsaros</td>
<td>Stable angina, n = 85 (ISR n = 12)</td>
<td>Peripheral vein Pre- and 24h post PCI</td>
<td>DES</td>
<td>ISR (Protocol angiography)</td>
<td>Pro- and active forms of MMP-2 and -9</td>
<td>Increased pre-PCI active MMP-9. Increased post-PCI active MMPs-2 and -9. Significant change in both pro-MMPs-2 and -9 and active MMPs-2 and -9 from pre- to post. Active MMPs-2 and -9 were correlated with late loss. No difference in pro- or active MMP-2, or pro-MMP-9 pre-PCI.</td>
</tr>
<tr>
<td>Ge</td>
<td>Stable, n = 40 (ISR n = 15)</td>
<td>Pre-interventional then day 1, 3, 7. Site not given in reference.</td>
<td>BMS</td>
<td>ISR (Protocol angiography)</td>
<td>Pro-MMPs-2, -9 and TIMP-1</td>
<td>Pro-MMP-2 was higher at day 1 in the ISR group than pre-procedurally, and higher than the non-ISR group. Pro-MMP-2 was not significantly different at other time points. Pro-MMP-9 was elevated over baseline in the ISR group at days 1 – 7. From day 3 – 7 pro-MMP-9 is higher in the ISR than the non-ISR group. Pro-MMP-9 correlated with multiple angiographic variables, including late loss. Changes in TIMP-1 did not reach significance.</td>
</tr>
<tr>
<td>Ye</td>
<td>Stable angina, n = 58 (revascularisations n = 13)</td>
<td>Peripheral vein, pre-PCI</td>
<td>PCI, not otherwise stated</td>
<td>Any revascularisation (Clinical, angiographically confirmed)</td>
<td>Total MMP-2, -3, -9 and TIMP-1</td>
<td>Total (i.e. activatable) MMP-9 was higher at baseline in those who had any revascularisation after PCI. No difference in total MMPs-2 or -3.</td>
</tr>
<tr>
<td>Hojo</td>
<td>Stable, n = 47, 7 controls (ISR = 10)</td>
<td>Coronary sinus over 24 hours. Pre- and post-PCI, then 4 and 24 hours.</td>
<td>BMS, PTCA and atherectomy</td>
<td>ISR (Protocol angiography in 62% of patients)</td>
<td>Pro-MMPs-1 and -2, Active MMP-2, TIMPs-1 and -2</td>
<td>Pro-MMP-1 below detection limit in 39/40. Pro-MMP-2 was elevated in the coronary sinus from 4 hours compared to pre-procedurally (not at post-intervention). Active MMP-2 was higher than baseline at 4h and 24h. 4h active MMP-9 correlated with late loss. No change in TIMP-2. TIMP-1 was higher at 24h.</td>
</tr>
<tr>
<td>Zemianskaia</td>
<td>PCI for stable angina (n = 78), (ISR n = 28)</td>
<td>Peripheral venous before PCI</td>
<td>PCI, not otherwise stated</td>
<td>ISR (Angiography or exercise test)</td>
<td>Pro-MMP-2? or Pro-MMP-9?</td>
<td>Pro-MMP-2 and -9 were significantly higher in those who would develop restenosis.</td>
</tr>
<tr>
<td>Kalela</td>
<td>History of CABG, asymptomatic 1.3 (range 0.38 – 1.4) years ago. n = 61 (vein graft stenosis n = 22)</td>
<td>Peripheral venous in patients with history of CABG before research angiogram, currently asymptomatic.</td>
<td>CABG with vein grafts</td>
<td>Asymptomatic vein graft stenosis (Protocol angiography)</td>
<td>Pro-MMP-9</td>
<td>There was no difference in pro-MMP-9 in those who had vein graft occlusions compared to those without. When those with a history of myocardial infarction (n = 30, restenosis n = 10) and stable disease (n = 31, restenosis n = 12) were compared separately, there were higher levels of pro-MMP-9 in the group with restenosis who initially presented with stable disease. There was a numerically higher level of pro-MMP-9 in those who initially presented with an MI but this did not reach statistical significance.</td>
</tr>
<tr>
<td>Jguenn-</td>
<td>CAD (n = 50) ISR during follow up (n = 19)</td>
<td>Peripheral venous. Pre-/post-interventional timing not given in reference.</td>
<td>PCI</td>
<td>ISR (Clinical)</td>
<td>Pro-MMP-12 TIMP-1</td>
<td>There was no difference in pro-MMP-12, but TIMP-1 was significantly lower in the group who would go on to develop ISR.</td>
</tr>
<tr>
<td>Soussi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Debris liberated during PCI, obtained through utilizing a distal protection device.
MMP-2 from four hours, the peak of which correlates with increased late loss.\textsuperscript{344} TIMP-1 is higher over the first 24 hours as a compensatory mechanism, with no change in TIMP-2.\textsuperscript{344} Early alterations in MMPs are detectable in the coronary sinus early after intervention, whereas there is no change in pro-MMP-9 in the peripheral circulation in the hour after balloon inflation,\textsuperscript{525} although pro-MMP-1 may be elevated in the peripheral venous system less than an hour after carotid stenting.\textsuperscript{537} However, systemic changes are eventually able to be detected, with elevated pro-MMPs-1,\textsuperscript{739} -2\textsuperscript{345} and -9\textsuperscript{345,739} at day one. Pro-MMP-2 normalises after day one, in contrast with pro-MMP-9, which is strongly elevated for a week after intervention.\textsuperscript{345} Circulating venous levels of both pro-MMPs -2 and -9 correlate with late loss and have been shown to be predictive of ISR in the first few days post-PCI.\textsuperscript{345} Active MMP-9 is elevated in both the coronary\textsuperscript{406} and peripheral circulation\textsuperscript{464} from 24 hours. Katsaros et al.\textsuperscript{464} describe increases in both the pro- and active- forms of MMPs-2 and -9 at 24 hours post-PCI, but demonstrate that the active forms of both are much more strongly associated with ISR than the pro- forms. In fact, while the increase in both pro-MMPs-2 and -9 are of similar magnitude regardless of ISR outcome, there is minimal change in active MMPs-2 and -9 in the group who did not develop restenosis.\textsuperscript{464} Pieved together, the temporal changes of MMPs are similar to that seen in animal studies, with greatly increased active MMPs-2 and -9 on the first day post-PCI.\textsuperscript{383} However, the study in this thesis is the first to link 3-month post-PCI changes in active MMP-9 to the development of restenosis. While mural MMP-9 protein remains high into the second month after murine arterial injury, this did not appear to be reflected in the level of MMP-9 activity, possibly because of increased TIMP-1 availability.\textsuperscript{383} Because of more rapid neointimal development in smaller animal models, this change at two months in mice likely corresponds to between three and six months in humans. However, Brown et al. showed that atherectomy specimens of ISR tissue had no MMP-9 antigen.\textsuperscript{393} Therefore, while our results suggest MMP-9 expression is elevated at three months in those developing ISR, levels may decrease to undetectable amounts at the time of ISR presentation. Our results are similar to that of Katsaros et al.,\textsuperscript{464} in that we demonstrate that a post-procedural increase of active MMP-9 is predictive of the development of ISR. However, the two studies had different methods, including their study used DES and protocol angiography, as well markedly different time frames. We measured active MMP-9 at index and three months, whereas Katsaros measured active MMP-9 at index and twenty-four hours. We observed that the level of active MMP-9
rose from baseline to three months, and this change was significantly greater in the ISR group than in controls.

What has not been displayed in animal models is an association between pre-interventional levels of MMPs and propensity to developing more prolific intimal hyperplasia. We previously showed that TIMP-1, and active MMPs-1, -3, and -9 were higher, and active MMP-2 lower, in a population of people who had a history of PCI with ISR. In the same population, there was a modest association with pro-MMP-9, but this did not reach significance. Kalela et al. saw elevated pro-MMP-9 in a group of patients with asymptomatic venous bypass graft stenosis, although this study design precludes attributing this to the restenotic process. A number of studies have now assessed pre-PCI MMPs in the prediction of restenosis, including pro-MMPs-2, -9, -12, active MMPs-2 and -9, as well as TIMP-1. Ye et al. measured the pre-PCI total fraction of MMPs-2, -3 and -9 that were able to be activated by APMA. The proportion of patients who achieved high cut off point of total MMP-9 was significantly higher in those who needed revascularisation, with 11/13 cases achieving this level compared to only half of patients with an uncomplicated course. However, circulating plasma levels were strangely not different between groups. Only one study has positively linked circulating pro-MMP-2 to the future development of restenoses, with the remaining studies showing a trend towards pro-MMP-2 having a protective effect. Active MMP-2 was numerically lower in those who would develop restenoses in the study by Katsaros. This is the same direction noted in the retrospective study (Chapter 3.2.2), but this effect was modest and would be expected to require a larger sample to detect a significant result. Pro-MMP-9 was slightly elevated in the groups that went on to develop ISR in a number of studies, but only one had a statistically significant relationship. There was no difference between ISR and control groups in the baseline level of pro-MMP-12. Two groups have reported the baseline level of TIMP-1 is numerically lower in those ISR, although only one of these studies reached statistically significance. In this study we found that TIMP-1 was similar between groups, but numerically higher in the ISR group. This is consistent with the findings of the retrospective study where TIMP-1 was robustly higher in those with ISR, and was predictive of those who had multiple ISR lesions. Both of our findings are in contrast to the findings of the other two studies discussed above.
We were unable to confirm the finding of Katsaros et al.\textsuperscript{464} that active MMP-9 was elevated before intervention amongst those who went on to develop ISR. Katsaros observed that the restenosis rate of lesions undergoing drug-eluting stenting was 7.7\% in the lower three quartiles of baseline active MMP-9, compared to a stunning 38.9\% for those in the highest quartile.\textsuperscript{464} We previously linked the circulating level of active MMP-3 to a history of ISR,\textsuperscript{530} but unfortunately this assay was removed from the market and was unavailable at the time of this current analysis. Ye et al.\textsuperscript{395} assessed the total fraction of MMP-3 able to be activated by APMA, and there was no difference in this result between those who required revascularisation compared to controls. Taken together, at present there is limited prospective evidence to support the idea that pre-interventional MMP measurements can predict the development of restenosis. While there are a number of very positive studies\textsuperscript{395,464} which suggest that the majority of patients with restenosis may be able to be stratified according to total- and active-MMP-9, respectively, the literature is consistent in support of the role of MMPs. If the findings associated with pro-MMPs are any indication, further studies will be required to show a conclusive result. Regardless, the study in this thesis was unable to replicate the findings that baseline active MMP-9 is predictive of the development of ISR.

There are a number of candidate sources of MMPs observed in clinical studies of ISR. Often it appears that authors uncritically assume that the origin is the injured artery and the developing neointimal lesion (the view we initially adopted at the beginning of this section, page 189). However, other possible sources include myocardium that is under abnormal wall stress or has infarcted (in the case of stable disease, perhaps from distal embolisation), or the result of multiple genetic polymorphisms (as discussed in Chapter 3.3.2.6). Two further possibilities are that circulating MMPs may derive from the recruitment of haematopoetic cells from the bone marrow, or that circulating MMPs represent a mechanism of disposal. MMP-9-mediated activation of soluble Kit-ligand is important for the release of cells from the bone marrow niche, with MMP-9 \textsuperscript{-/-} mice having delayed reconstitution of circulating white blood cells after depopulation with 5-fluorouracil.\textsuperscript{281} The majority of \textit{MMP9 -/-} mice died due to leukopaenia-related causes after 5-fluorouracil administration, whereas there were no fatalities in the \textit{MMP9 +/-} group. While MMP-9 was not measured in the plasma of this model, liberation of bone marrow cells was associated with a detectable increase of circulating soluble Kit indicating that systemic-level changes in protein expression occur.\textsuperscript{281} Systemic active MMP-9 measurements are detectable at least one day after PCI\textsuperscript{464} and Inoue
Prospective association of MMP markers and the development of ISR

*et al.* have shown that active MMP-9 correlate with the later recruitment of CD34 positive cells, which appear to be recruited by granulocyte-colony stimulating factor. While it has not been delineated exactly how bone marrow derived cells contribute to the tissue component of ISR (as discussed in Chapter 1.4.1.2), they may also be part of a generic inflammatory response, with leukocyte activation and systemic elevations of innate immune cells being evoked after PCI for around one week and associated with the development of ISR. Thus it is clear that PCI may be associated with systemic changes in inflammatory markers. Many of these studies were performed in people who had stable coronary disease, so these changes cannot be explained by myocardial changes. However, the tissue volume of the stented lesion and developing neointima is very small, casting doubt on whether the lesion itself is the source of these changes. Interestingly, these observations of increased circulating inflammatory cells do not appear to extend to drug-eluting stents. Cytostatic drug release causing local inhibition of inflammatory molecule generation and consequent inflammatory cell recruitment supports the idea that the injured arterial section itself is the stimuli for these changes.

Finally, the MMPs observed in the discussed studies may represent a form of clearance. Of note, this mechanism is not necessarily mutually exclusive with the other sources. It is known that MMPs are cleared via autoproteolysis and cellular re-uptake, but the full extent of MMP inactivation and breakdown in *vivo* is not understood. One circulation-specific excretion mechanism has been suggested, that of protein-binding to albumin and α-2 macroglobulin. α-2 macroglobulin is able to bind many proteinases including non-TIMP bound MMPs. It appears to be an important proteinase inhibitor in serum. Interestingly, α-2 macroglobulin complexes with MMPs at a faster rate constant than TIMPs do, with MMP-1 preferentially binding to α-2 macroglobulin in the presence of both TIMP and α-2 macroglobulin. α-2 macroglobulin is an H-shaped protein and the mechanism of this inhibition appears to be binding enzymes in such a way that the active site faces inwards. This is accomplished by the target enzyme proteolysing a vulnerable “bait region” which leads to a conformational change. In this way, α-2 macroglobulin can bind and inhibit multiple different proteinases without needing to bind specifically to their active site. One corollary of this is that MMPs that enter the circulation will only become bound to α-2 macroglobulin if they were in the active form.
While some authors have claimed that no MMP activity is present in the circulation,\textsuperscript{437} this may be due to the use of anticoagulants which chelate metal ions on which MMP activity is dependent.\textsuperscript{709} Furthermore, earlier ELISAs were less sensitive and some studies found that the majority of subjects had undetectable levels of some MMPs.\textsuperscript{575}

TIMPs are endogenous inhibitors of MMPs, binding in 1:1 stoichiometry. The assay we used was a substrate-lysis based assay after MMP capture at a non-active site epitope, and did not detect MMPs inhibited by TIMPs. However, we also measured the levels of TIMP-1, and found that the increase in active MMP-9 predicted the development of ISR independently of TIMP-1, indicating that the increase in active MMP-9 is not inhibited by TIMP-1.

### 7.3.2. Active MMP-9 as a clinical predictor of ISR

In Chapter 5 the relationship between index clinical presentation and MMP variables was discussed. While index presentation did not appear to be associated with acute changes in active MMP-9, the increase over three months was greater in the STEMI group, independently of ISR. Hence there may be a role for presentation-specific cut off points in the prediction of ISR. While this study was underpowered to detect a difference between ISR and controls amongst clinical presentation sub-groups, this was explored by performing \textit{post hoc} analyses after removing participants with index clinical presentations associated with greater variability, namely STEMI and unstable angina. While the results of these analyses need to be interpreted carefully, in both instances the predictive utility of increasing active MMP-9 appeared to increase relative to that of the whole cohort, where no cut-off point emerged as predictive of ISR. The point of pursuing these analyses was to demonstrate the potential utility of presentation-specific cut-off points. The best way to deal with a biomarker that may be confounded by variables such as time from symptom onset,\textsuperscript{700} clinical presentation\textsuperscript{700} and myocardial dysfunction\textsuperscript{695,750} would be to quantify the effect from each of these (\textit{i.e.} by including measurements across the spectrum of each of these variables, having serial measurements and having a large sample), and presenting an adjustment factor for each. Unfortunately we were unable to confirm the contribution of these effects in this study.

At present, there are no biochemical markers for either the pre-interventional stratification or post-interventional monitoring of patients undergoing PCI. However, a meta-analysis of studies in bare-metal stent patients has indicated that pre-procedural high-sensitivity C-reactive protein is associated with increased risk of ISR,\textsuperscript{661} although there was
evidence for publication bias and significant heterogeneity. Optimally, a randomized trial with a biomarker-driven protocol would be done. This would allow definite conclusions about the benefit of high-sensitivity C-reactive protein to be drawn. While there may be a role for presentation-specific levels of active MMP-9 in ISR prediction, our ability to derive such a model from this dataset is limited. The limited number of cases in this study, and lack of association between pre-PCI active MMP-9 and ISR precluded us from utilizing the models described in Chapter 4.

7.3.3. Clinical and demographics factors and ISR in the prospective study

The ISR group in the present study was on average older, with larger waist circumferences and body mass indices. Age has previously been identified as a univariate predictor of restenosis, but likely is a surrogate for other factors with direct links with ISR, such as type II diabetes. The association between measures of body habitus and ISR was previously noted and discussed in the retrospective study (Chapter 11.5). The difference in the present study is that it appears to be a much stronger effect. This is likely because with fewer cases in this study, the group average was markedly shifted by a few participants with greater body masses. The apparent trend between impaired renal function and ISR has been noted previously, but has not been confirmed in larger studies.

Because clinical presentation has previously been demonstrated to be associated with alterations in MMP levels, we frequency matched clinical presentation between the ISR and the control group. The clinical presentations did not match perfectly as the controls were matched to both the ISR group and the other events group. The lack of association between other classical risk factors for ISR in this study likely reflect the power of this study to reliably detect differences of this size.

7.4. Limitations

Limitations of this study included despite involving a relatively large cohort, there was only a small number of cases. This study only examined patients treated with bare-metal stents. The recruitment for this study began before DES were widely available in New Zealand. However, up to 50% of all interventions worldwide are still done using BMS.

Change in active MMP-9 from baseline was included as a secondary outcome only. That multiple a priori hypotheses were tested raises the risk that the positive association
between the change in active MMP-9 at three months and ISR is a false positive. If this association were corrected for multiple testing using the Bonferroni-Dunn method, it would not meet the criteria for significance.

The study in this thesis used clinically-driven angiographically-proven ISR as the outcome. This means that patients with asymptomatic angiographic ISR are not included in the ISR group, and may be included in the control group. This may have caused a type II error and prevented us from detecting true associations.

Index samples were drawn immediately prior to PCI, whereas in other studies they were drawn within a certain time from presentation. This may have led to an increase in variability, which precluded us confirming the results from some other studies.

### 7.5. Conclusions

This is the first study to link plasma active MMP-9 to the development of ISR in an all-comers population undergoing bare-metal stenting, specifically including patients with unstable disease and acute myocardial infarction. The increase of active MMP-9 from pre-intervention to three months was greater amongst those who subsequently developed ISR, and this appeared to be independent of clinical and demographic factors. The index level of active MMP-9 was associated with the follow up in-stent percent stenosis. The association of active MMP-9 and ISR would need to be confirmed in larger studies. Monitoring of the MMP-9 activity after percutaneous coronary intervention may allow early detection of ISR, but adjustment for the presence of STEMI may be needed and presentation-specific cut off points may allow better prognostic information.
8. Association of MMP markers and cardiovascular variables

8.1. Introduction

In contrast to the build up of vascular smooth muscle and ECM that causes ISR, the underlying pathophysiology of acute coronary events involves breakdown of the connective tissue of the plaque shoulder. Both of these processes require ECM remodelling, and the matrix-metalloproteinase family have been shown to have an important role. \(^{30}\)

The seminal paper by Galis et al. \(^{363}\) demonstrated the up-regulation of MMPs-1, -3 and -9 in the shoulder area of unstable plaque which suggest an active role in the progression towards rupture. Lowered circulating pro-MMPs-3 and -9 have been linked to STEMI, \(^{632}\) and experimental over-expression of active-, but not pro-, MMP-9 consistently causes plaque rupture in mice. \(^{426}\)

Moreover, as well as a contributory role in the development of breakdown and rupture through the lysis of ECM, matrix metalloproteinases may also have a role in promoting thrombus formation. Morishige et al. \(^{376}\) demonstrated, in a porcine model, that MMP-9 over-expression was associated with a greater likelihood of persistent thrombus at the site of experimental injury. This highlights MMP-9 as a putative risk factor for stent thrombosis, but also has potential ramifications for thrombosis-related pathology in general. For example a predisposition towards higher MMP-9 production may cause more severe thrombotic occlusion with plaque disruption.

The lesions which rupture to cause acute coronary syndrome are usually detectable by intravascular ultrasound in the years before presentation, and are often small and have unstable features. \(^{753}\) However, the presence of multiple unstable features has a low predictive value for which plaques will rupture, \(^{753}\) suggesting that even morphological assessment of lesions within the coronary tree does not allow accurate prediction of future events.

While animal and pathobiological studies have confirmed a role for MMPs in the development and progression of atherosclerosis, these insights have not yet been developed into clinically useful tools. Some investigators are currently developing molecular imaging
techniques which may allow spatial and biochemical information (i.e. fibrous cap morphology and MMP expression data) to be gained during angiography with a specially designed catheter. However, circulating MMPs appear to be responsive to various plaque features, with higher levels in triple vessel disease and in the presence of unstable plaque, perhaps indicating that the changes in plaque phenotype in the months before presentation are associated with changes in the circulating levels of MMPs.

The comparisons in this study were designed to test associations between MMPs and the incidence of cardiovascular disease in three ways. The first investigated whether pre-interventional levels of active MMP-9 and TIMP-1 were associated with the development of cardiovascular events. This was based on the idea that circulating levels of these proteins may reflect a predisposition to the development of disease like that hypothesised for ISR in Chapter 3.3.2.6. The second was designed to assess the longitudinal profile of active MMP-9 and TIMP-1, comparing the baseline levels with those at three- and six-months. This was to test whether changes in these proteins evoked by PCI or ischaemia would predict the development of future events. Finally, this study aimed to assess whether active MMP-9 and TIMP-1 measurements change in the months before incident cardiovascular events. This was tested by comparing the sample closest in temporal relationship to the development of symptoms, before the incident cardiovascular event, to that of controls. The cardiovascular events included are a group of heterogeneous conditions with different pathophysiologies including progression of atherosclerotic plaque, erosion and rupture of fibrous caps with thrombosis and stent thrombosis. As a pilot study, each condition was analysed separately, with the outcome being a measurement of the effect size associated with the difference in circulating markers.

MMP expression appears to be an important pathophysiological mechanism of adverse myocardial remodelling. Multiple studies have examined the role of MMPs in systolic dysfunction, but MMPs may be important in diastolic dysfunction as well, with increased expression of MMPs in both mouse models and clinical studies. Of note, pro-MMP-9 appears to be one of the MMPs most strongly associated with diastolic dysfunction. We previously showed that active MMP-9 was associated with diastolic dysfunction. However, there were a number of limitations to this study, including a retrospective design, where active MMP levels were linked to echocardiography studies undertaken previously, and limited assessment of diastolic dysfunction.

The aims of this chapter were to assess whether the development of cardiovascular
events was associated with alterations in active MMP-9 and TIMP-1: (1) before intervention, (2) over three- and six-months after intervention, and (3) in the months before presentation with these conditions. This study also (4) aimed to assess whether active MMP-9 and TIMP-1 were associated with cardiovascular risk factors, and finally, (5) whether the active form of MMP-9 was associated with systolic and diastolic myocardial dysfunction.

8.2. Results

8.2.1. Description of clinical population

Of those approached, 402 out of 494 were included in the study (81.4%; see Chapter 5, Figure 5.1). At one year, in addition to the 35 participants who had presented with ISR (as described in chapter 7) a further 30 (7.5%) participants suffered other (non-ISR) cardiovascular events. Ninety-eight patients who were asymptomatic at one year were randomly selected, after stratification by index clinical presentation, to both the ISR and the non-ISR cardiac events groups.

Those who had suffered a cardiac event during the follow up year were similar in terms of demographics to the control group (Table 8.1). Those in the cardiac events group had more severe coronary artery disease (Table 8.2), but were otherwise similar in terms of clinical and angiographic variables. All patients with cardiovascular events that did not cause death underwent angiography. The majority of those with progressive coronary artery disease underwent revascularisation, either with PCI or bypass grafting. Seven underwent medical therapy. The in-stent percent stenosis was recorded, but because some patients underwent angiography very early (i.e. two cases of definite stent thrombosis causing STEMI at days two and seven post-PCI), this reading may be falsely low. There was no association of active MMP-9 or TIMP-1 with the percentage ISR stenosis of those patients undergoing angiography for the evaluation of other coronary events.

The median time to onset of symptoms was 213 days and the closest time points to symptom onset are displayed in Table 8.3. The median time from the last sample taken to the onset of symptoms was 81 days.
### Table 8.1 Baseline demographic variables stratified by one-year outcome

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control n = 98</th>
<th>Cardiac event n = 30</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>61.0 ± 10.3</td>
<td>64.0 ± 11.6</td>
<td>0.17</td>
</tr>
<tr>
<td>Sex, male</td>
<td>76 (77.6%)</td>
<td>27 (90.0%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>98.7 ± 12.4</td>
<td>99.8 ± 7.8</td>
<td>0.67</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.7 ± 4.9</td>
<td>27.7 ± 3.6</td>
<td>0.96</td>
</tr>
<tr>
<td>Ethnicity*</td>
<td></td>
<td></td>
<td>0.084</td>
</tr>
<tr>
<td>NZ European</td>
<td>78 (79.6%)</td>
<td>29 (96.7%)</td>
<td></td>
</tr>
<tr>
<td>Mäori/Pacific Island</td>
<td>5 (5.1%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>15 (15.3%)</td>
<td>1 (3.3%)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.69 ± 1.05</td>
<td>4.57 ± 1.14</td>
<td>0.62</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.69 ± 0.73</td>
<td>1.72 ± 1.20</td>
<td>0.86</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.85 ± 0.92</td>
<td>2.68 ± 1.12</td>
<td>0.46</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.07 ± 0.28</td>
<td>1.10 ± 0.34</td>
<td>0.61</td>
</tr>
<tr>
<td>hs-CRP, mg/L†</td>
<td>2.7 (1.1 – 4.2)</td>
<td>2.7 (1.8 – 3.8)</td>
<td>0.61</td>
</tr>
<tr>
<td>Hypertension</td>
<td>69 (70.4%)</td>
<td>22 (73.3%)</td>
<td>0.76</td>
</tr>
<tr>
<td>Diabetes</td>
<td>14 (14.3%)</td>
<td>1 (3.3%)</td>
<td>0.19</td>
</tr>
<tr>
<td>Pack years</td>
<td>9.7 (0 – 26.0)</td>
<td>4.0 (0 – 19.0)</td>
<td>0.58</td>
</tr>
<tr>
<td>eCrCl &lt; 60mL/min</td>
<td>16 (16.3%)</td>
<td>2 (6.7%)</td>
<td>0.23</td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td>32 (32.7%)</td>
<td>11 (36.7%)</td>
<td>0.67</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>52 (53.1%)</td>
<td>20 (66.7%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Ca²⁺ antagonist</td>
<td>13 (13.3%)</td>
<td>7 (23.3%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Statin</td>
<td>58 (59.2%)</td>
<td>17 (56.7%)</td>
<td>0.84</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td>22 (22.4%)</td>
<td>8 (26.7%)</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Results displayed as mean ± standard deviation, number (%) and median (interquartile range). *Chi² test for trend. †hs-CRP values ≥ 10 mg/L excluded (n = 37) due to presumed acute inflammation. ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; BMI = body mass index; eCrCl = estimated creatinine clearance, Cockraft-Gault model; HDL = high-density lipoprotein; hs-CRP = high-sensitivity C-reactive protein.
### Table 8.2 Baseline clinical variables stratified by one year outcome

<table>
<thead>
<tr>
<th></th>
<th>Control n = 98</th>
<th>Cardiac event n = 30</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Index presentation</strong>*</td>
<td></td>
<td></td>
<td>0.90</td>
</tr>
<tr>
<td>Stable Angina</td>
<td>23 (23.5%)</td>
<td>7 (23.3%)</td>
<td></td>
</tr>
<tr>
<td>Unstable Angina</td>
<td>15 (15.3%)</td>
<td>3 (10.0%)</td>
<td></td>
</tr>
<tr>
<td>NSTEMI</td>
<td>36 (36.7%)</td>
<td>12 (40.0%)</td>
<td></td>
</tr>
<tr>
<td>STEMI</td>
<td>24 (24.5%)</td>
<td>8 (26.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>CAD severity</strong>*</td>
<td></td>
<td></td>
<td>0.026</td>
</tr>
<tr>
<td>1VD</td>
<td>45 (45.9%)</td>
<td>6 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>2VD</td>
<td>31 (31.6%)</td>
<td>16 (53.3%)</td>
<td></td>
</tr>
<tr>
<td>3VD</td>
<td>22 (22.4%)</td>
<td>8 (26.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Time to PCI, days†</strong></td>
<td>1 (0 – 3)</td>
<td>2 (0 – 3)</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Index ACC/AHA score</strong>*</td>
<td></td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td>A</td>
<td>10 (10.2%)</td>
<td>2 (6.7%)</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>30 (30.6%)</td>
<td>7 (23.3%)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>31 (31.6%)</td>
<td>9 (30.0%)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>27 (27.6%)</td>
<td>12 (40.0%)</td>
<td></td>
</tr>
<tr>
<td>Reference diameter, mm</td>
<td>3.06 ± 0.62</td>
<td>2.97 ± 0.39</td>
<td>0.50</td>
</tr>
<tr>
<td>MLD, mm</td>
<td>0.72 ± 0.43</td>
<td>0.86 ± 0.45</td>
<td>0.16</td>
</tr>
<tr>
<td>Diameter stenosis</td>
<td>76.1% ± 13.4%</td>
<td>71.2% ± 14.4%</td>
<td>0.09</td>
</tr>
<tr>
<td>Lesion length, mm</td>
<td>16.4 (11.8 – 27.2)</td>
<td>17.7 (10.5 – 24.8)</td>
<td>0.57</td>
</tr>
<tr>
<td>Stent diameter, mm</td>
<td>3.07 ± 0.46</td>
<td>3.09 ± 0.35</td>
<td>0.85</td>
</tr>
<tr>
<td>Stent length, mm</td>
<td>24.0 (16.0 – 36.0)</td>
<td>25.5 (15.0 – 39.0)</td>
<td>0.88</td>
</tr>
<tr>
<td>Number of stents</td>
<td>1.5 ± 0.8</td>
<td>1.5 ± 1.0</td>
<td>0.94</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>47.5% ± 10.9%</td>
<td>53.3 ± 5.2</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Results displayed as mean ± standard deviation, number (%) and median (interquartile range).

* \( \chi^2 \) test for trend. † Time to PCI in days from initial hospital admission for the initial symptomatic episode. ACC/AHA = modified American College of Cardiology/American Heart Association score; CAD = coronary artery disease; MLD = minimal lumen diameter; NSTEMI = non-ST elevation myocardial infarction; STEMI = ST elevation myocardial infarction; VD = vessel disease.
Table 8.3 Characteristics of presentation with cardiovascular events in prospective cohort

<table>
<thead>
<tr>
<th>Cardiac events</th>
<th>n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Presentation with cardiac event</strong></td>
<td></td>
</tr>
<tr>
<td>Stable angina</td>
<td>10 (33.3%)</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>8 (26.7%)</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>STEMI</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td>Sudden death</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td><strong>Aetiology</strong></td>
<td></td>
</tr>
<tr>
<td>Atherosclerotic progression</td>
<td>22 (73.3%)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>10 (45.5%)</td>
</tr>
<tr>
<td>Acute coronary syndrome</td>
<td>12 (54.5%)</td>
</tr>
<tr>
<td>Stent thrombosis*</td>
<td>7 (23.3%)</td>
</tr>
<tr>
<td>Possible</td>
<td>3 (42.9%)</td>
</tr>
<tr>
<td>Definite</td>
<td>4 (57.1%)</td>
</tr>
<tr>
<td>Myocardial rupture</td>
<td>1 (3.3%)</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Target vessel revascularisation</td>
<td>5 (16.7%)</td>
</tr>
<tr>
<td>Non-target vessel revascularisation</td>
<td>11 (36.7%)</td>
</tr>
<tr>
<td>Multi-vessel revascularisation</td>
<td>3 (10.0%)</td>
</tr>
<tr>
<td>Medical therapy</td>
<td>7 (23.3%)</td>
</tr>
<tr>
<td>Deceased†</td>
<td>4 (13.3%)</td>
</tr>
<tr>
<td><strong>Number of lesions, n = 26 (19 patients)</strong></td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td><strong>Percent diameter stenosis in-stent, n = 26</strong></td>
<td>10% (0 – 30%)</td>
</tr>
<tr>
<td><strong>Time to symptom onset, days</strong></td>
<td>213 (96 – 311)</td>
</tr>
<tr>
<td><strong>Last time point</strong></td>
<td></td>
</tr>
<tr>
<td>Index</td>
<td>5 (16.7%)</td>
</tr>
<tr>
<td>Three months</td>
<td>8 (26.7%)</td>
</tr>
<tr>
<td>Six months</td>
<td>17 (56.6%)</td>
</tr>
<tr>
<td><strong>Last sample to symptom onset, days</strong></td>
<td>81 (30 – 155)</td>
</tr>
</tbody>
</table>

Results are mean ± standard deviation, median (interquartile range) or number (percent).

* Academic Research Consortium classification of stent thrombosis.
† Presentation with cardiovascular event was sudden death in four patients, therefore no additional treatment was performed.
8.2.2. Association of active MMP-9 and TIMP-1 measurements and the development of non-ISRCT cardiac events

Neither the levels of at any time point, nor the changes in active MMP-9 or TIMP-1 had statistically significant relationships with non-ISRCT cardiac events (Table 8.4). When the last measurement before clinical presentation was considered, both the levels and the changes of active MMP-9 and TIMP-1 were not statistically significantly different between groups. As there was a wide range of times from the last measurement to clinical presentation [median 81 days (IQR 30 – 155), Table 8.3], this variable was a potential confounder. However, there was no correlation between either active MMP-9 and TIMP-1 and the time from last measurement to clinical presentation.

The longitudinal changes of active MMP-9 and TIMP-1 for each individual are displayed for those with incident cardiac events and for controls are displayed in Figure 8.1 (active MMP-9) and Figure 8.2 (TIMP-1). There is a wide variation in the trajectories of both active MMP-9 and TIMP-1, as demonstrated by the plots of the control groups (Figure 8.1 and Figure 8.2). All groups (including the control group) appear to have individual measurements with either increased or decreased levels of active MMP-9 or TIMP-1 at three months, compared to baseline.

As the outcomes comprised a heterogeneous group of conditions, each was then considered separately. In order to detect whether there was any signal of association between the development of cardiac events and the last measurements of active MMP-9 and TIMP-1 before presentation, the average levels for each group of incident cardiac disease was compared to the control group. Each case was matched to controls based on initial clinical presentation and diastolic dysfunction category (Table 8.5). Not all controls were able to be matched to cases because of differences in diastolic dysfunction, so for this comparison, only 67 controls were used. There appeared to be modest differences in the last measurements of active MMP-9 and TIMP-1 between the controls and those with incident cardiovascular events. However, all apparent changes were very small, as indicated by modest effect sizes (Cohen’s $d = 0.02 – 0.37$), requiring large sample sizes (greater than $n = 63$ per arm) to detect the observed differences with 90% power. Similar results were obtained when comparing those with non-ISRCT events to randomly selected controls. Overall, the effect sizes associated with active MMP-9 and TIMP-1 before presentation with incident cardiovascular disease
Table 8.4 Circulating levels of active MMP-9 and TIMP-1 at index, three and six months by one-year outcome

<table>
<thead>
<tr>
<th></th>
<th>Control n = 98*</th>
<th>Cardiac event n = 30</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Index active MMP-9, ng/mL</strong></td>
<td>0.59 (0.28 – 0.81)</td>
<td>0.40 (0.17 – 0.82)</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>3-month active MMP-9, ng/mL</strong></td>
<td>0.82 (0.39 – 1.27)</td>
<td>0.74 (0.41 – 1.10)</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>6-month active MMP-9, ng/mL</strong></td>
<td>0.87 (0.49 – 1.18)</td>
<td>0.76 (0.50 – 1.02)</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>3-month Δ active MMP-9, ng/mL</strong></td>
<td>0.24 ± 0.83</td>
<td>0.32 ± 0.44</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>6-month Δ active MMP-9, ng/mL</strong></td>
<td>0.20 ± 0.83</td>
<td>0.14 ± 0.32</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Index TIMP-1, ng/mL</strong></td>
<td>105.7 ± 24.0</td>
<td>97.5 ± 29.4</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>3-month TIMP-1, ng/mL</strong></td>
<td>103.5 ± 27.6</td>
<td>103.3 ± 26.7</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>6-month TIMP-1, ng/mL</strong></td>
<td>104.4 ± 23.8</td>
<td>107.6 ± 27.0</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>3-month Δ TIMP-1, ng/mL</strong></td>
<td>-2.8 ± 2.7</td>
<td>1.2 ± 34.1</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>6-month Δ TIMP-1, ng/mL</strong></td>
<td>-1.7 ± 2.4</td>
<td>6.2 ± 19.1</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Last active MMP-9, ng/mL</strong></td>
<td>0.72 (0.37 – 1.18)</td>
<td>0.61 (0.34 – 0.92)</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>Last TIMP-1, ng/mL</strong></td>
<td>104.2 ± 24.3</td>
<td>101.7 ± 32.5</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Index–last Δ active MMP-9, ng/mL</strong></td>
<td>0.14 ± 0.65</td>
<td>0.15 ± 0.35</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>Index–last Δ TIMP-1, ng/mL</strong></td>
<td>-0.8 ± 18.7</td>
<td>4.2 ± 30.2</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Results displayed as median (interquartile range) and mean ± standard deviation. Results by Mann Whitney U and unpaired t-test. Three- and six-month change were calculated by subtracting each individuals index measurement from the three- and six-month values, respectively. *n displayed is for index measurement.
Figure 8.1 Serial changes in active MMP-9 by presentation with cardiac event
Figure 8.2 Serial changes in TIMP-1 by presentation with cardiac event
Table 8.5 Last measurements of active MMP-9 and TIMP-1 by incident clinical events compared to matched controls

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 67)</th>
<th>Stable angina (n = 10)</th>
<th>Unstable angina (n = 8)</th>
<th>NSTEMI (n = 4)</th>
<th>STEMI (n = 4)</th>
<th>Sudden death (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Last active MMP-9*</td>
<td>0.55 (0.25–1.02)</td>
<td>0.61 (0.51–0.82)</td>
<td>0.27 (0.08–0.63)</td>
<td>0.44 (0.24–0.77)</td>
<td>0.87 (0.40–1.24)</td>
<td>0.82 (0.65–1.82)</td>
</tr>
<tr>
<td>Cohen’s d effect size</td>
<td>–</td>
<td>0.23</td>
<td>0.21</td>
<td>0.07</td>
<td>0.20</td>
<td>0.37</td>
</tr>
<tr>
<td>n for 90% power</td>
<td>–</td>
<td>95</td>
<td>109</td>
<td>1078</td>
<td>127</td>
<td>38</td>
</tr>
<tr>
<td>Last TIMP-1*</td>
<td>105.8 ± 24.7</td>
<td>97.5 ± 13.2</td>
<td>105.2 ± 39.4</td>
<td>85.7 ± 62.0</td>
<td>109.6 ± 11.4</td>
<td>110.7 ± 30.5</td>
</tr>
<tr>
<td>Cohen’s d effect size</td>
<td>–</td>
<td>0.42</td>
<td>0.01</td>
<td>0.21</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>n for 90% power</td>
<td>–</td>
<td>120</td>
<td>63 117</td>
<td>116</td>
<td>539</td>
<td>675</td>
</tr>
</tbody>
</table>

Results are median (interquartile range), n and mean ± standard deviation.

*Measurements of active MMP-9 and TIMP-1 with the closest temporal relationship to the subsequent clinical presentation (ng/mL)

Sample size calculations are the number of participants required per arm to achieve 90% power.
were small. Thus, it is unlikely that measurements of active MMP-9 and TIMP-1 in the months before presentation with incident cardiovascular disease would be prognostically useful. The small effect sizes observed may reflect the small volume of remodelling plaque relative to the individual. However, the most likely explanation is that the observed variation is due to chance. This study could not confirm an association between measurements of active MMP-9 and TIMP-1 in the months before presentation with incident cardiovascular disease. Thus, changes in circulating active MMP-9 appear to display specificity for ISR, and may not be significantly altered by the development of non-ISR cardiovascular events.

8.2.3. Association of active MMP-9 and TIMP-1 with coronary risk factors

The associations of active MMP-9 and TIMP-1 with coronary risk factor variables are displayed in Table 8.6 and Table 8.7, respectively. Associations between active MMP-9, TIMP-1 and prescribed medications at hospital admission are displayed in Table 8.8. Categorical variables have the average levels displayed for each category and continuous variables are displayed as correlations. Female sex was associated with significantly higher active MMP-9 concentrations, but this was explained by increased rates of diastolic dysfunction amongst females (see section 8.2.4), with no association on multivariate modelling for age and development of ISR. Patients with impaired renal function (estimated creatinine clearance < 60 mL/min, n = 27) had increased active MMP-9 (Table 8.6). This was statistically significant only at the three-month time point, but appeared to be independent of a history of STEMI, ISR and diastolic dysfunction, as well as sex and age. Both diabetes (Table 8.7) and treatment with ACE-I/ARB (Table 8.8) were associated with increased TIMP-1. Diabetes was associated with higher TIMP-1 at three and six months and a trend association of similar magnitude at baseline. Treatment with ACE-I/ARB at the time of admission was associated with increased TIMP-1. The associations between TIMP-1 and ACE-I/ARB prescription were positive but not statistically significant at other time points. There was no difference in active MMP-9 or TIMP-1 in those participants with high sensitivity C-reactive protein ≥ 10 mg/L compared to those < 10 mg/L.
Table 8.6 Association of active MMP-9 with coronary risk factor variables

<table>
<thead>
<tr>
<th></th>
<th>Index n = 163</th>
<th>3 months n = 145</th>
<th>6 months n = 121</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>0.12</td>
<td>0.14†</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.51 (0.22 – 0.72)</td>
<td>0.73 (0.41 – 1.12)</td>
<td>0.72 (0.43 – 1.11)</td>
</tr>
<tr>
<td>Female</td>
<td>0.72 (0.47 – 1.22)</td>
<td>1.33 (0.74 – 2.11)</td>
<td>1.16 (0.66 – 1.51)</td>
</tr>
<tr>
<td><strong>Waist, cm</strong></td>
<td>0.06</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>0.09</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ European</td>
<td>0.51 (0.23 – 0.81)</td>
<td>0.82 (0.40 – 1.22)</td>
<td>0.76 (0.46 – 1.18)</td>
</tr>
<tr>
<td>Māori/Pacific Island</td>
<td>1.03 (0.50 – 1.47)</td>
<td>1.23 (0.62 – 1.91)</td>
<td>1.22 (0.95 – 1.56)</td>
</tr>
<tr>
<td>Other</td>
<td>0.65 (0.34 – 0.75)</td>
<td>0.94 (0.66 – 2.10)</td>
<td>0.76 (0.50 – 1.07)</td>
</tr>
<tr>
<td><strong>Dyslipidaemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.55 (0.23 – 0.87)</td>
<td>0.85 (0.53 – 1.23)</td>
<td>0.85 (0.50 – 1.17)</td>
</tr>
<tr>
<td>No</td>
<td>0.54 (0.28 – 0.74)</td>
<td>0.74 (0.34 – 1.46)</td>
<td>0.76 (0.45 – 1.14)</td>
</tr>
<tr>
<td><strong>Total cholesterol, mmol/L</strong></td>
<td>0.02</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides, mmol/L</strong></td>
<td>0.07</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>LDL, mmol/L</strong></td>
<td>-0.01</td>
<td>0.01</td>
<td>-0.07</td>
</tr>
<tr>
<td><strong>HDL, mmol/L</strong></td>
<td>-0.03</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>hs-CRP, mg/L</strong></td>
<td>0.14</td>
<td>0.04</td>
<td>-0.01</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.56 (0.27 – 0.82)</td>
<td>0.83 (0.41 – 1.23)</td>
<td>0.82 (0.50 – 1.19)</td>
</tr>
<tr>
<td>No</td>
<td>0.51 (0.26 – 0.81)</td>
<td>0.82 (0.41 – 1.32)</td>
<td>0.75 (0.48 – 1.14)</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.63 (0.48 – 0.98)</td>
<td>1.19 (0.63 – 1.87)</td>
<td>0.82 (0.50 – 1.19)</td>
</tr>
<tr>
<td>No</td>
<td>0.51 (0.22 – 1.32)</td>
<td>0.80 (0.41 – 1.23)</td>
<td>0.75 (0.48 – 1.14)</td>
</tr>
<tr>
<td><strong>Pack years</strong></td>
<td>0.02</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>eCrCl &lt; 60mL/min</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.74 (0.24 – 1.43)</td>
<td>1.44 (0.59 – 2.12)</td>
<td>1.04 (0.63 – 1.28)</td>
</tr>
<tr>
<td>No</td>
<td>0.51 (0.26 – 0.75)</td>
<td>0.82 (0.41 – 1.18)</td>
<td>0.76 (0.47 – 1.16)</td>
</tr>
</tbody>
</table>

Results are Spearman’s correlation (*), and median (interquartile range).

Red indicates highly statistically significant (p < 0.01); blue indicates statistically significant (p < 0.05) and pink indicates a trend (p <0.1).

† p value < 0.1; ‡ p value < 0.05; § p value < 0.01.

BMI = body mass index; eCrCl = estimated creatinine clearance, Cockraft-Gault model; HDL = high-density lipoprotein; hs-CRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein.
Table 8.7 Association of TIMP-1 with coronary risk factor variables

<table>
<thead>
<tr>
<th></th>
<th>Index n = 160</th>
<th>3 months n = 145</th>
<th>6 months n = 120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong>*</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>104.7 ± 28.2</td>
<td>108.2 ± 27.3</td>
<td>107.1 ± 28.0</td>
</tr>
<tr>
<td>Female</td>
<td>105.4 ± 25.3</td>
<td>101.9 ± 27.7</td>
<td>104.8 ± 24.9</td>
</tr>
<tr>
<td><strong>Waist, cm</strong>*</td>
<td>0.01</td>
<td>0.01</td>
<td>粉色 0.03†</td>
</tr>
<tr>
<td><strong>BMI, kg/m^2</strong></td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ European</td>
<td>105.8 ± 28.4</td>
<td>103.4 ± 28.0</td>
<td>105.2 ± 23.7</td>
</tr>
<tr>
<td>Māori/Pacific Island</td>
<td>97.7 ± 26.5</td>
<td>94.8 ± 20.2</td>
<td>102.2 ± 38.6</td>
</tr>
<tr>
<td>Other</td>
<td>101.5 ± 21.3</td>
<td>107.0 ± 29.0</td>
<td>107.0 ± 30.6</td>
</tr>
<tr>
<td><strong>Dyslipidaemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>107.6 ± 31.0</td>
<td>105.0 ± 29.2</td>
<td>106.6 ± 26.2</td>
</tr>
<tr>
<td>No</td>
<td>101.1 ± 21.6</td>
<td>100.8 ± 25.2</td>
<td>103.4 ± 24.5</td>
</tr>
<tr>
<td><strong>Total cholesterol, mmol/L</strong>*</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Triglycerides, mmol/L</strong>*</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>LDL, mmol/L</strong>*</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>HDL, mmol/L</strong>*</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>hs-CRP, mg/L</strong>*</td>
<td>0.01</td>
<td>0.01</td>
<td>粉色 0.05†</td>
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<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>104.7 ± 27.6</td>
<td>103.7 ± 28.9</td>
<td>105.9 ± 25.9</td>
</tr>
<tr>
<td>No</td>
<td>105.3 ± 27.6</td>
<td>102.3 ± 24.9</td>
<td>104.1 ± 24.9</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>111.2 ± 34.1</td>
<td>116.5 ± 38.0</td>
<td>128.1 ± 29.8</td>
</tr>
<tr>
<td>No</td>
<td>104.0 ± 26.5</td>
<td>101.5 ± 25.6</td>
<td>102.7 ± 23.9</td>
</tr>
<tr>
<td><strong>Pack years</strong>*</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>eCrCl &lt; 60mL/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>102.6 ± 19.5</td>
<td>100.7 ± 24.4</td>
<td>102.7 ± 29.9</td>
</tr>
<tr>
<td>No</td>
<td>105.6 ± 28.6</td>
<td>103.7 ± 27.3</td>
<td>105.6 ± 23.1</td>
</tr>
</tbody>
</table>

Results are Pearson’s correlation (*), median (interquartile range) and mean ± standard deviation. Red indicates highly statistically significant (p < 0.01); blue indicates statistically significant (p < 0.05) and pink indicates a trend (p <0.1).
† p value < 0.1; ‡ p value < 0.05; § p value < 0.01.

BMI = body mass index; eCrCl = estimated creatinine clearance, Cockraft-Gault model; HDL = high-density lipoprotein; hs-CRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein.
Table 8.8 Association of active MMP-9 and TIMP-1 with prescribed medications before index admission

<table>
<thead>
<tr>
<th></th>
<th>Index n = 163</th>
<th>3 months n = 145</th>
<th>6 months n = 121</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active MMP-9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.53 (0.19 – 0.84)</td>
<td>0.85 (0.53 – 1.53)</td>
<td>0.75 (0.50 – 1.16)</td>
</tr>
<tr>
<td>No</td>
<td>0.55 (0.28 – 0.82)</td>
<td>0.82 (0.41 – 1.25)</td>
<td>0.85 (0.47 – 1.18)</td>
</tr>
<tr>
<td>Aspirin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.55 (0.26 – 0.85)</td>
<td>0.82 (0.45 – 1.23)</td>
<td>0.79 (0.48 – 1.15)</td>
</tr>
<tr>
<td>No</td>
<td>0.54 (0.26 – 0.74)</td>
<td>0.83 (0.37 – 1.36)</td>
<td>0.77 (0.54 – 1.19)</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.53 (0.22 – 0.82)</td>
<td>0.81 (0.41 – 1.23)</td>
<td>0.79 (0.34 – 1.18)</td>
</tr>
<tr>
<td>No</td>
<td>0.56 (0.31 – 0.75)</td>
<td>0.85 (0.41 – 1.32)</td>
<td>0.77 (0.50 – 1.17)</td>
</tr>
<tr>
<td>Ca(^{2+}) antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.84 (0.53 – 1.18)</td>
<td>0.94 (0.57 – 1.21)</td>
<td>0.92 (0.34 – 1.18)</td>
</tr>
<tr>
<td>No</td>
<td>0.51 (0.24 – 0.72)</td>
<td>0.81 (0.40 – 1.36)</td>
<td>0.76 (0.50 – 1.17)</td>
</tr>
<tr>
<td>Statin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.53 (0.22 – 0.87)</td>
<td>0.83 (0.51 – 1.23)</td>
<td>0.79 (0.49 – 1.22)</td>
</tr>
<tr>
<td>No</td>
<td>0.55 (0.33 – 0.76)</td>
<td>0.81 (0.34 – 1.32)</td>
<td>0.77 (0.44 – 1.12)</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.49 (0.07 – 0.75)</td>
<td>0.81 (0.47 – 1.08)</td>
<td>0.75 (0.45 – 0.99)</td>
</tr>
<tr>
<td>No</td>
<td>0.56 (0.31 – 0.86)</td>
<td>0.82 (0.41 – 1.38)</td>
<td>0.87 (0.49 – 1.22)</td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>111.9 ± 31.9</td>
<td>107.6 ± 27.2</td>
<td>111.0 ± 28.7</td>
</tr>
<tr>
<td>No</td>
<td>101.2 ± 24.2</td>
<td>100.9 ± 27.7</td>
<td>102.2 ± 23.1</td>
</tr>
<tr>
<td>Aspirin</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Yes</td>
<td>105.2 ± 26.8</td>
<td>103.9 ± 27.0</td>
<td>105.9 ± 26.5</td>
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<tr>
<td>No</td>
<td>104.3 ± 29.1</td>
<td>102.1 ± 29.2</td>
<td>104.2 ± 23.7</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>105.3 ± 28.1</td>
<td>105.3 ± 26.7</td>
<td>105.7 ± 24.9</td>
</tr>
<tr>
<td>No</td>
<td>104.4 ± 27.0</td>
<td>100.9 ± 28.7</td>
<td>104.8 ± 26.3</td>
</tr>
<tr>
<td>Ca(^{2+}) antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>100.8 ± 26.4</td>
<td>106.2 ± 34.4</td>
<td>109.9 ± 34.9</td>
</tr>
<tr>
<td>No</td>
<td>105.8 ± 27.8</td>
<td>102.7 ± 26.2</td>
<td>104.3 ± 23.1</td>
</tr>
<tr>
<td>Statin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>107.1 ± 25.5</td>
<td>106.0 ± 29.5</td>
<td>106.2 ± 25.9</td>
</tr>
<tr>
<td>No</td>
<td>101.8 ± 30.0</td>
<td>99.7 ± 24.7</td>
<td>104.1 ± 25.1</td>
</tr>
<tr>
<td>Long-acting nitrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>110.6 ± 20.3</td>
<td>106.0 ± 29.5</td>
<td>106.2 ± 25.9</td>
</tr>
<tr>
<td>No</td>
<td>103.0 ± 29.4</td>
<td>99.7 ± 24.7</td>
<td>104.1 ± 25.1</td>
</tr>
</tbody>
</table>

Results are median (interquartile range) and mean ± standard deviation.

*Red indicates highly statistically significant (p < 0.01); †blue indicates statistically significant (p < 0.05) and ‡pink indicates a trend (p < 0.1). ACE-I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker.
When TIMP-1 measurements, age, sex, development of ISR and treatment with ACE-I/ARB were included in a multivariate model, the association between diabetes and TIMP-1 concentration remained significant, indicating that the association of TIMP-1 and ACE-I/ARB medication was a surrogate for a history of diabetes. Rates of diabetes were higher in those with diastolic dysfunction (see section 8.2.4). However, when diastolic dysfunction was included with diabetes in a multivariate model, there was no significant association with either variable and TIMP-1, meaning that the independence of this association could not be established. Active MMP-9 appeared to be increased in patients with a history of diabetes, but the association did not reach statistical significance. Active MMP-9 and TIMP-1 levels were similar across other clinical and demographic measures, including patient-reported histories of hypertension and dyslipidaemia, ethnicity, smoking history and lipids.

Active MMP-9 was higher at index in those on treatment with Ca\(^{2+}\) antagonists, with non-significantly higher levels at three and six months. However, those receiving Ca\(^{2+}\) antagonists were older and more likely to be female and the relationship between active MMP-9 and Ca\(^{2+}\) antagonists was not independent of these variables.

### 8.2.4. Association of MMPs with diastolic dysfunction

Of the 163 patients in the prospective study, a sub-set of 119 patients were included in a comparison of diastolic dysfunction and active MMP-9 and TIMP-1. Compared to those who did not have an echocardiographic evaluation of diastolic dysfunction, there was no difference in age, sex, index clinical presentation and the development of further clinical events in those included in this analysis.

Because there were few participants with severe diastolic dysfunction (n = 6), patients with moderate and severe diastolic dysfunction were included in the same category. Patients with worse diastolic dysfunction were older, more likely to have diabetes mellitus and less likely to be male, otherwise they had similar clinical and demographic details (Table 8.9). Of note, while they all recently underwent PCI, the rates of acute coronary syndrome and acute myocardial infarction were similar between the two groups, and there was no preponderance towards those who suffered clinical events towards having worse diastolic dysfunction.

The echocardiographic profiles for the groups are displayed in Table 8.10. Ejection fractions were similar in both groups. With more severe diastolic dysfunction, there was higher peak early and atrial filling, shorter deceleration time and E/A ratio, shorter deceleration time, lower e’ and a greater E/e’.
Table 8.9 Demographic and clinical factors for diastolic dysfunction

<table>
<thead>
<tr>
<th></th>
<th>Normal n = 24</th>
<th>Mild n = 19</th>
<th>Mild-Moderate n = 42</th>
<th>Moderate or Severe n = 34</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>53.8 ± 8.0</td>
<td>61.3 ± 9.0</td>
<td>66.1 ± 10.1</td>
<td>63.7 ± 11.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Sex, male</td>
<td>23 (95.8%)</td>
<td>18 (94.7%)</td>
<td>35 (83.3%)</td>
<td>21 (61.8%)</td>
<td>0.0054</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>98.2 ± 9.5</td>
<td>103.3 ± 8.6</td>
<td>101.5 ± 11.3</td>
<td>100.7 ± 12.1</td>
<td>0.46</td>
</tr>
<tr>
<td>Hypertension</td>
<td>17 (70.8%)</td>
<td>14 (73.7%)</td>
<td>29 (70.7%)</td>
<td>22 (64.7%)</td>
<td>0.90</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>12 (50.0%)</td>
<td>9 (47.4%)</td>
<td>24 (57.1%)</td>
<td>21 (61.8%)</td>
<td>0.74</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>8 (19.0%)</td>
<td>4 (11.7%)</td>
<td>0.031</td>
</tr>
<tr>
<td>Smoking pack years*</td>
<td>16.4 (0.0 – 30.0)</td>
<td>13.0 (0.0 – 25.6)</td>
<td>3.8 (0.0 – 13.3)</td>
<td>11.7 (0.0 – 30.0)</td>
<td>0.46</td>
</tr>
<tr>
<td>ACE-I or ARB</td>
<td>4 (16.7%)</td>
<td>4 (21.1%)</td>
<td>19 (45.2%)</td>
<td>13 (38.2%)</td>
<td>0.063</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>10 (41.7%)</td>
<td>8 (42.1%)</td>
<td>27 (64.3%)</td>
<td>18 (52.9%)</td>
<td>0.23</td>
</tr>
<tr>
<td>Ca^{2+} channel antagonist</td>
<td>1 (4.2%)</td>
<td>3 (15.8%)</td>
<td>8 (19.1%)</td>
<td>7 (20.6%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Statin</td>
<td>12 (50.0%)</td>
<td>7 (36.8%)</td>
<td>30 (71.4%)</td>
<td>20 (58.8%)</td>
<td>0.064</td>
</tr>
<tr>
<td>Triple vessel disease</td>
<td>3 (12.5%)</td>
<td>1 (5.3%)</td>
<td>11 (26.2%)</td>
<td>8 (23.6%)</td>
<td>0.10</td>
</tr>
<tr>
<td>Index clinical presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>SA</td>
<td>5 (20.8%)</td>
<td>3 (15.8%)</td>
<td>15 (35.7%)</td>
<td>10 (29.4%)</td>
<td></td>
</tr>
<tr>
<td>UA</td>
<td>1 (4.2%)</td>
<td>5 (26.3%)</td>
<td>6 (14.3%)</td>
<td>5 (14.7%)</td>
<td></td>
</tr>
<tr>
<td>NSTEMI</td>
<td>10 (41.7%)</td>
<td>10 (52.6%)</td>
<td>14 (33.3%)</td>
<td>11 (32.4%)</td>
<td></td>
</tr>
<tr>
<td>STEMI</td>
<td>8 (33.3%)</td>
<td>1 (5.3%)</td>
<td>7 (16.7%)</td>
<td>8 (23.5%)</td>
<td></td>
</tr>
<tr>
<td>Clinical event</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>Control</td>
<td>17 (70.8%)</td>
<td>12 (63.1%)</td>
<td>23 (54.8%)</td>
<td>22 (64.7%)</td>
<td></td>
</tr>
<tr>
<td>ISR</td>
<td>2 (8.4%)</td>
<td>3 (15.8%)</td>
<td>12 (28.5%)</td>
<td>9 (26.5%)</td>
<td></td>
</tr>
<tr>
<td>Non-ISR event</td>
<td>5 (20.8%)</td>
<td>4 (21.1%)</td>
<td>7 (16.7%)</td>
<td>3 (8.8%)</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± standard deviation, median (interquartile range) or number (percentage). Results by ANOVA or Kruskall-Wallis and Chi².
### Table 8.10 Echocardiographic and MMP data for diastolic dysfunction

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild</th>
<th>Mild-Moderate</th>
<th>Moderate or Severe</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 24</td>
<td>n = 19</td>
<td>n = 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>53.5 ± 7.1</td>
<td>54.2 ± 7.7</td>
<td>54.1 ± 6.1</td>
<td>54.2 ± 7.4</td>
<td>0.98</td>
</tr>
<tr>
<td>Peak E, cm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.60 (0.55 – 0.70)</td>
<td>0.50 (0.43 – 0.60)</td>
<td>0.60 (0.50 – 0.70)</td>
<td>0.85 (0.70 – 0.90)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Peak A, cm/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50 (0.40 – 0.60)</td>
<td>0.70 (0.60 – 0.70)</td>
<td>0.70 (0.60 – 0.80)</td>
<td>0.70 (0.60 – 0.80)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.27 ± 0.26</td>
<td>0.77 ± 0.17</td>
<td>0.88 ± 0.23</td>
<td>1.41 ± 0.65</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DT, ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>214.0 ± 29.6</td>
<td>274.0 ± 65.3</td>
<td>257.6 ± 88.4</td>
<td>181.8 ± 29.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>92.3 ± 14.3</td>
<td>102.0 ± 32.7</td>
<td>99.1 ± 17.8</td>
<td>93.4 ± 12.9</td>
<td>0.23</td>
</tr>
<tr>
<td>IVS, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.13 ± 0.15</td>
<td>1.19 ± 0.21</td>
<td>1.22 ± 0.22</td>
<td>1.19 ± 0.19</td>
<td>0.27</td>
</tr>
<tr>
<td>PW, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>1.10 (0.95 – 1.20)</td>
<td>1.10 (1.00 – 1.20)</td>
<td>1.05 (1.00 – 1.20)</td>
<td>1.10 (1.00 – 1.20)</td>
<td>0.60</td>
</tr>
<tr>
<td>LA, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>68.9 ± 21.3</td>
<td>57.9 ± 20.5</td>
<td>66.7 ± 22.2</td>
<td>63.2 ± 23.0</td>
<td>0.36</td>
</tr>
<tr>
<td>e’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.090 ± 0.016</td>
<td>0.075 ± 0.020</td>
<td>0.057 ± 0.012</td>
<td>0.064 ± 0.019</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E/e’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.6 (5.7 – 8.6)</td>
<td>7.1 (6.3 – 8.0)</td>
<td>10.0 (10.0 – 12.5)</td>
<td>12.7 (11.3 – 16.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hs-CRP, mg/L*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.1 (0.7 – 4.0)</td>
<td>2.2 (1.1 – 3.7)</td>
<td>2.8 (1.4 – 4.9)</td>
<td>2.7 (1.4 – 5.4)‡</td>
<td>0.67</td>
</tr>
<tr>
<td>Index active MMP-9, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.23 (0.10 – 0.60)</td>
<td>0.64 (0.31 – 0.87)†</td>
<td>0.51 (0.22 – 0.72)†</td>
<td>0.72 (0.49 – 1.31)‡</td>
<td>0.0003</td>
</tr>
<tr>
<td>3-month active MMP-9, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.55 (0.39 – 0.82)</td>
<td>0.85 (0.32 – 1.01)</td>
<td>0.64 (0.33 – 1.07)</td>
<td>1.47 (0.86 – 2.21)†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>6-month active MMP-9, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.61 (0.34 – 0.87)</td>
<td>0.95 (0.65 – 1.12)†</td>
<td>0.72 (0.26 – 1.12)</td>
<td>1.02 (0.62 – 1.64)</td>
<td>0.059</td>
</tr>
<tr>
<td>Index TIMP-1, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>97.1 ± 17.9</td>
<td>98.9 ± 28.0</td>
<td>112.8 ± 26.3†</td>
<td>104.5 ± 18.7</td>
<td>0.038</td>
</tr>
<tr>
<td>3-month TIMP-1, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>103.3 ± 25.7</td>
<td>87.5 ± 18.0†</td>
<td>115.5 ± 34.2</td>
<td>98.5 ± 23.0</td>
<td>0.0024</td>
</tr>
<tr>
<td>6-month TIMP-1, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>95.5 ± 18.3</td>
<td>103.2 ± 27.3</td>
<td>113.3 ± 31.1</td>
<td>102.8 ± 24.6†</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Results are mean ± standard deviation or median (interquartile range). DT = deceleration time of early wave; E/A = early/atrial ratio of trans-mitral blood flow; E/e’ = ratio of the early trans-mitral flow to the peak velocity of the mitral annulus; e’ = peak velocity of mitral annulus; LA = left atrial wall thickness; IVRT = isovolumetric relaxation; IVS, interventricular septum thickness; PW, = thickness of posterior ventricular wall. *High sensitivity C-reactive protein measurements greater than 10 mg/L were excluded (n=37) due to presumed acute inflammation. † p <0.05 compared to Normal. ‡ p < 0.001 compared to Normal.
Figure 8.3 Serial active MMP-9 measurement by diastolic dysfunction categories
Levels of active MMP-9 are plotted for each individual at index, three- and six months, with lines connecting the values. Results are by multiple regression, clustering for each individual to preserve the independence of observations, with active MMP-9 level transformed by natural logarithm. Each comparison included the whole cohort from the prospective study, including controls, the ISR group and the non-ISR events group.
Figure 8.4 Serial TIMP-1 measurements by diastolic dysfunction categories
Levels of TIMP-1 are plotted for each individual at index, three- and six months, with lines connecting the values. Results are by multiple regression, clustering for each individual to preserve the independence of observations. Each comparison included the whole cohort from the prospective study, including controls, the ISR group and the non-ISR events group. Other comparisons were not statistically significant with \( p > 0.15 \).
The active MMP-9 and TIMP-1 levels for each diastolic dysfunction subgroups are displayed in Table 8.10. Active MMP-9 levels were significantly higher with more severe diastolic dysfunction at all time points (Table 8.10, Figure 8.3). This was highly significant at the index and three-month time point, but lost significant at six months. Using logistic regression the relationship between active MMP-9 level at index was independently associated with moderate or severe diastolic dysfunction when adjusting for age, sex, clinical presentation and events at one year (for a 1ng/mL increase in active MMP-9: OR 2.1 (95% CI 1.3 – 3.6; p = 0.0055). Utilizing a cut off point of ≥ 0.9 ng/mL the unadjusted odds ratio was 5.9 (95% CI 2.2 – 15.7; p = 0.0003) and after adjusting for age, sex, clinical presentation and events at one year the odds ratio was 4.4 (95% CI 1.5 – 12.6; p = 0.006).

Index active MMP-9 levels were also significantly higher with all individual diastolic dysfunction categories compared to those with normal function, indicating that it may be a sensitive marker for early changes (Table 8.10).

In contrast to the association of both active MMP-9 and TIMP-1 with diastolic dysfunction, there was no association between either active MMP-9 or TIMP-1 with systolic dysfunction. There was no correlation between left ventricular ejection fraction and active MMP-9 or TIMP-1 at any time point.

This finding corroborates our previous finding that active MMP-9 was increased in diastolic dysfunction. TIMP-1 levels were significantly altered by diastolic dysfunction category (Table 8.10), but this was mainly driven by increased levels in the mild-moderate group compared to all other groups (Figure 8.4). Diastolic dysfunction could have confounded the relationship between change in active MMP-9 and ISR. Therefore, diastolic dysfunction was included in a logistic regression model, and the association between change in active MMP-9 and ISR remained significant.
8.3. Discussion

8.3.1. Active MMP-9 and TIMP-1 are not associated with the development of incident cardiovascular events after coronary stenting

The main finding of this chapter was that, in comparison to the association of active MMP-9 with the development of ISR, there does not appear to be an association between either active MMP-9 or TIMP-1 and the development of other cardiovascular events. There was no association either of levels measured before coronary intervention, or the change at three and six months. Furthermore, there was no association between incident cardiovascular events and active MMP-9 or TIMP-1 in the months before the presentation with the event. In the majority of cases, the time between the last blood sample measurement and the development of symptoms was between one and five months. It is possible that there may be more acute changes before presentation. The time frame of plaque remodelling before erosion and rupture has only recently begun to be delineated, as the necessary tools have not been available. In recent years, insights have been gained from carotid magnetic resonance imaging, and intravascular ultrasound of coronary arteries, where imaging evidence of plaque rupture is highly predictive of brain ischaemia, and unstable features have prognostic value for acute coronary syndrome. While the thrombotic component of acute myocardial infarction appears to evolve over hours, it is clear that plaques remodel over months to years, and can regress in response to treatment.

8.3.2. Involvement of MMPs in the pathophysiology of coronary events

The development of stable coronary artery disease is caused by progression of atherosclerotic plaque to the point that on physical exertion there is insufficient myocardial perfusion whereas unstable disease and acute coronary syndrome are caused by the disruption of the plaque surface through erosion or rupture, and the formation of thrombus. The growth of stable atherosclerotic stenoses is a complex process with continued expansion of lipid core and fibrous cap, constrictive remodelling, along with plaque disruption with super-imposed healing. While much research has focussed on the role MMPs play in the unstable plaque, multiple MMPs are present in atherosclerotic arteries which are absent from the normal arterial wall, with evidence of increased MMPs-1, -2, -3, -8, -9 and -14 in
atherosclerotic lesions. Only MMPs-2 and -3 appear to be present in normal arteries. Both positive and constrictive wall remodelling appear to rely on the activity of MMPs and over-expression of MMP-9 promotes outward remodelling. Therefore a relative deficiency may contribute to the progression of atherosclerosis via constrictive remodelling at the site of a lesion. The progression from unstable atherosclerotic plaque to rupture and acute coronary syndrome most likely includes degradation of the fibrous cap ECM by MMPs. MMP-1 is elevated in carotid plaques with evidence of old intra-plaque haemorrhage. MMPs-1, -3 and -9 protein and activity are up-regulated in the shoulder region of human plaque and unstable atheromatous lesions. Mouse knockouts of MMPs-3, -9 and -12 have less fibrous layers, indicating a more unstable phenotype. Experimental up-regulation of active MMP-9, but not pro-MMP-9, reliably induces widespread plaque rupture in atherosclerotic mice. Similar MMPs appear to be associated with both the development of atherosclerosis and the progression of unstable plaques. This is probably explained by differences in the levels of MMPs, particularly MMP-9, which may be higher in unstable plaques, and additionally the location, with MMPs in the shoulder region contributing to instability.

Stent thrombosis appears to develop from an imbalance in the local haemostatic environment, with the initial tissue damage and endothelial denudation leading to very high rates of early stent thrombosis (up to 20% of patients) before the development of contemporary anti-platelet regimens. However, patient factors such as diabetes mellitus, and procedural factors such as stent under-expansion, also play a role. The association of endothelial defects and stent thrombosis is further confirmed by the association of poor re-endothelialisation and the need for prolonged anti-platelet therapy with drug-eluting stenting. MMPs have not been specifically linked to coronary stent thrombosis, but there is significant interplay between MMPs and thrombus formation. Experimental over-expression of MMP-9 promotes thrombus, and MMP-2 is involved in propagating platelet activation, with attenuated thrombus formation in MMP-2 -/- mice. Furthermore, platelet activation and thrombus formation are associated with the release of MMPs, and the plasminogen activating system appears to be important for MMP function. Therefore a potential association between stent thrombosis and MMPs could be causal in either direction.
8.3.3. Clinical studies of circulating MMPs and cardiovascular prognosis

Others have found an overall modest relationship between pro-MMP-9 and cardiovascular events. A number of clinical and secondary prevention, and population-based studies have examined the role of one-off measurements of MMPs and the subsequent development of incident coronary events. The primary prevention population-based studies have included nearly 300,000 patient years of follow up. Two studies reported the incidence of cardiovascular death, representing 281 cardiovascular deaths and 1608 controls. Elevated TIMP-1 levels were associated with an increased risk of death during the study period (HR 1.22 [95% CI 1.09 – 1.37] and 1.97 [95% CI 1.53 – 2.53] for each standard deviation rise). Pro-MMP-9 was less robustly associated with cardiovascular death, with a hazard ratio of 1.09 [95% CI 0.97 – 1.23] for each standard deviation rise. The other study was able to detect pro-MMP-9 in only 30% of participants, and there was a modest association of detectable levels with incident cardiovascular death (HR 1.24 [95% CI 0.62 – 2.49]).

Of note, pro-MMP-9 had a stronger relationship with non-cardiovascular mortality than cardiovascular mortality (HR 1.11 [95% CI 1.02 – 1.22] vs. HR 1.09 [95% CI 0.97 – 1.23], per standard deviation increase) in a cohort of elderly Swedish men, although both associations were modest. TIMP-1 was more closely correlated with cardiovascular than non-cardiovascular death (HR 1.22 [95% CI 1.09 – 1.37] vs. HR 1.04 [95% CI 0.93 – 1.16], per standard deviation increase).

When the endpoint of “any cardiovascular event” was considered, each standard deviation increase of TIMP-1 was associated with a hazard ratio of 1.36 (95% 1.09–1.71), and myocardial infarction (HR 1.14 [95% CI 0.98–1.33] and stroke (HR 1.18 [95% CI 1.04–1.35]). However, Weiss et al. saw no difference in TIMP-1 levels amongst Norwegian males who developed the composite endpoint of any cardiovascular event.

Pro-MMP-9 was associated with the development of combined cardiovascular events (OR 1.37 [95% CI 1.04 – 1.82, for top vs. bottom tertile], OR 1.93 [95% CI 1.13–2.30, for top vs. other quartiles] and HR 1.50 (95% CI 0.87–2.58, for the presence of detectable levels). The top tertile compared to the bottom tertile of pro-MMP-9 was associated with an OR of 1.53 (95% CI 1.09 - 2.13) for the development of myocardial infarction, and OR of 1.36 (95% CI 0.92, 2.00) for stroke. However, pro-MMP-9 was not associated with cardiovascular events in the Women’s Health Initiative trials (OR 1.06 [95% CI 0.92 –
Association of MMP markers and cardiovascular variables

1.22) or with incident myocardial infarction or stroke in a cohort of Swedish males (HRs 0.98 [95% CI 0.83–1.17] and 1.00 [95% CI 0.86–1.17]) respectively. The findings above are probably consistent with a modest association both of pro-MMP-9 and TIMP-1 with cardiovascular disease, however the heterogeneity of outcomes reported precludes a meaningful meta-analysis. Of note, pro-MMP-9 may be more strongly associated with non-cardiovascular death than cardiovascular death.

A broader range of MMPs have been investigated in secondary prevention and clinical studies, with pro-MMPs-1, 779 -2, 478,704,720 -3, 443,704,720 -9, 398,478,704,720,776,778,780 -12, 706 total (i.e. fraction available for activation) MMPs-2, 704 -3704 and -9704 and active MMPs -2781 and -9, 781 as well as TIMPs-1, 706,776,777,779 -2, 777 -3777 and -4777 all being measured in patients with cardiovascular disease and linked to the development of future events. Ye et al.395 measured total (i.e. fraction available for activation) MMPs-2, -3 and -9 in a patient population who underwent revascularisation with PCI and CABG, and subsequently correlated these markers with the need for any revascularisation. However, the aetiology of the stenoses in the vast majority of those who required revascularisation was restenosis (both bare metal stent and vein graft restenosis), so this study will not be discussed further here (but is included in Chapter 7.3.1 in discussion of circulating MMPs and restenosis).

As active MMP-9 and TIMP-1 were investigated in this study these proteins will be discussed first. Also, as pro-MMP-9 and TIMP-1 have been the most commonly investigated markers, there is a large body of associated literature. One interesting study showed that pro-MMP-9 measurements at the time of angiography, in a population with stable coronary disease undergoing delayed PCI, were predictive of the angiographic progression of coronary stenoses over a time period of just under five months.478 Progressive narrowing of stenoses initially identified at the first angiogram occurred in 28% of patients, and those with higher levels of pro-MMP-9 at baseline were significantly more likely to have a further narrowing (OR 2.7 [95% CI 1.2 – 6.4]). This association was independent of cardiovascular risk factors.

One comprehensive study linked variation of circulating pro-MMP-9 to both functional genetic markers and outcome in patients undergoing angiography for stable or unstable angina.398 They found that the C-1562T polymorphism in the promoter region (previously shown to increase MMP9 expression one and a half-fold) explained at least a moderate amount of the variation in circulating pro-MMP-9 (a correlation coefficient was
not given). Circulating pro-MMP-9 was associated with cardiovascular death, with a hazard ratio of 1.4 (95% CI 1.2 – 1.8) and ~85% of those with levels in the highest quartile survived at four years, compared to ~95% of those in the lowest two quartiles. While cardiovascular death was strongly associated with circulating pro-MMP-9 there was no association of the promter polymorphism and cardiovascular death. Other common polymorphisms were not associated with alterations in pro-MMP-9. Associations between pro-MMP-9 levels and cardiovascular events may be non-genetically determined, or determined by genes other than the MMP9 gene itself. Total MMP-9 levels were elevated in patients with stable disease who went on to have further events, with nearly half of all cases having elevated levels, compared to 28% of controls. The authors did not measure pro-MMP-9, so the difference in predictive value is difficult to determine. Dhillon et al., Apple et al., and Beygui et al. found that levels of pro-MMP-9 were similar amongst those with acute coronary syndrome who went on to have incident myocardial infarction, heart failure or cardiovascular death.

Only one study has attempted to show whether pro-MMP-9 contributes to prediction of events using a validated risk prediction model, the GRACE score (which consists of age, heart rate, blood pressure, serum creatinine, heart failure, ECG changes, cardiac enzymes and cardiac arrest). However, there was no association with pro-MMP-9 and incident cardiovascular events in that cohort, a group of patients with acute coronary syndrome. Despite this, there was an association between lower pro-MMP-9 and a composite of incident heart failure and death, and pro-MMP-9 contributed modestly to the predictive value of the GRACE score for this outcome (area under the curve for GRACE score = 0.775, vs. area under the curve for GRACE score + pro-MMP-9 quartiles = 0.788; a 1.7% relative improvement of discrimination). Pro-MMP-9 was lower in patients with cardiac mortality or incident myocardial infarction in a cohort of male patients undergoing angiography and followed for two years, but this was not independent of risk factors.

TIMP-1 was independently associated with cardiac mortality and incident myocardial infarction, as well as all cause mortality in a group of male patients undergoing angiography. Those with the highest quartile of TIMP-1 at angiography had a survival rate of 72% at two years, compared to over 95% survival for the lowest quartile and there appeared to be a strong dose-response relationship. Patients with higher TIMP-1 were older, and more had presented with myocardial infarction or heart failure. When
adjusting for these factors, TIMP-1 was still associated with cardiac mortality and incident myocardial infarction, but the associations were attenuated.\textsuperscript{776} Two studies examined circulating TIMP-1 after myocardial infarction,\textsuperscript{777,779} and both found that TIMP-1 was associated with a composite end point of cardiovascular events (total n = 1546, follow up > 1.5 years). Manhenke \textit{et al.}\textsuperscript{779} recorded 28 cardiovascular deaths and 79 cases of incident heart failure. The number of events was not given by Kelly \textit{et al.}\textsuperscript{777} TIMP-1 was more strongly associated with cardiovascular death than the composite of cardiovascular death and incident heart failure, and was not associated with recurrent myocardial infarction.\textsuperscript{779} Kelly \textit{et al.}\textsuperscript{777} also measured TIMPs-2 and -4 at the same time as TIMP-1, and found that all TIMPs were independent of each other, and the GRACE score in the prediction of a composite of cardiovascular events. The addition of TIMPs-1, -2 and -4 to the GRACE score raised the discriminatory capacity of the GRACE score (area under the curve for GRACE score = 0.69, vs. area under the curve for GRACE score + TIMPs-1, -2 and -4 = 0.72, a 4.4\% relative improvement of discrimination).\textsuperscript{777}

Pro-MMP-3 was higher in those with cardiovascular death, and this was not explained by incident heart failure or myocardial infarction.\textsuperscript{720} However, the median NT-proBNP was much higher in the group with events, and pro-MMP-3 levels were moderately correlated (Spearman’s rho = 0.23), although they were not significantly elevated in those with a diagnosis of heart failure. As discussed in Chapter 5.3.1 pro-MMP-3 levels before hospital discharge were predictive of the degree of worsening of left ventricular functioning,\textsuperscript{443} but the association of pro-MMP-3 did not remain significant on multivariate adjustment. Wu \textit{et al.}\textsuperscript{704} measured the total (\textit{i.e.} fraction available for activation) MMP-3 in patients with stable disease, finding that levels were significantly higher amongst those who had events, with 80\% of cases having high levels compared to less than 60\% of controls. The association of total MMP-3 with cardiovascular events was independent of the other factors that were associated with cardiovascular events in this study, including high sensitivity C-reactive protein and level of total MMP-9. Total MMP-3 appeared to be able to identify a high-risk subgroup; only 40\% of those having an elevated level of total MMP-3 survived event-free at 36 months, compared to 80\% of those with low levels of total MMP-3.\textsuperscript{704}

Pro-MMP-2 was associated with cardiac death amongst patients with acute coronary syndrome, who were followed for a median of one and a half years.\textsuperscript{720} This appeared to be particularly prominent amongst those with the highest pro-MMP-2 levels, as nearly 20\% of
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those with levels in the top quartile died from cardiovascular disease in this time period, compared to 10% of those with lower levels. This was not explained by an association of pro-MMP-2 with either incident myocardial infarction or development of heart failure. However, pro-MMP-2 was higher in those with heart failure at index. Hence the relationship between pro-MMP-2 and cardiovascular death could be mediated through the presence of heart failure. In contrast to this finding, pro-MMP-2 was not elevated in patients who had progression of coronary stenoses on angiography;\textsuperscript{478} and total (\textit{i.e.} fraction available for activation) MMP-2 levels were similar between those with and without further cardiovascular disease in a group with stable coronary artery disease.\textsuperscript{704} Only one study has examined pro-MMP-1, a prospective study following 233 patients with acute myocardial infarction.\textsuperscript{779} The authors found that levels of pro-MMP-1 were not different according to the development of cardiovascular death, incident heart failure or recurrent myocardial infarction.

Taking these findings together, it has not been conclusively established that pro-MMP-9 is associated with the secondary development of coronary disease. It appears that pro-MMP-9 was associated with the development of further events in the cohorts without acute myocardial infarction, and no association was able to be confirmed in any of the studies of patients with acute myocardial infarction. In the primary prevention studies a fairly consistent, although modest, relationship of pro-MMP-9 with incident cardiovascular disease was noted. When the primary prevention studies are compared with those performed in patients with prevalent coronary disease, the observed findings are consistent with a modest association in both cases, but with insufficient power to detect an association in the smaller secondary prevention studies.

A number of large population-based studies have linked TIMP-1 levels with the incidence of cardiac death, as well as individual cardiovascular end points. Only one study has assessed the role of TIMPs-2 and -4 and outcomes in cardiovascular disease, but suggested strong, independent roles for all of TIMPs-1, -2 and -4.\textsuperscript{777} This indicates that the governing factors for each TIMP are different, and are each associated with cardiovascular disease in different ways.

MMP-3 has been associated consistently with cardiovascular events, with both the pro- and total- forms appearing to have prognostic information.\textsuperscript{443,704,720} MMP-3 may be associated with adverse cardiac remodelling after myocardial infarction. Interestingly, while
there was no association between markers of left ventricular dysfunction at index, pro-MMP-3 levels correlated with the worsening of ejection fraction when re-assessed months later.443

Only one study has looked the levels of circulating pro-MMP-1 and cardiovascular disease prognosis, with no evidence for association.779 While a number of studies have looked at the associations of pro-MMP-2, the findings have been inconsistent.478,704,720 These results are consistent with either no association between the levels of pro-MMPs-1 and -2 and incidence of coronary disease, or weaker associations than those seen for pro-MMPs-3, -9, total-MMP-3 and TIMPs.

One of the explanations for elevated MMPs with incident coronary disease is the mechanism of cardiac remodelling and heart failure. This may be either a cause or a consequence, i.e. MMPs are elevated with the development of heart failure which subsequently leads to death, or myocardial ischaemia raises the risk for further myocardial ischaemia but also leads to infarction which precipitates remodelling and heart failure. This view is supported by the findings of Wagner et al.781 and Manhenke et al.779 who found that pro-MMP-9 predicted the development of heart failure, and that associations between TIMP-1 and cardiovascular death disappear after adjustment for BNP. A large study by Dhillon et al.720 observed that despite elevated pro-MMP-2 being associated with future cardiac death, there was no difference in pro-MMP-2 in those who did not die, but suffered either recurrent myocardial infarction or incident heart failure. However, the difference in levels of pro-MMP-2 between the heart failure group and the control group (medians 25.4 vs. 21.8 ng/mL, \( p = 0.55 \)) was similar in magnitude to the difference between the cardiac death group and the controls (medians 26.8 vs. 21.8 ng/mL, \( p = 0.006 \)). The reason for the vast difference in level of significance is unclear, but may reflect a greater variation in the heart failure group.

The exclusion of patients with heart failure may therefore explain why there was no difference in active MMP-9 or TIMP-1 measurements by cardiovascular outcome. This study observed a low rate of incident heart failure in this cohort, with only two individuals developing confirmed heart failure during one year of follow up. The reason for this is likely to be two-fold. Patients with heart failure at index were excluded from this study, which could have led to the cohort having lower risk for the development of further heart
failure events. Secondly, this may represent false negatives, as this study relied on patient report of hospitalisation before hospital notes were reviewed.

The outcomes assessed in this chapter were heterogeneous, so further exploratory analysis by individual aetiology of events was undertaken. The sample size of this study was too small to confirm any relationships, but instead the aim was to quantify the effect size. The result of this analysis is consistent with either no change, or very small, signal of changes in active MMP-9 and TIMP-1 with the development of further coronary lesions.

These findings suggest that these characteristics of individual atherosclerotic plaques do not affect the circulating levels of active MMP-9 and TIMP-1. This would be more accurately quantified by serial invasive monitoring of individual plaques, for example with intravascular ultrasound or optical coherence tomography. It is more likely that measurements of MMPs reported to have associations with plaque characteristics in the literature, as well as in the retrospective study (Chapter 3.2.3, Table 3.9), reflect systemic atherosclerotic plaque phenotype rather than that of individual plaques. For example, the expression of total (i.e. that available for activation) MMP-8 in carotid atherosclerosis undergoing endarterectomy independently predicts plaque rupture in other areas of the body. Therefore, the change in active MMP-9 appears to have specificity for ISR.

8.3.4. Association of vascular risk factors and MMPs in the prospective study

The relationships between MMPs and vascular risk factors amongst the participants in the prospective study were explored in this chapter. Many separate comparisons were made, and if adjustment for multiple testing were carried out, none would reach statistical significance. Hence, all of these results should be viewed as hypothesis generating.

The main findings were that active MMP-9 appeared to be elevated in participants with renal impairment, and TIMP-1 was elevated in participants with a history of diabetes mellitus. A number of studies have examined the association between circulating MMPs and possible confounding by cardiovascular medications and risk factors, and animal models have investigated the roles of MMPs in mechanistic studies of hypertension.

In this study, patients with impaired renal function had higher levels of active MMP-9. This is interesting as an MMP-9-related protein, neutrophil gelatinase-associated lipocalcin
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(NGAL), appears to be a useful marker of acute kidney injury. While the association between active MMP-9 and impaired renal function was modest, the observation may also have mechanistic implications for the measurement of active MMP-9, as increased circulating NGAL may promote the activation of MMP-9. A role for MMPs in a variety of kidney diseases has been established, including that of diabetic nephropathy, as suggested by the association with prescription of renin/angiotensin system antagonists in this study. Ban et al. noted increased total-MMP-7 in a cross sectional study of patients with renal disease secondary to diabetes. It appears there may be different MMP profiles with different aetiologies of renal failure. Other reports suggest decreased genetic and enzymatic expression of MMP-9 in rat models of diabetes. However in clinical studies, microalbuminuria is associated with increased pro-MMP-9 in plasma and urine, and changes in pro-MMP-9 appear to occur before microalbuminuria develop. Furthermore, pro-MMP-9 appears to correlate with reduction of microalbuminuria after treatment with ACE inhibitors. There was no association between active MMP-9 and diabetes in the retrospective study (Chapter 3.2.3, Table 3.9), but the reason for this discrepancy is not clear. Active MMP-9 has not otherwise been measured in people with renal impairment. Data on the specific aetiology of renal impairment in this study was not collected. In contrast to the retrospective study (Chapter 3.2.3, Table 3.9) there was no association between TIMP-1 and renal impairment in the present study.

The finding of increased TIMP-1 levels in association with diabetes in this study is in agreement with the association found in the retrospective study, where TIMP-1 levels were also higher in patients with diabetes mellitus (Chapter 3.2.3, Table 3.9). There was a trend towards higher active MMP-9 levels with diabetes, but this did not achieve statistical significance. Circulating plasma pro-MMP-9 has been reported to be higher in patients with diabetes, but a similar association with active MMP-9 could not be confirmed in this study.

There are probably multiple sources of altered MMPs in diabetes, with pro-MMPs-2 and -9 in the urine correlating with HbA1c and diabetic nephropathy indicating a renal source. Increased gene expression of MMP2 and MMP9 correlates with increased production of collagen type one in the vasculature of diabetic rats, and increased MMP-2 and -9 activity in the renal arteries of patients undergoing renal transplantation compared to those of the kidney donors. MMP-2 and -9 activities in the renal vessel walls were
highly correlated with in vivo arterial stiffness, quantified by non-invasive micromanometry.\textsuperscript{797}

Treatment with ACE-I/ARBs was also associated with increased TIMP-1, but it appeared that this was a surrogate for the presence of diabetes. As other anti-hypertensive drugs were not associated with TIMP-1 concentration, the renin/angiotensin system antagonists may have been prescribed for their renoprotective effects in this population.

There was no association of either active-MMP-9 or TIMP-1 with smoking, lipids or measures of body mass in this study. The associations of MMPs with conventional coronary risk factors was previously discussed in Chapter 3.3.5. Briefly, a number of studies saw small increases of pro-MMP-9 with various risk factors, including hypertension, obesity, smoking and dyslipidaemia.\textsuperscript{571-573,575,576} This is consistent with findings for active MMP-9, where a number of risk factors are associated with small increases in active MMP-9, but are not statistically significant on their own.

This study was unable to confirm relationships between lipids, smoking and hypertension with active MMP-9 or TIMP-1. While alterations in pro-MMP-9 have previously been linked to renal failure, this is the first study to link active MMP-9 to renal failure but appropriate control variables were not included in this study. This study was able to confirm the finding that TIMP-1 levels are elevated in the plasma of individuals with diabetes mellitus.

\subsection{8.3.5. Association between MMPs and diastolic dysfunction}

We found that active MMP-9, but not TIMP-1, was strongly associated with categories of diastolic dysfunction, and this relationship appeared to be independent of clinical and demographic variables. This finding is in agreement with our previous study,\textsuperscript{750} which is now confirmed prospectively, and including additional measures of diastolic dysfunction utilizing Tissue Doppler imaging. Diastolic dysfunction is common in the community,\textsuperscript{445} and is strongly associated with progression to heart failure\textsuperscript{798} and mortality.\textsuperscript{445}

Mouse models of diastolic heart failure show up-regulated MMPs and collagen gene expression.\textsuperscript{446} This has been confirmed in clinical studies, with decreased myocardial MMP-1 protein\textsuperscript{799} (but another studying finding elevated circulating pro-MMP-1)\textsuperscript{447} and elevated circulating levels of pro-MMPs-2,\textsuperscript{447,448,691} -3,\textsuperscript{447} -7,\textsuperscript{447} -8,\textsuperscript{447} -9\textsuperscript{447,448,691} and TIMPs-1\textsuperscript{447,448,691} -2\textsuperscript{447} and -4\textsuperscript{447} with diastolic heart failure. However, of these marker,
those with the greatest contribution to discrimination of diastolic function appear to be TIMP-1 and pro-MMP-9.\textsuperscript{447}

However, we observed changes not in symptomatic diastolic heart failure, but rather pre-clinical dysfunction in asymptomatic patients. Füth et al.\textsuperscript{800} demonstrated that pro-MMP-2 was higher in patients undergoing angiography for stable coronary artery disease who had pseudonormal (mild-moderate dysfunction).\textsuperscript{471} In the previous study, there was a trend towards increased TIMP-1 levels, but this did not achieve statistical significance.\textsuperscript{750} However, there was no association between pro-MMP-9 or active MMPs-1, -2 or -3 and diastolic dysfunction in our previous study.\textsuperscript{750}

Pro-MMP-9 and TIMP-1 were not correlated with diastolic dysfunction measurements in elderly participants in one population-based study\textsuperscript{573} and pro-MMP-7 but not TIMP-1 was elevated in patients with diabetes and diastolic dysfunction, but the results were not presented for each category of diastolic dysfunction, so it is unclear where the changes in pro-MMP-7 are occurring.\textsuperscript{498} In contrast to the above studies, both pro-MMP-9 and TIMP-1 were associated with echocardiographic measures in studies of hypertensive patients.\textsuperscript{691,801,802}

Saglam et al.\textsuperscript{802} showed that both pro-MMPs-3 and -9 were associated with multiple indices of worse diastolic dysfunction (including E/A ratio and e’), whereas Tayebjee et al. found an association between pro-MMP-9 and E/A ratio, but not e’,\textsuperscript{801} and TIMP-1 associated with E/A ratio and e’.\textsuperscript{801} Ahmed et al.\textsuperscript{691} saw that TIMP-1, but not pro-MMPs-2 or -9, was associated with e’, although this may be due to the higher levels of TIMP-1 amongst those with heart failure.

Martos et al.\textsuperscript{448} reported that there was no difference in pro-MMP-9 across diastolic dysfunction categories, although levels were much higher in patients with diastolic heart failure. The disparity between their findings with regard to the two MMP-9 isoforms indicate that the active form of MMP-9 is a more sensitive indicator of diastolic function. Their finding that pro-MMP-9 is greatly elevated in diastolic heart failure, along with the association between pro-MMP-9 and Doppler measurements, in the studies described above add weight to the contention that MMP-9 has an important role in diastolic dysfunction.\textsuperscript{801,802}

Because the study was designed to investigate the relationship between active MMPs and both ISR and other coronary events, the study population was disproportionately
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weighted with ISR cases. While they did not appear to have a preponderance towards any category of diastolic dysfunction, they were included in the multiple regression model, and the association between active MMP-9 and phases of diastolic dysfunction remained significant.

Due to the study design in this thesis, with all patients recently undergoing PCI for atherosclerotic disease (< 2 weeks before echocardiography), we cannot rule out that at least part of the observed results are an acute process such as myocardial stunning, which is also associated with a proteolytic state. However, stunning typically resolves within days after ischaemia whereas the changes we noted appeared to be sustained over six months, and we noted no association between diastolic dysfunction and index presentation indicating that the changes were not due to acute myocardial infarction. Therefore it is likely that the alterations of active MMP-9 are due to chronic diastolic dysfunction.

The expression profile of MMPs in experimental models of systolic and diastolic heart failure differ, with greater MMP-9 activity in diastolic heart failure, particularly in the middle layer of the left ventricle. Systolic heart failure was associated with a more moderate elevation of active MMP-9, expressed evenly throughout the ventricular wall. In a clinical study of systolic heart failure the prevalence of diastolic dysfunction measures was higher with elevated pro-MMP-9 (and pro-MMP-3). Furthermore, elevation of pro-MMPs-3 and -9 were independently related to prognosis, and appear to have a synergistic effect in predicting survival in the setting of systolic heart failure. Whereas previous studies have linked alterations of pro-MMPs to diastolic heart failure and more severe categories of diastolic dysfunction (E/e’ > 15), we observed that active MMP-9 was significantly different for all (Canadian Consensus Classification) categories of dysfunction, including asymptomatic dysfunction. Markers of collagen metabolism have been shown to be sensitive to treatment in heart failure. While B-type natriuretic peptide has been established as a strong prognostic marker, there is some evidence that pro-MMP-9 level may allow better stratification, at least in symptomatic patients with concurrent coronary disease. Although it still needs to be independently confirmed, active MMP-9 level may allow risk stratification with pre-clinical diastolic dysfunction.

While the association between active MMP-9 and diastolic dysfunction is interesting in its own right, this observation is also within this chapter in-order to highlight it as a form of ‘positive control’ for the activity ELISA. One of the issues in measuring different forms
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of MMPs is that there is no gold standard per se. While there is difficulty in establishing the validity from a biochemical point of view, here we have shown that active MMP-9 is not only associated with developing ISR, but also with the phase of diastolic dysfunction. Both ISR and diastolic dysfunction have strong pre-clinical arguments for the involvement of MMP activity, and therefore the observed associations essentially corroborate the biological relevance of the activity ELISA we used.

8.4. Limitations

There were a number of limitations of the section of this study looking at non-ISR cardiovascular outcomes. Firstly, there were small numbers of events, particularly when the analysis was split by the presumed aetiology of the disease (e.g. atherosclerotic progression, plaque rupture, stent thrombosis). Secondly, the composite outcome coronary events included heterogeneous outcomes: progression of stable disease, incident acute coronary syndrome, stent thrombosis and cardiovascular death. Therefore, an exploratory analysis by the outcome of each event was performed. Given the small numbers, so the aim of this comparison was to detect whether there was any signal associated with active MMP-9 or TIMP-1 and the ECM remodelling associated with the development of cardiovascular events. There was an inconsistent time from the last sampling time point to the onset of symptoms with cardiovascular outcome (interquartile range of 30 to 150 days) because of the unpredictable nature of incident cardiovascular events. While there did not appear to be an association of active MMP-9 or TIMP-1 with this variation, it may have prevented us from detecting an association with cardiovascular events. Additionally, the length of time from the last sampling time point to onset of symptoms with the cardiovascular outcome was relatively long, with a median time of nearly three months. While this is probably within the time frame of lesion remodelling before clinical presentation, it is still a relatively long time and this study was not able to rule out altered levels of active MMP-9 and TIMP-1 over shorter time intervals.

The exploratory analysis of associations between active MMP-9 was limited by multiple testing. The associations seen would not reach criteria for statistical significance if the \( \alpha \) value were corrected for multiple comparisons. Instead of concluding that there were no associations, the present study used a conservative interpretation, taking each finding in the context of the wider literature, and suggesting that novel associations were hypothesis
generating, rather than confirmatory. Lastly, the present study did not collect variables specifically to measure diabetes and kidney-specific confounding. Neither the aetiology or the chronicity of renal failure was collected.

A major limitation of the diastolic dysfunction study was that there was only one echocardiography and tissue Doppler study, and it may have been confounded by the recent ischaemic event and PCI. It would have been optimal to include a longitudinal component to the diastolic dysfunction assessment in order to determine whether active MMP-9 levels give any information about the prognosis of diastolic dysfunction severity.

8.5. Conclusions

Circulating active MMP-9 and TIMP-1 were not altered before intervention, or in the months following PCI, in those who went on to develop cardiovascular events. The changes over three months were not associated with the development of cardiovascular events, therefore evoked changes in active MMP-9 after PCI appear to be specific for the development of ISR. Furthermore, there was no signal that active MMP-9 or TIMP-1 were altered before presentation with incident cardiovascular events. Admittedly this study was underpowered to detect such an association if this did exit.

Impaired renal function was associated with elevated circulating active MMP-9, but not TIMP-1. Elevated pro-MMP-9 has been reported in patients with impaired renal function, but this is the first time that active MMP-9 has been associated with impaired renal function. This finding should be confirmed in a study recording confounding variables for renal disease, as well as an accurate quantification of the aetiology of renal disease.

Circulating plasma TIMP-1 was increased in patients with a history of diabetes, which has been observed in a number of previous studies. Active MMP-9 was altered in patients receiving Ca\textsuperscript{2+} channel antagonists, and TIMP-1 was higher in patients receiving medications inhibiting the renin/angiotensin system, but these observations appeared to be explained by confounding. There did not appear to be an association with other medications, tobacco smoking, hypertension or lipid variables. These associations may have been obscured by small sample size or by the fact that many participants in this study were on treatment for cardiovascular risk factors.

More severe grades of diastolic dysfunction were associated with higher levels of active MMP-9, and TIMP-1 was elevated with mild-moderate diastolic dysfunction, but not
either less nor more severe disease. Neither active MMP-9 or TIMP-1 levels were associated with left ventricular ejection fraction. Active MMP-9 may be involved in adverse remodelling in diastolic dysfunction and TIMP-1 may initially protect against the development of diastolic dysfunction, with impaired production of TIMP-1 being associated with severe disease.
9. General Discussion

9.1. Overview

ISR has been the limiting complication of percutaneous coronary intervention,\footnote{811} and while drug-eluting stents have reduced this problem, somewhere between 5\% and 10\% of interventions need to be repeated due to ISR.\footnote{812} ISR is caused by the characteristic hyperplastic response of the artery wall to injury.\footnote{64} The neointimal lesion is hypo-cellular compared to atherosclerosis,\footnote{393} and is composed of synthetic-phenotype VSMCs,\footnote{813} fibronectin, proteoglycans and collagens I and III.\footnote{107,112}

The overall objective of this thesis was to evaluate whether the active fraction of MMPs, as assessed by activity ELISA, were altered in the peripheral circulation of patients with a propensity to develop ISR, and during the development of ISR. MMPs have been linked to the migration of VSMCs, and are up-regulated in experimental arterial injury.\footnote{131,132} Animal studies suggest that genetic over-production of MMPs leads to over-production of intimal hyperplasia.\footnote{524} Other groups have linked increased pro-MMPs-2 and -9, total MMP-9 and altered TIMP-1 to clinical restenosis.\footnote{345,395,737} However, enzyme activation is an important regulatory step governing MMP function, which is not included when assaying the pro- or total forms.\footnote{283} While zymography, utilizing electrophoretic migration coupled with functional substrate degradation, has previously been used to assay MMP activity, this technique has important limitations, including disruption of TIMP-binding and activation of latent MMPs \textit{in vitro}.\footnote{330} We used ELISA-based activity assays, which captured the specific MMP species, and measured the endogenous enzymatic activity through cleavage of a colour-forming substrate.

We hypothesized that active MMP measurements might give an indication of the propensity to form hyperplastic intimal lesions, or leak from the developing neointima in the months after stenting. Hence, alterations of active MMPs might be predictive of the development of ISR before, during and after percutaneous coronary intervention.

These hypotheses were tested through two clinically-based studies. Initially a retrospective, case-control study compared levels of active MMPs in asymptomatic patients who had all previously undergone percutaneous coronary intervention, half of whom had
subsequently developed ISR. In this study, a panel of active MMPs was measured cross-sectionally in patients with and without a history of ISR, in an attempt to identify which active MMP species were altered in those with ISR.

Secondly, we undertook a prospective cohort study, including patients undergoing percutaneous coronary intervention, and took blood samples before intervention, then serially over the course of one year. In this study, the serial changes in plasma active MMP-9 and TIMP-1 were characterized, with respect to index clinical presentation, myocardial function and the development of ISR and other coronary outcomes.

The findings in each of the chapters have already been discussed with respect to the literature. The aim of this chapter is to give an overall picture of the work, discussing implications that have arisen, limitations and possible future directions of study.

9.2. Major findings and their implications

9.2.1. Active MMPs-3 and -9, and TIMP-1 in patients predisposed to ISR

MMP activation is important for VSMC migration and the development of intimal hyperplasia.\textsuperscript{343,367,369,377} When MMP genes are knocked out,\textsuperscript{377} or MMP activity inhibited,\textsuperscript{343,367,369} the ability of VSMCs to migrate in response to damaging stimuli and to contribute to intimal hyperplasia is limited.

The role of a number of MMP gene single nucleotide polymorphisms in ISR have been investigated,\textsuperscript{814-817} although without conclusive results. A direct measurement of active MMPs may represent the net sum of genetic alterations in regulatory mechanisms that predispose to increased production and activation, and may reflect an endogenous susceptibility to more severe intimal hyperplasia and ISR.

In the retrospective study, we observed that both active MMPs-3 and -9, and TIMP-1 were elevated in those with a history of bare-metal stent restenosis, compared to controls who underwent PCI and did not have clinical events after one year. The potential origins of the activated proteins are VSMCs\textsuperscript{347} and inflammatory cells,\textsuperscript{131} and elevated levels in patients without active disease may represent part of the endogenous propensity to mount a healing response. This is most likely influenced by a variety of polymorphisms in an array of genes, for example, those involved in the production of stimulating growth factors and receptors, functional mutations in matrix metalloproteinase genes themselves as well as other enzymes capable of activating matrix metalloproteinases.
Katsaros et al. studied 85 patients undergoing DES implantation for stable coronary artery disease, and found that circulating active MMP-9, but not active MMP-2, was higher in those who would develop angiographic restenosis. Their results suggest a potential role for active MMP-9 in the risk stratification of patients undergoing PCI, with 40% of those having active MMP-9 measurements in the highest quartile developing angiographic ISR, compared to only 6.3% of those in the lower quartiles. However, they had a very low number of cases, with only 12 patients developing angiographic ISR in their study.

Figure 9.1 portrays the interplay between patient and lesional factors with technical, mechanical and biological factors and the location of the ISR lesion. The postulated relationship of ISR within this context is that altered MMP levels may reflect the sensitivity of the neointimal response, with a greater endogenous response potentially producing a larger amount of neointima in reaction to a given injury. Hence, the diagram predicts that a large endogenous response is contributory, but not necessary, for the development of ISR. For instance, in a patient with triple vessel disease and prior bypass grafting coupled with poor operator technique, ISR may occur at the stent edge with little endogenous susceptibility.

In the prospective study, the measurements of active MMP-9 and TIMP-1 were similar between those who would go on to develop ISR and the controls. Unfortunately, the commercial assay for active MMP-3 was unavailable at the time this analysis was undertaken. We were unable to confirm the finding that absolute levels of active MMP-9 were higher in the ISR group, despite being adequately powered to detect the ~40% difference observed in the retrospective study. Levels of TIMP-1 were elevated to a similar degree in the ISR group.
levels in the prospective study (15.6% higher). However, this result did not achieve statistical significance in the prospective study. This may be due to differences in the study design. In the retrospective study, blood samples were taken from asymptomatic patients with a history of coronary stenting both with and without ISR. In the prospective study, blood samples were taken from currently symptomatic patients, immediately before the PCI procedure. These differences may have contributed to a lack of association through a variety of mechanisms. Firstly, active disease and clinical presentations with acute coronary syndrome could have led to greater variability in sample measurements. However, we could not confirm that circulating levels of active MMP-9 or TIMP-1 were altered before PCI by the type of clinical presentation. Alternatively, the observed associations in the retrospective study could be due to bias not present in the prospective study. For example, there may be a survival bias, with patients with ISR less likely to choose to be involved in the study. However, it is not clear if that would translate into differential levels of active MMPs between groups.

Finally, there may be a number of reasons why the retrospective and prospective studies are not comparable. For example, there have been changes in stent design which lower rates of restenosis, and the lack of availability of DES in the time period of the first

Figure 9.1 Postulated inter-relationship between ISR risk factors Modified from Costa and Simon. Bold arrow = strong relationship; middle arrow = moderate relationship; dotted arrow = mild relationship.
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study allows the possibility that a sub-group high risk patients were included in the retrospective – but not the prospective – study.

Novel biomarkers should be assessed in the context of established risk variables. Inclusion of active MMPs-3 and -9, and TIMP-1 alongside clinical and demographic factors known to be associated with ISR improved the ability of the model to predict those with ISR, beyond the established risk factors alone. If the associations of active MMPs-3 and -9, and TIMP-1 with ISR could be established before intervention, our results that suggest their inclusion into ISR risk models may allow identification of very high ( >35%) and very low-risk (<2.5%) individuals.

9.2.2. Active MMP-9, but not TIMP-1, is influenced by index clinical presentation over three- and six months

A number of studies have linked changes in various circulating pro-form MMPs with different clinical presentations, noting that pro-MMP-9 is higher with acute coronary syndromes and myocardial infarction, and is associated with plaque rupture. Therefore, clinical presentation at index is an important potential confounder of any relationship between pre-interventional MMPs and ISR. However, in the study in this thesis there was no difference in active MMP-9 or TIMP-1 levels at the time of angiography.

When the levels of active MMP-9 and TIMP-1 were assessed longitudinally, active MMP-9, but not TIMP-1, appeared to increase over three- and six-months in those who initially presented with ST elevation myocardial infarction. To eliminate the effect of ISR development on circulating levels of active MMP-9, this analysis was repeated in the control group only, confirming that active MMP-9 rose over three- and six-months after PCI in those who had ST elevation myocardial infarction compared to the stable angina group. Webb et al. previously showed increased pro-MMP-9 levels out to six months after acute myocardial infarction, which was associated with increasing left ventricular end diastolic volume. Thus, the observed changes probably relate to left ventricular remodelling after loss of contractile myocardial tissue.

9.2.3. Active MMP-9, but not TIMP-1, increases over three months in patients developing ISR

MMPs appear to have an active role in the development of ISR, with MMP levels in the developing neointima being greatly increased shortly after the time of injury until the
mature lesion forms. Furthermore, MMP-9 is important in the liberation of sequestered inflammatory cells from the bone marrow niche, which have been shown to be elevated during the development of ISR.

In our prospective study, levels of active MMP-9 had a greater rise in the group who were developing ISR compared to those who did not have clinical events. This is consistent with two possible interpretations. The first is that the changes in active MMP-9 are directly related to the developing neointima, perhaps either leaking from the elevated levels in the neointimal tissue, or from the bone marrow where MMP-9 is involved in cleaving cell-matrix bonds.

The second is that the increase of active MMP-9 is from an alternate source, with the levels being reflective of an endogenous predisposition to mounting a healing response, but with a ‘trigger’ leading to a systemic phenotypic manifestation, that being increased circulating active MMP-9. One suggestion for the location of the alternate source is the myocardium, with active MMP-9 produced during remodelling in response to ischaemia associated with the clinical presentation or the PCI procedure. In contrast to the association between increasing levels of active MMP-9 and ISR, there was no association between levels or changes of active MMP-9 or TIMP-1 in the circulation of those developing cardiovascular events.

9.2.4. Active MMP-9 and TIMP-1 are associated with diastolic dysfunction

Diastolic dysfunction is the abnormal functioning of the heart due to impaired relaxation. One of the characteristic pathophysiological features, from a structural point of view, is abnormal accumulation of ECM, in particular increased fibrillar collagens, with increased sub-endocardial collagen. MMPs have been implicated in diastolic heart failure. Altered active MMP-9 and TIMP-1 measurements are present within those with echocardiographic evidence of diastolic dysfunction, with active MMP-9 appearing to be a sensitive marker of mild dysfunction. Whereas active MMP-9 levels progressively increase across more severe diastolic dysfunction categories, TIMP-1 levels are higher in those with mild/moderate dysfunction than either less severe, or more severe dysfunction. The discrepancy between active MMP-9 and TIMP-1 may represent altered mechanisms of MMP/TIMP regulation with severe compared to mild/moderate diastolic dysfunction.
9.2.5. Active MMP-9 and TIMP-1 are stable in storage, and are not subject to seasonal variation

There has been controversy over the optimum pre-analytical strategy for the measurement of circulating MMPs. Notably, Rouy et al.\textsuperscript{709} described declining levels of both protein and the functional activity of MMP-9 with storage at -80°C, decreasing to ~1% of baseline at four years. Because they studies in this thesis relied measuring MMPs that had been stored for up to three years at -80°C, the findings of Rouy et al. could potentially be a critical limitation. However, in our own results, there was no effect of storage time at -80°C and the assayed concentration of active MMP-9 or TIMP-1. Furthermore, there was no seasonal variation in levels of active MMP-9 or TIMP-1, an effect that has been noted with other circulating biomarkers.\textsuperscript{718} Hence, neither storage time or seasonal variation appear to be important pre-analytical confounders of the associations in the studies in this thesis.

9.3. The effect of anticoagulant, time to centrifugation and sample storage on MMPs

There has been some controversy over the optimal pre-analytical strategies for assessing MMPs. We measured pro-MMP-9 and TIMP-1 using EDTA as an anticoagulant, which reportedly gives pro-MMP-9 readings that were highly consistent with other plasma measurements.\textsuperscript{334,490} Correlations have not been reported for TIMP-1. EDTA appears to keep measurements of pro-MMP-9 more stable than serum or heparin, but is associated with around 20% in assayed concentration when stored at 4°C for 24 hours.\textsuperscript{510} We measured active MMPs-1, -2, -3 and -9 in heparin, which may have caused a decrease in apparent concentration (although only MMP-2 and -9 have been tested).\textsuperscript{501} There is some evidence to suggest that increasing concentration of heparin and increasing blood-drawing-to-centrifugation time increases assayed concentration,\textsuperscript{690} although this was not confirmed in another study.\textsuperscript{511}

In the retrospective study, extra effort was made to recruit patients who had developed ISR. Many blood samples were drawn in the community, and while these samples were stored at 4°C and processed the same day, it is possible that, on average, samples from ISR patients had longer blood drawing-to-centrifugation times. Hence, it is possible that ISR samples had artificially higher MMP and TIMP readings. However, the median pro-MMP-9 in the ISR group was 21.3 versus 24.2 ng/mL in the control group.
(Chapter 3.2.2, Table 3.5). This is around 12% lower in the ISR group, in contrast to the predicted increase of pro-MMP-9 that has been noted with longer blood-drawing-to-centrifuge times when collected in EDTA.\textsuperscript{510}

Hence, while it is impossible to rule out a potential confounder due to differences in sample collection time, it appears unlikely that it has made a significant difference in this study. It is possible that inter-sample variation was increased due to this effect, and this could mask some of the association. This is particularly important for the association of pro-MMP-9 with a history of ISR, which was of borderline statistical significance. However, as active MMP-3, -9 and TIMP-1 all had very robust associations, masking associations due to inter-sample variation did not impair our ability to detect these relationships.

In the prospective study any variance at index sample collection would affect controls and cases equally due to the prospective nature of the study. Secondly, when levels of active MMP-9 and TIMP-1 at index, three- and six-months were compared in the controls presenting with stable angina, the levels were similar at all three time points. We cannot rule out a real effect from the qualitative differences in the pre-analytical protocol of the three- and six-month time point samples due to the design of the study. However, if present it would appear to be minor, rather than the many fold changes which have been described for the pro-forms.\textsuperscript{511}

Hence, (1) there is no evidence for a systematic difference in MMP measurements between cases and controls, (2) our primary results were obtained by measuring the endogenous active forms, and despite reports that heparinised plasma changes assayed concentrations compared to citrate plasma, there is no data to support any effect of blood-drawing-to-centrifuge time change in the active form.\textsuperscript{334} However, (3) increased inter-sample variability due to pre-analytical factors could have reduced the statistical power of our comparisons, which may have contributed to the lack of association between MMP markers and ISR in the prospective study.

9.3.1. Discordance in active MMPs between the retrospective and prospective studies

In the retrospective study, the average concentrations of active MMP-9 and TIMP-1 in the control group were 1.4 (0.9 – 1.9) and 225.6 ± 52.9 ng/mL, respectively (Chapter 3.2.2, Table 3.5), whereas in the prospective study they were 0.59 (0.28 – 0.81) and 104.4 ±
23.8 ng/mL, respectively (Chapter 7.2.4, Table 7.5). Thus, in the prospective study, the levels of both active MMP-9 and TIMP-1 appeared to be halved in comparison to the retrospective study. Possible reasons for the differences in active MMP-9 and TIMP-1 include dilutional error or differences in antibody-analyte sensitivity. The differences observed are unlikely to be due to different pre-analytical strategies, as the times from blood drawing-to-centrifugation were most likely similar between the retrospective and prospective studies. Between the time that the analyses of the retrospective and the prospective studies were performed, the company producing the assays was bought by another company, and at the same time we noted, in our own quality controls, that there was substantial inter-assay variability between some of the assay plates provided by the company.

Nevertheless, in both studies quality controls were satisfactory. These included having a standard curve run in duplicate on each ELISA plate, high degrees of reproducibility within duplicate controls, and mixing cases and controls from across the study on every plate. These quality control measures indicate that both studies have high degrees of internal validity, even if the measured levels of MMP variables differ between the two studies.

9.4. Limitations

One of the limitations of this thesis was that, in both studies, a clinical definition of restenosis was used. This has three specific consequences. Firstly, that those patients with asymptomatic angiographic restenosis were not included in the ISR group of either study. Secondly, patients with asymptomatic angiographic restenosis may have been included in the control groups of either study. Thirdly, as we did not have protocol angiography, we could not detect the distribution of late loss amongst the whole population.

The implications of these consequences are that the definition of the ISR phenotype was less rigorous, and may have obscured biological relationships between circulating MMPs and ISR. The second implication of using a clinical definition is that because we could not detect the distribution of late loss, we could not correlate circulating MMPs to less severe degrees of restenosis. These problems may have been avoided if a group of patients who underwent follow up angiography were used as a control group. However, at this institution,
the only patients undergoing follow up angiography were those who developed new symptoms. Therefore those who had negative follow up angiography were patients who had complained of new non-cardiac chest pain, who may be systematically different to the group as a whole. Therefore the inclusion of these patients may introduce a selection bias, and it was elected to randomly select from the asymptomatic population instead.

Another potential limitation of these studies is the sampling method. The anticoagulants used to prevent clotting in the MMP samples (heparin for activity ELISAs, EDTA for pro-MMP-9 and TIMP-1) has been shown to interact with the apparent concentration of some MMPs, and it appears that some forms of MMPs degrade with long-term storage. However, there was no evidence for degradation in our samples, and it is unknown whether the anticoagulants used impaired our ability to detect relationships between ISR and the MMP markers. Unfortunately, after finding that levels of active MMP-3 were associated with a history of ISR, the assay for active MMP-3 was not available at the time of analysis for the prospective study.

Due to financial restrictions, the number of active MMP-9 and TIMP-1 samples we could include in the prospective study was limited. To maximise the utility we randomly selected three controls for each case, so not all the population was represented. However, as the controls were selected in an unbiased way, it is unlikely they are different to the population as a whole.

Patients undergoing drug-eluting stenting were excluded from both studies. The retrospective study did not include patients undergoing drug-eluting stenting, as the participants were recruited before drug-eluting stents were shown to be effective. Patients with drug-eluting stents were not included in the prospective study for two reasons. Firstly, low numbers of drug-eluting stents were being used at our institution at the beginning of recruitment (around 20% of patients were receiving drug-eluting stents). Secondly, due to the reduced rates of ISR with drug-eluting stents, we would have needed a significantly larger study to reach the same study power, utilizing a clinical restenosis definition. The exclusion of patients with DES limits the applicability of our results to clinical practice, as DES are an important and widely used anti-restenotic therapy.59

The hypothesis that the change in active MMP-9 from baseline to three months would be greater amongst those developing ISR was included as a secondary hypothesis. This finding would not stand up to correction for multiple testing if all hypotheses were
considered. Therefore, these results should be considered to be hypothesis generating, and should be confirmed in future research.

Throughout this thesis, smoking history has been quantified by pack years. This lifetime measure of tobacco exposure may overestimate current exposure. This is a potential limiting factor, as current smoking may have an inverse relationship with ISR. However, this is unlikely to have confounded the relationship between active MMP-9, TIMP-1 and ISR, as there was no association between these markers and smoking history.

In the prospective study, active MMP-9 and TIMP-1 were associated with diastolic dysfunction. While we had longitudinal measurements of these markers, we only had one echocardiography study, shortly after index PCI. Longitudinal studies could investigate whether levels and changes of MMPs relate to progressive impairment of diastolic function. Finally, the levels of active MMP-9 and TIMP-1 were quantitatively different between the retrospective and prospective studies, with an apparent halving of the assayed concentration in the prospective study. The reason for this change is not clear, but may reflect different antibody sensitivity. While internal controls were intact in both sets of analysis, this raises the possibility of reduced generalizability between these two studies.

9.5. Future directions

A number of interesting future questions have been raised by this thesis. We showed that the active form of MMP-9 specifically, and not the pro-form, was associated with a history of ISR. However, it is unknown which pathways contribute to the in vivo activity of circulating MMP activity in humans, both in general and in ISR. Mouse models implicate the necessity of plasminogen for MMP activation in experimental arterial injury. However, mouse models do not fully replicate the human phenotype of coronary artery disease, for example, the absence of large lipid cores, and stent strut protrusion into necrotic lipid cores is an important pathophysiological step in ISR development.

An extension of the search for the source of active MMPs might be a Mendelian randomization study of single nucleotide polymorphisms leading to alterations in active MMPs-3 and -9, and TIMP-1. This could provide some insight into whether the circulating levels of these proteins were genetically-driven, or whether they were determined by non-genetic factors. One implication of such a study would be that active MMPs may either be
risk factors (*i.e.* causally linked to ISR development) or they may be risk markers (*i.e.* on the causal pathway between the true aetiiological factor and ISR).

A further project that should be undertaken is to assay the active MMP-3 profile before intervention and during the development of ISR in the prospective study, should the assay for active MMP-3 become available again. Our findings suggested that active MMP-3 was elevated in those with a history of ISR in the retrospective study, but subsequently the commercial assay was withdrawn from supply.

One possibility is to combine imaging probes specific to MMP activity for *in vivo* monitoring of ISR. Currently, gadolinium-linked non-selective MMP probes are available for molecular magnetic resonance imaging\(^{824}\) and can detect changes in MMPs associated with phenotypic changes in experimental atherosclerosis.\(^{825}\) However, as our results suggest that the level of inactive enzymes is less important than the active levels, so activity-linked probes may provide further information. Furthermore, including the anatomical distribution of active MMPs may decrease the confounding of active MMP levels from extra-coronary sources.\(^{826}\)

Our results, and those of Katsaros *et al.*\(^{464}\) may have implications for the future development of drug eluting stents. Specific MMP inhibition during neointimal development, in addition to other anti-restenotic therapy from next-generation drug eluting stents, could result in better management of ISR risk. The results from animal models suggest that increased neointimal VSMC proliferation negates the reduced neointimal volume caused by the MMP inhibitors reducing VSMC migration.\(^{342}\) However, perhaps short term elution of MMP inhibitors to prevent the initial migration of VSMC could be combined with lower doses of anti-proliferative drugs. This strategy might allow better healing of the endothelial deficit,\(^{164,169}\) and while still allowing MMP-dependent contraction of the neointimal ECM in the maturing lesion.

There is no gold standard measurement of MMP activity. The preparation of samples for zymography interferes with MMP/TIMP complexes and alters the pattern of endogenous activity.\(^{330}\) MMPs bind to various extracellular\(^{274}\) and plasma proteins,\(^{283}\) and it is not known how these alter the sensitivity and specificity of the MMP-antibody relationships in ELISAs. MMP ELISAs are developed using recombinant pro-MMP and APMA-activated MMP species.\(^{333}\) Knowledge of MMP biology could allow better MMP activity assays to be designed. MMPs must be in their active form to bind to \(\alpha_2-\)
macroglobulin. This is because the mechanism of $\alpha_2$-macroglobulin binding is via endogenous proteolysis of a vulnerable region in the $\alpha_2$-macroglobulin protein by the target enzyme, which leads to conformational change of the $\alpha_2$-macroglobulin and trapping of the target enzyme. Hence, determination of specific MMP/$\alpha_2$-macroglobulin complexes could allow further insights into the activity of MMPs that are leaked into the circulation. Thus the knowledge of MMP biology may allow better assays to be developed in the future.

One important topic would be the question as to whether these observations extend to ISR of DES. While we did not empirically test this, as only patients treated with BMS were included in the study in this thesis, the findings of Katsaros et al. support this idea. NIH is the the main pathological factor in both DES and BMS restenosis. Conceptually, a reliable answer to this question depends firstly on the cellular origin of MMP-9 protein and activity in restenosis, which has been discussed elsewhere. Briefly, if altered active MMPs indicate the susceptibility of an individual to form intimal hyperplasia in response to an injury, and if active MMPs leak out of the developing neointima, then both of these associations between active MMPs and ISR may potentially extend to patients treated with DES. The rationale for this statement is that the cytostatic effects of DES would most likely minimize the production of active MMPs from these processes, unless ISR was developing. However, if the origin of active MMPs is the net expression from all vascular beds and indicates general plaque complexity, or if ischaemia or the PCI procedure itself elicits production of active MMPs from a source other than the stented lesion; then the risk of ISR with DES may be independent of measurements of circulating active MMPs.

We observed that active MMP-9 was progressively elevated with asymptomatic diastolic dysfunction, being elevated in even the mildest category of diastolic dysfunction compared to normal. TIMP-1 was also altered, but with a different pattern. TIMP-1 was highest in the mild/moderate category, compared to both those with less severe and more severe disease. The reason for this discrepancy between active MMP-9 and TIMP-1 is unclear, but may represent different regulatory mechanisms of MMPs between mild/moderate and severe diastolic dysfunction. A useful study would be to assay serial measurements of active MMP-9 and TIMP-1 alongside serial echocardiograms, in order to assess whether the levels or changes of active MMP-9 and TIMP-1 were predictive of worsening diastolic dysfunction.
9.6. Conclusions

In conclusion, circulating active MMP-3 and -9, and TIMP-1, may be associated with a propensity to form intimal hyperplasia with bare-metal coronary stenting. The addition of active MMPs-3 and -9, and TIMP-1 to clinical and demographic risk factors could allow improved ISR risk stratification of patients, although this was unable to be confirmed in the prospective study, in which pre-interventional measurements were taken. However, the results from the prospective study suggest that after percutaneous coronary intervention, an elicited increase of active MMP-9, but not TIMP-1, is greater in patients developing bare-metal stent restenosis. Additionally, active MMP-9 levels were altered in the months after clinical presentation, with STEMI, and with worse diastolic dysfunction. Adjustment for these variables may be necessary if active MMP-9 is to be used as a predictor of ISR development. Active MMP-9 and TIMP-1 are stable in storage at -80°C in heparin plasma for up to three years, and are not subject to seasonal variation.

In summary, measurement of active MMPs and TIMP-1 in patients undergoing PCI may allow improved risk stratification, as well as early detection of bare-metal stent ISR. While further research is needed to confirm these relationships and extend them to drug-eluting stenting, applying active MMPs and TIMP-1 to clinical populations may allow improved management of patients undergoing PCI.
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Bibliography


11. Appendix

11.1. Retrospective study

11.1.1. Information sheet

Information Sheet

Study of the Genetics of Coronary Artery Disease

We would like to invite you into a study on coronary artery disease. We wish to identify any genes which may be associated with the development of various forms of vascular disease including coronary artery disease (sometimes referred to as heart disease). To do this we require a sample of DNA from individuals with this condition. This will be compared to DNA from patients with other forms of vascular disease (such as abdominal aortic aneurysm). This will enable us to identify any genetic differences between these conditions.

We will ask you to answer a simple questionnaire and to collect a blood sample from which the genetic analysis can be done. We will also examine your abdomen and neck using ultrasound to determine the health of your aorta (the major artery in the body). Ultrasound is a harmless and completely painless form of examination. The ultrasound examination and blood sampling will be performed by fully trained staff members. The blood collected will be used for testing of genetic markers for a range of different genes thought to be involved in the formation of vascular disease and will be stored for a maximum of seven years. At the end of the study the DNA will be disposed of in New Zealand.

Participation in this study is confidential; the blood will not be linked to you in any way, nor will material that could identify you be used in any reports on this study. All information held about the participants in the study is secured within the Department of Medical and Surgical Sciences, situated within the Dunedin Public Hospital.

If you have any queries or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Services Consumer Advocate, telephone (xx) xxx xxxx or xxxx xxx xxx. If there is a specific Māori issue/concern please contact Linda Grennell at xxxx xxx xxx.

In the future we hope that this research will help people who may be at risk from vascular disease in later life, so that earlier treatment options can be offered before the serious complications of this disease occur. The results of this study will be passed on to The Ngai Tahu Māori Health Research Unit, The National Heart Foundation, Te Hotu Manawa Māori and GPs. Individuals, however, will not be able to be identified.

This study is under the supervision of Professor André van Rij, Professor of Surgery, Dunedin School of Medicine.

This study has received ethical approval from the Otago Ethics Committee.

Contact information: Dr Michael Williams, Cardiologist, C/- Cardiology Research at xx xxx xxxx extension xxxx
Dunedin Hospital
11.1.2. Consent form

**Consent Form**

Study of Genetic Markers for the Susceptibility to Coronary Artery Disease

- I have read and understand the information sheet for volunteers participating in the study designed to investigate genetic markers for susceptibility to vascular disease.
- I have had the opportunity to discuss this study and to ask questions which have been answered to my satisfaction.
- I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time and this will in no way affect my future health care.
- I understand that my participation in this study is confidential and that no material which could identify me will be used in any reports on this study.
- I have had time to consider whether to take part.
- I know whom to contact if I have any questions about the study.
- I __________________________(full name) hereby consent to take part in this study.

Date ________________
Signature ____________________________________________________

Full names of Researchers:  Professor André van Rij, Dr Michael Williams, Dr Gregory Jones, Mrs Vicky Phillips, Mrs Rebecca Oskam-Schmidt and Ms Linda Gulliver.

Contact Phone Number:  Dr Michael Williams, Cardiologist, C/- Cardiology Research (xx) xxx xxxx extension xxxx Dunedin Hospital

Project explained by:
Project role:
Signature: __________________________
11.1.3. Questionnaire

**The Genetics of Coronary Artery Disease**

<table>
<thead>
<tr>
<th>National Number:</th>
<th>Age at recruitment:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of birth:</td>
<td>Age at first event:</td>
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Ethnicity: 

Gender:  

Height (m): 

Weight (kg): 

Waist (cm): 

Hip (cm): 

**Smoking**

Do you smoke?

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<td>Number of cigarettes per day?</td>
<td>Have you ever smoked? Y / N</td>
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<td>For how many years?</td>
<td>Cigarettes per day?</td>
</tr>
<tr>
<td>Other: For how many years did you smoke</td>
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Have you ever had/or been treated for? How were you treated?

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<td>Y / N</td>
</tr>
<tr>
<td>High Cholesterol?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Angina/ Heart attack?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Stroke?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Abdominal aortic aneurysm?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Leg cramps or pains when walking?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Operation to the arteries in the legs?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>Y / N</td>
</tr>
<tr>
<td>Varicose veins?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Ulcers from Varicose veins?</td>
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**Medications**

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<th>Aspirin</th>
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<td>Ca2+ antagonists</td>
<td>ACE inhibitors</td>
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<tr>
<td>Nitrates</td>
<td>Aldosterone antagonists</td>
<td>AngII Receptor antagonists</td>
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### 11.1.4. Questionnaire continued

Cardiovascular Events

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<td>Y/N</td>
<td>Date</td>
</tr>
<tr>
<td>Diagnostic cardiac catheter</td>
<td>Y/N</td>
<td>Date</td>
</tr>
<tr>
<td>Percutaneous coronary intervention</td>
<td>Y/N</td>
<td>Date</td>
</tr>
<tr>
<td>CABG</td>
<td>Y/N</td>
<td>Date</td>
</tr>
<tr>
<td>Rapid restenosis</td>
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11.2. Prospective study

11.2.1. Information sheet

Information Sheet
Heart Artery Stent Renarrowing Study

Investigators: Dr Michael Williams, Cardiologist
Cardiology Department, Dunedin Hospital,
Telephone xxxxxxx, Ext xxxx

A/Prof Gerard Wilkins, Cardiologist
Cardiology Department, Dunedin Hospital,
Telephone xxxxxxx

Dr Greg Jones, Senior Research Fellow,
Section of Surgery, University of Otago
Telephone xxxxxxx, Ext xxxx

You are invited to take part in a study looking at factors which may affect heart (coronary) artery stent renarrowing (reblockage). Please feel free to decline to take part in this study and you do not have to give any reason for your decision.

About the study
What are the aims of the study?
We want to find new predictors of renarrowing of heart (coronary) artery stents which occurs in up to 15% of persons in the 2 to 6 month period after stent insertion. The results of this study may help us decide which persons are best suited to insertion of stents or other forms of treatment such as medicine or surgery.

How were participants selected for the study, and who selected them?
We will approach participants whose cardiologist has arranged coronary angiography (X-ray pictures of their heart arteries) for consideration of stent insertion.

How many participants will be involved?
We aim to recruit 800 participants.
Appendix

11.2.2. Information sheet continued

Where will the study be held?
The study will take place in the Cardiology Department, Dunedin Hospital and Section of Surgery, University of Otago.

How long will the study take?
The initial meeting to explain the study will take approximately 20 minutes. There will be 3 subsequent visits of 15 minutes each.

What will happen during the study?
You will be asked to complete a short questionnaire before the procedure of coronary angiography (X-ray pictures of your heart arteries). Immediately before insertion of the stent your cardiologist will take a specimen of blood (about two tablespoonfuls) from the tube that has been placed in your leg for the angiography procedure.

Three further samples of blood will be taken at further visits 3, 6 and 12 months after the stent procedure.

The blood collected will be used for genetic and other testing of markers which may indicate an increased likelihood of developing stent renarrowing (scar tissue inside the stent). At the end of the study the extracted genetic information and remaining blood will be disposed of in New Zealand.

Participation in this study is confidential; the blood and genetic information will not be linked to you in any way, nor will material that could identify you be used in any reports on this study. All information held about the participants in the study is secured within the Department of Medical and Surgical Sciences, situated within Dunedin Hospital.

Who can take part in the study?
If you are 20 years or older, and are having coronary angiography performed with consideration of stent insertion, you are eligible for inclusion in the study. However, we will not be able to take you in the study if you have severe lung disease or are unable to have a stent inserted.

Are there any risks involved in the study?
Taking samples of blood is a safe procedure and there is a very low risk of injury from this test.

Compensation
In the unlikely event of a physical injury as a result of your participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.
11.2.3. Information sheet continued

What will I get from the study?
There are no direct benefits to you from participating in the study.

Will there be any costs?
Parking your car may involve you in costs that we will pay. A nearby parking building may be the most convenient parking place. You will be reimbursed for any bus and taxi fares needed to transport you to the study centre.

Will my GP know if I am in the study?
If you wish, your GP will be informed of your participation in the study.

What will happen at the end of the study?
After completion of the study your results will be available to you and your cardiologist and GP if you wish. We will be available to explain your results to you.

Your participation in our study is entirely voluntary (your choice). You do not have to take part in this study, and if you choose not to take part this will not affect any future care or treatment. If you do agree to take part you are free to withdraw from the study at any time, without having to give a reason. This will in no way affect your future or continuing health care.

In the future we hope that this research will help people who may be at risk from vascular disease in later life, so that earlier treatment options can be offered before the serious complications of this disease occur. The results of this study will be passed on to The Ngai Tahu Māori Health Research Unit, The National Heart Foundation, Te Hotu Manawa Māori and GPs. Individuals will not be able to be identified.

Where can I get more information about the study?
Contact: Dr Michael Williams, telephone xxxxxxx, Ext xxxx or Dr Greg Jones, telephone xxxxxxx, Ext xxxx

If you have any queries or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Services Consumer Advocate, telephone (xx) xxx xxxx or xxxx xxx xxx. If there is a specific Māori issue/concern please contact Linda Grennell at xxxx xxx xxx.

This study has been approved by the Lower South Regional Ethics Committee.
11.2.4. Consent form

Consent Form

Heart Artery Stent Renarrowing Study

• I have read and understand the information sheet for volunteers participating in the study designed to investigate genetic markers for susceptibility to vascular disease.
• I have had the opportunity to discuss this study and to ask questions which have been answered to my satisfaction.
• I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time and this will in no way affect my future health care.
• I understand that my participation in this study is confidential and that no material which could identify me will be used in any reports on this study.
• I have had time to consider whether to take part.

• I know whom to contact if I have any questions about the study.
• I __________________________(full name) hereby consent to take part in this study.

Date ________________
Signature ____________________________________________________

Full names of Researchers: Dr Michael Williams, Dr Gerard Wilkins, Dr Gregory Jones, Ms Rebecca Oskam-Schmidt, Mr Gregory Tarr.

Contact Phone Number: Dr Michael Williams, Cardiologist, Dunedin Hospital
C/- Cardiology Research (xx) xxx xxxx ask for extension xxxx
## Appendix

### 11.2.5. Index questionnaire

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>CSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>National Number: ________</td>
<td>Gender: F / M</td>
</tr>
<tr>
<td>Age at recruitment: ________</td>
<td>Address: ________________</td>
</tr>
<tr>
<td>Phone number: ( )</td>
<td></td>
</tr>
<tr>
<td>Height (m): ________</td>
<td>Ethnicity: New Zealand European</td>
</tr>
<tr>
<td>Weight (kg): ________</td>
<td>Maori</td>
</tr>
<tr>
<td>Waist (cm): ________</td>
<td>Samoan</td>
</tr>
<tr>
<td>Hip (cm): ________</td>
<td>Cook Island Maori</td>
</tr>
<tr>
<td>Indian</td>
<td>Tongan</td>
</tr>
<tr>
<td>Other (such as DUTCH, JAPANESE, TOKELAUA)</td>
<td>Niuean</td>
</tr>
<tr>
<td>Smoking</td>
<td>Chinese</td>
</tr>
</tbody>
</table>

### Do you smoke?

<table>
<thead>
<tr>
<th>11.2.5.1. Yes</th>
<th>11.2.5.2. No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cigarettes per day?</td>
<td>Have you ever smoked? Y / N</td>
</tr>
<tr>
<td>For how many years?</td>
<td>Cigarettes per day ?</td>
</tr>
<tr>
<td>Other:</td>
<td>For how many years did you smoke?</td>
</tr>
</tbody>
</table>

### Disease history

<table>
<thead>
<tr>
<th>Have you ever had/or been treated for?</th>
<th>How were you treated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes? (insulin dependent/oral med at PCI)</td>
<td>Y / N</td>
</tr>
<tr>
<td>Hypertension (High blood pressure)?</td>
<td>Y / N</td>
</tr>
<tr>
<td>High cholesterol?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Varicose veins?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Ulcers from varicose veins?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>Y / N</td>
</tr>
<tr>
<td>Operation to arteries in legs?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Leg cramps or pains when walking?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Stroke?</td>
<td>Y / N</td>
</tr>
<tr>
<td>Abdominal aortic aneurysm?</td>
<td>Y / N</td>
</tr>
</tbody>
</table>

### Medications

<table>
<thead>
<tr>
<th>Statins (admission/discharge)</th>
<th>Beta-blockers (admission/discharge)</th>
<th>AngII antagonists (admission/discharge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrates (admission/discharge)</td>
<td>Ca2+ antagonists (admission/discharge)</td>
<td>Nitrates (admission/discharge)</td>
</tr>
<tr>
<td>Aspirin (admission/discharge)</td>
<td>ACE inhibitors (admission/discharge)</td>
<td>Aldosterone antagonists (admission/discharge)</td>
</tr>
<tr>
<td>Creatinine (serum) (µmol/L)</td>
<td>Urea (serum) (mmol/L)</td>
<td></td>
</tr>
</tbody>
</table>
## 11.2.6. Angiographic assessment

**Angiographic assessment**

To be completed by Cardiologist/ Cardiology Research Nurse

<table>
<thead>
<tr>
<th>National Number:</th>
<th>Age:</th>
<th>Gender: F / M</th>
</tr>
</thead>
</table>

**Presentation (Circle):**
- STEMI
- Non-STEMI or Non-ST elevation ACS
- Stable Angina

**Date of hospital admission:** __/__/___  **Date of PCI:** __/__/___

<table>
<thead>
<tr>
<th>Pre TIMI Flow</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post TIMI Flow</td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ECHO performed</th>
<th>Yes / No</th>
<th>Ejection Fraction</th>
</tr>
</thead>
</table>

### Primary Angiographic Assessment

<table>
<thead>
<tr>
<th>Lesion length (Total, mm)</th>
<th>Artery name</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Index ACC/AHA Lesion Score</th>
<th>A</th>
<th>B1</th>
<th>B2</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Diameter (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal Luminal Diameter (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stent lengths (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stent length (total, mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stent diameter (average, mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stent diameter (minimum, mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of Sites stented</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
</table>

**Comments:**

**DES** | YES / NO | 1 | 2 | 3 | 4 | 5 | 6 | of | 1 | 2 | 3 | 4 | 5 | 6 |

Circle DES segment above (artery name)

### Follow-up Angiographic assessment (ISR PATIENTS ONLY)

<table>
<thead>
<tr>
<th>Mehran Score (Maximum)</th>
<th>IA</th>
<th>IB</th>
<th>IC</th>
<th>ID</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Percentage coronary ISR stenosis (Maximum)</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Multiple ISR Segments</th>
<th></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Number of ISR Sites</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Time to recurrence of symptoms</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
</table>

**Comments:**
11.2.7. Follow up questionnaire

Heart Artery Stent Renarrowing Study follow up CRF

Date: (DD/MMM/YYYY)
Follow up: [ ] 3 months [ ] 6 months [ ] 12 months
Patient ID: CSR

1. Has the patient experienced new or recurrent angina since last contact? [ ] YES [ ] NO
   If YES: Angina Status (CCSC)+: [ ] I [ ] II [ ] III [ ] IV

2. Worst Angina pattern since last contact is:
   If Unstable: [ ] Worsening exertional angina [ ] Rest Angina [ ] Pain during MI ONLY [ ] Post MI angina

3. Adverse Events: [ ] YES [ ] NO
   If YES: Type of Event
   [ ] Death [ ] MI (Q wave) [ ] MI (non-Q wave)
   [ ] Emergent CABG [ ] Target Vessel Revascularisation

4. Did other Adverse Events occur? [ ] YES [ ] NO
   If YES explain:

5. Taking aspirin? Usage since last visit
   Continuously | Interruption of >1 wk |
   Discontinued |

6. Taking antiplatelet? If stopped was antiplatelet taken for 6 months [ ] YES [ ] NO
   Comments:

7. Any changes to medications since last contact? [ ] YES [ ] NO
   Comments:

8. Closest SCL to get blood test:

Researcher signature: _________________________ Date: (DD/MMM/YYYY)
### 11.3. Modified ACC/AHA score

<table>
<thead>
<tr>
<th>Table 11.1 Definition of modified ACC/AHA score criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type A</strong></td>
</tr>
<tr>
<td>Discrete (&lt;10 mm length)</td>
</tr>
<tr>
<td>Concentric</td>
</tr>
<tr>
<td>Readily accessible</td>
</tr>
<tr>
<td>Smooth contour</td>
</tr>
<tr>
<td>No major branch involvement</td>
</tr>
<tr>
<td>Little or no calcification</td>
</tr>
<tr>
<td>Less than totally occlusive</td>
</tr>
<tr>
<td>Nonangulated segment, &lt;45°</td>
</tr>
<tr>
<td>Not ostial in location</td>
</tr>
<tr>
<td>Absence of thrombus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Type B Lesions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular (10-20 mm length)</td>
</tr>
<tr>
<td>Moderate tortuosity of proximal segment</td>
</tr>
<tr>
<td>Moderate to heavy calcification</td>
</tr>
<tr>
<td>Ostial in location</td>
</tr>
<tr>
<td>Irregular contour</td>
</tr>
<tr>
<td>B1 = 1 criteria, B2 = ≥ criteria</td>
</tr>
<tr>
<td>Eccentric</td>
</tr>
<tr>
<td>Moderately angulated segment, &gt;45° &lt;90°</td>
</tr>
<tr>
<td>Total occlusion, &lt;3 months old</td>
</tr>
<tr>
<td>Bifurcation lesions requiring double guide wires</td>
</tr>
<tr>
<td>Some thrombus present</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Type C Lesions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse (&gt;2 cm length)</td>
</tr>
<tr>
<td>Extremely angulated segments &gt;90°</td>
</tr>
<tr>
<td>Inability to protect major side branches</td>
</tr>
<tr>
<td>Excessive tortuosity of proximal segment</td>
</tr>
<tr>
<td>Total occlusion, &gt;3 months old</td>
</tr>
<tr>
<td>Degenerated vein grafts with friable lesions</td>
</tr>
</tbody>
</table>

Modified from Ellis *et al.* 474,828
11.4. Mehran scoring of ISR pattern.

**ISR Pattern I: Focal**

- **Type IA:** Articulation or Gap
- **Type IB:** Margin
- **Type IC:** Focal Body
- **Type ID:** Multifocal

**ISR Patterns II, III, IV: Diffuse**

- **ISR Pattern II:** Intra-stent
- **ISR Pattern III:** Proliferative
- **ISR Pattern IV:** Total Occlusion

Figure 11.1 Mehran score grading of ISR patterns
From Mehran *et al.*

Table 11.2 Definitions of Mehran patterns

<table>
<thead>
<tr>
<th>Class I: Focal ISR group</th>
<th>Lesions are &lt;10 mm in length and</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1A: positioned at the unscaffolded segment</td>
</tr>
<tr>
<td></td>
<td>1B: the proximal or distal margin (but not both)</td>
</tr>
<tr>
<td></td>
<td>1C: the body of the stent</td>
</tr>
<tr>
<td></td>
<td>1D: combination of these sites (multifocal ISR)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Class II: “Diffuse intrastent”</th>
<th>Lesions are &gt;10 mm in length and</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>are confined to the stent(s)</td>
</tr>
<tr>
<td></td>
<td><em>without</em> extending outside the margins of the stent(s)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Class III: “Diffuse proliferative”</th>
<th>Lesions are &gt;10 mm and</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>length and extend beyond the margin(s) of the stent(s)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Class IV: ISR with “total occlusion.”</th>
<th>Lesions have a TIMI flow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grade of 0.</td>
</tr>
</tbody>
</table>

From Mehran *et al.*

475
11.5. Clinical and demographic factors associated with in-stent restenosis in the retrospective study

This study had a case-control design, and recruited a retrospective cohort of patients with a history of ISR, and a cohort of patients undergoing coronary angiography during a similar time period as controls. There were clear differences between the ISR and non-ISR groups. These included baseline coronary disease severity; the number, length and diameter of stents inserted; baseline classification of angiographic disease by the modified American College of Cardiology/American Heart Association (ACC/AHA) score; HDL-cholesterol; high sensitivity C-reactive protein; and measures of adiposity. The ISR group was also prescribed significantly more medication than the non-ISR group.

These variables broadly agree with those described in the literature, with two major deviations: the lack of association between diabetes and ISR, and the increase in medications in the ISR group.

Diabetes is known to increase the risk of both ISR\textsuperscript{25} and death\textsuperscript{24} after PCI. In a post hoc analysis of the BARI trial, patients with diabetes had lower rates of mortality if they had been randomized to the CABG arm.\textsuperscript{829} However, the more recent CARDia trial, which is the first trial to randomize patients with diabetes to PCI or CABG, demonstrated similar rates of all cause mortality by treatment arm.\textsuperscript{830} It appears that diet-controlled diabetes is associated with minimal risk ISR, but those using insulin and oral anti-hypoglycaemic medication have approximately 40% increased risk.\textsuperscript{518} We did not differentiate between the method of diabetes control, but those with diet-controlled diabetes are probably a small proportion of the total number of patients with diabetes undergoing PCI.\textsuperscript{46,518} The lack of association seen in this study may be due to an alteration in clinical cardiology practice. Thus, the patients in this study with diabetes may be a distinct subpopulation of all diabetic patients with symptomatic coronary disease, if others were preferentially referred for bypass grafting. Due to the retrospective nature of the study in this thesis, survivor bias may also have blunted the association between ISR and diabetes.

Women account for around one-third of coronary stent recipients.\textsuperscript{831} In our data set, females did not have a higher risk of ISR, despite tending to be older, and having more diabetes, smaller vessels and triple vessel disease. In a large study it appears that female sex is protective against ISR, despite higher rates of diabetes, narrow vessel diameter and being older.\textsuperscript{44}
Markers of obesity, waist, BMI and WHR were all significantly associated with ISR. Obesity has been recognised as a risk factor for ISR. The necessity for re-intervention appears to be higher in patients who were overweight\textsuperscript{832} or obese\textsuperscript{832,833} with both BMS\textsuperscript{832,833} and DES.\textsuperscript{834} Furthermore, there appears to be a biological gradient where greater BMI is associated with higher rates of ISR.\textsuperscript{833}

In recent decades, evidence for an association between inflammation and CAD has emerged, leading to an understanding of the importance of inflammation in the development of atherosclerosis.\textsuperscript{24,835} Much work has been done investigating the use of high sensitivity C-reactive protein in a broad range of vascular diseases,\textsuperscript{836} most prominently for primary prevention of coronary artery disease.\textsuperscript{211,669,837} Inflammation plays a significant role in all phases of the development of ISR,\textsuperscript{127,838} and it seems plausible that alterations of inflammatory systems may affect risk for ISR.\textsuperscript{839} Consistent with this, circulating high sensitivity C-reactive protein has been implicated as a risk marker for ISR, with elevated levels found in the ISR group of the present study, and in previous reports.\textsuperscript{254,840,841} However, this relationship may be lost with drug-eluting stent treatment.\textsuperscript{842} Elevated pre-PCI high sensitivity C-reactive protein has also been linked to non-ISR cardiac events.\textsuperscript{843}

In the study in this thesis, the only difference in lipid markers between stent and ISR groups was a decreased level of HDL-cholesterol in those with a history of ISR. While dyslipidaemia as a binary variable does not appear to be associated with ISR,\textsuperscript{46,79} HDL cholesterol appears to play a role in the development of intimal hyperplasia. Low HDL levels appeared to be predictive of restenosis one year after carotid stenting,\textsuperscript{844} and both restenosis and other cardiac events in 66 diabetic ISR patients.\textsuperscript{845} It has been postulated that HDL may display anti-restenotic effects due to anti-coagulant and anti-inflammatory effects.\textsuperscript{846}

The modified ACC/AHA score is derived from the angiographic appearance of the lesion. While originally developed to estimate the success rate of a given intervention,\textsuperscript{474,475} it has been found to affect the rate of ISR as well.\textsuperscript{136,847} In our dataset, an overall trend was found, with rates of ISR increasing with higher ACC/AHA score. The ACC/AHA score has been grouped in different ways in the literature, with either A and B1 grouped as “simple” versus B2 and C as “complex”\textsuperscript{136,847} or comparing class C versus all other lesions.\textsuperscript{138,519}

Although an alternate angiographic lesion score exists, the Society for Cardiovascular and Angiographic Interventions classification system, it appears to be better suited to
predicting procedure success and complication rate, and it has not yet been evaluated in terms of predictive ability for ISR.

The number of coronary vessels that are diseased is a commonly used indicator of the severity of coronary disease; it was originally derived to predict mortality with revascularisation. Subsequently it was used to determine whether PCI or surgery was a more appropriate therapy and, for much of the early history of PCI, multi-vessel disease has been a relative contra-indication to percutaneous therapy. However, with increasing confidence in the BMS era, multivessel PCI was performed, and with DES, multivessel revascularisation is commonplace.

The number of stents placed was significantly associated with ISR in the studies in this thesis, which is in agreement with the ISR prediction literature. The number of stents placed is consistently associated with increasing restenosis rate. The length of stent implanted is also consistently associated with ISR. The susceptibility of these lesions to developing ISR can be linked to the fact that longer stents will expose more arterial wall to trauma. In this study, both the total and the average length of individual stents implanted were strongly associated with ISR.

The average diameter of stents implanted is a strong predictor of ISR, which is reflected in our findings. Geometrically speaking, area scales as the square of the diameter, so the effect of a given amount of intimal hyperplasia is magnified in smaller arteries. A coronary artery with a luminal diameter of four millimetres will need one and seven-ninths times the amount of intimal hyperplasia to produce a narrowing of the same degree in an artery with a luminal diameter of three millimetres.

Finally, there have been a number of factors that have been implicated in risk for ISR which were not measured in the present study including: procedural problems; history of restenosis; stent design; lesions in the left anterior descending (LAD) coronary artery; and ostial locations. Overall, our findings regarding the clinical and demographic risk factors for ISR are in broad agreement with the literature.

11.6. Extrapolation of retrospective study data to approximate a population with a 10% risk of ISR

The observed numbers of study participants, according to case/control status and split by quartiles of risk score is displayed in Table 11.3. The extrapolated numbers for a hypothetical
population with 10% risk of restenosis and the estimated risk of restenosis for each quartile of risk score are also given.

To approximate the rates of restenosis within each category from the case control study, the number of controls in each category was multiplied nine-fold. From the original case control (which had a ratio of approximately one case to one control) this gives approximately nine controls to every case (151 * 9 = 1,359 controls; 1,359 controls + 152 cases = 1511 total population; (152 cases / 1511 total population) * 100% = 10.1% “risk” of restenosis). To estimate the risk in each quartile of risk score, the number of controls in each cell was multiplied by nine and the rate calculated.
## Table 11.3 Approximation of risk score stratification in a hypothetical population with an ISR rate of 10%

<table>
<thead>
<tr>
<th>Quartile of risk score</th>
<th>Observed numbers of cases/controls in retrospective study</th>
<th>Hypothetical derived population with 10% restenosis</th>
<th>Restenosis rate in each quartile in hypothetical population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1</td>
<td>Q2</td>
<td>Q3</td>
</tr>
<tr>
<td>PRESTO score alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>46</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>ISR</td>
<td>23</td>
<td>33</td>
<td>38</td>
</tr>
<tr>
<td>PRESTO + active MMPs-3,-9 and TIMP-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>47</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td>ISR</td>
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<td>26</td>
<td>44</td>
</tr>
<tr>
<td>ITVR score alone</td>
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<td></td>
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</tr>
<tr>
<td>Controls</td>
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<td>46</td>
<td>36</td>
</tr>
<tr>
<td>ISR</td>
<td>22</td>
<td>31</td>
<td>41</td>
</tr>
<tr>
<td>ITVR + active MMPs-3,-9 and TIMP-1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
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<td>43</td>
<td>44</td>
</tr>
<tr>
<td>ISR</td>
<td>12</td>
<td>28</td>
<td>37</td>
</tr>
</tbody>
</table>
11.7. Published manuscripts

11.7.1. Active matrix metalloproteinases 3 and 9 are independently associated with coronary artery in-stent restenosis

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A R T I C L E   I N F O

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Restenosis
Matrix metalloproteinases
Plasma
Coronary stent

A B S T R A C T

Objective: This study aimed to determine whether plasma levels of active matrix metalloproteinases (MMP) are predictors of in-stent restenosis (ISR) in New Zealand patients treated with bare-metal coronary stents.

Methods: A group of 151 patients with a history of ISR were compared with 151 symptom free 1-year post-stenting patients (non-ISR). Demographic and angiographic characteristics were collected. Plasma samples were analyzed for the active forms of MMP-1, -2, -3 and -9 as well as tissue inhibitor of metalloproteinases (TIMP-1) using ELISA-based isoelectric focusing assays.

Results: Both active MMP-9 and active MMP-1 were independently associated with history of ISR. Elevated levels of both active MMP-3 and -9 had an adjusted odds ratio of 11.1 (95% CI: 4.35, p<0.0001) for association with ISR, with 91% of ISR patients having such levels versus 11% on non-ISR. The addition of both of the MMP biomarkers significantly increased the area under the curve (AUC) of a receiver operator characteristic (ROC) analysis incorporating the significant demographic and angiographic variables (AUC 0.85 versus 0.79, p<0.005).

Conclusion: Measures of plasma active MMP isoenforms appear to be independently associated with ISR, and assessment of multiple MMP markers yields cumulative utility.

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1. Introduction

In-stent restenosis (ISR) is regarded to be a major limiting factor of bare-metal coronary stenting [1], and while drug-eluting stents have reduced the rates of ISR, they are still significant [2]. Concern over very late late stent thrombosis and bleeding with prolonged dual antiplatelet therapy has led to proposals that patients be risk profiled for selective use of drug-eluting stents [3]. The ability to more reliably predict the risk of ISR would be an important part of the decision making process to optimise patient management.

Models of arterial injury have revealed that matrix metalloproteinase (MMP) genes [4] and plasma concentrations are up-regulated in a time and location specific manner [5,6]. Over-expression of tissue inhibitors of metalloproteinases (TIMP) reduced smooth muscle cell (SMC) migration [7], and MMP-9 in particular is critical for the proliferation and migration of SMC [8]. The levels of various MMPs appear to be raised after stent implantation in humans [9,10], and there is some evidence that MMPs are linked to restenosis of carotid endarterectomy [11] and after coronary stenting [12]. The association of MMPs and vascular injury is consistent in both animal and human studies, and suggest that levels of MMPs may be increased with the development of restenosis after coronary stent implantation.

In this study, circulating plasma levels of four active isoenforms (MMP-1, -2, -3 and -9) and TIMP-1 were assayed inpatients who had previously undergone bare-metal stent placement to determine if individual or combined levels of these isoenforms were predictors of ISR.

2. Materials and methods

2.1. Subjects

Patients with coronary bare-metal stent placements were recruited retrospectively from the Dunedin Hospital Cardiology Clinical database as previously described [13]. A group of 152 patients with a history of symptomatic, angiographically proven, ISR were compared with 151 patients who were angina free for more than 1 year following their stent placement (non-ISR). At the time of study all subjects in the ISR group had undergone revascularization with either repeat percutaneous intervention or
cervical artery bypass surgery and were then free of symptoms and cardiovascular events for >5 months. Participants were predominantly of New Zealand European ethnicity (97%), with the remainder being New Zealand Māori [35]. Detailed demographic details are shown in Supplemental Table 1. Multivariate adjusted, cardiovascular risk factors and medication use were recorded for the participants. All patients gave written informed consent before being recruited.

All subjects included in the 184 group had angiographic restenosis confirmed by their treating cardiologist and clinically driven revascularisation. A second experienced cardiologist reassessed all the ISRI group coronary angiograms and independently confirmed the presence of restenosis. Coronary artery disease was quantified by the number of vessels with >50% stenosis by visual assessment and the American College of Cardiology/American Heart Association (ACC/AHA) classification [14] was used to quantify the morphology of baseline coronary lesions. The follow-up angiographic definition of in-stent restenosis was diameter stenosis >50% of the vessel reference diameter in ≥1 projections, by visual assessment. The most severe view was used to categorize restenosis by the Mehran classification [15]. Clinical and demographic variables were recorded as previously described [13].

2.2 Sample analysis

EDTA plasma samples were analyzed for high-sensitivity C-reactive protein (hs-CRP), HbA1c, TNF-α, and high sensitivity assay, using and creatinine, lipid profiles (enzymatic-colorimetric method; Roche Diagnostics), and total MMP-1 [26], and ACE activity. ACE (Healthcare Life Sciences, RPN2611 and RPN2614). Glomerular filtration rate (GFR) was calculated according to the Cockroft-Gault formula [16] with patients being classified as having ‘poor’ kidney function when GFR was less than 60 mL/min. Endogenous plasma MMP-1, -2, -3, and -9 were measured in heparin plasma samples using an ELISA-based proteolytic activity assay system (GE Healthcare Life Sciences, RPN2645, RPN2631, RPN2638 and RPN2634). This system measures the endogenous activity of the specific matrix metalloproteinase. The ratio of pro-MMP-9 to active enzyme indicated the proportion of pro MMP-9 to active enzyme. The active enzyme percent of both activity and ELISA assay was >5%.

Table 1

<table>
<thead>
<tr>
<th>MMP-1 (μg/mL)</th>
<th>ISRI n = 152</th>
<th>ISRI n = 152</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1</td>
<td>2.6 (1.4–4.0)</td>
<td>2.6 (1.4–4.8)</td>
</tr>
<tr>
<td>Active MMP-2</td>
<td>16.2 (10.0–18.8)</td>
<td>13.5 (12.1–16.7)</td>
</tr>
<tr>
<td>Active MMP-3</td>
<td>7.1 (4.0–12.6)</td>
<td>7.5 (5.9–12.7)</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>1.6 (0.5–1.8)</td>
<td>2.3 (1.4–3.2)</td>
</tr>
<tr>
<td>ProMMP-9 (ng/mL)</td>
<td>2.1 (1.1–1.5)</td>
<td>2.3 (1.5–1.6)</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>2.0 (1.5–2.0)</td>
<td>2.0 (1.5–2.0)</td>
</tr>
<tr>
<td>Ratio of pro-MMP-9/TIMP-1</td>
<td>0.09 (0.08–0.10)</td>
<td>0.09 (0.08–0.10)</td>
</tr>
<tr>
<td>Ratio of active MMP-9/pro-MMP-9</td>
<td>0.09 (0.08–0.10)</td>
<td>0.09 (0.08–0.10)</td>
</tr>
<tr>
<td>Elevated MMP-3 and -9 (Sanger, one/both)</td>
<td>6.2 (3.4–8.2)</td>
<td>6.2 (3.4–8.2)</td>
</tr>
<tr>
<td>Results are expressed as medians and (interquartile range) Elevated active MMP-3 &gt; 3 ng/mL, active MMP-9 &gt; 2ng/mL.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3 Statistical analysis

StatView version 5.01 (SAS Institute) was used to perform statistical analysis. The distribution of continuous variables (kurtosis and skewness) was assessed and compared with either the Mann-Whitney U or ANOVA with the Fisher protected least significant difference test. Multiple logistic regression was used to evaluate the interaction between variables and MMPs in correlation with in-stent restenosis. A stepwise entry procedure was applied to identify significant or suggestive (p < 0.15) confounders of either patient group or MMP level. Results were given as mean ± SD except for Gaussian variables, which were expressed as medians and interquartile ranges. Odds ratios were expressed with 95% confidence intervals. A p-value of less than 0.05 was considered statistically significant.

The mROC (Unite de biostatistiques, CICAL Val d’Aurelle, V1.0) software package was used to perform multiple receiver operating characteristic curves simultaneously [16].

3. Results

3.1 Demographic factors associated with ISRI

Patients with ISR had significantly greater waist circumference, BMI and high-sensitivity CRP, significantly lower HDL-cholesterol and were more medicated compared with those with no history of ISR (Online Table 1). Patients with low ISR also had a higher rate of triple vessel disease and significantly more complex ACC/AHA lesion score and stent characteristics (Table 2). There were no other significant differences between the two patient groups demographic variables.

3.2 Plasma MMP Levels

Plasma active MMP-1 and -9 and TIMP-1 were significantly higher in patients with a history of ISR (Table 1). Adjusting for confounding factors indicated that they were also independently associated with a history of ISR (Table 2).

By univariate analysis, levels of active MMP-2 were significantly lower in patients with a history of ISR, however this

### Table 2

<table>
<thead>
<tr>
<th>MMP-1 (μg/mL)</th>
<th>ISRI n = 152</th>
<th>ISRI n = 152</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1</td>
<td>2.6 (1.4–4.0)</td>
<td>2.6 (1.4–4.8)</td>
</tr>
<tr>
<td>Active MMP-2</td>
<td>16.2 (10.0–18.8)</td>
<td>13.5 (12.1–16.7)</td>
</tr>
<tr>
<td>Active MMP-3</td>
<td>7.1 (4.0–12.6)</td>
<td>7.5 (5.9–12.7)</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>1.6 (0.5–1.8)</td>
<td>2.3 (1.4–3.2)</td>
</tr>
<tr>
<td>ProMMP-9 (ng/mL)</td>
<td>2.1 (1.1–1.5)</td>
<td>2.3 (1.5–1.6)</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>2.0 (1.5–2.0)</td>
<td>2.0 (1.5–2.0)</td>
</tr>
<tr>
<td>Ratio of pro-MMP-9/TIMP-1</td>
<td>0.09 (0.08–0.10)</td>
<td>0.09 (0.08–0.10)</td>
</tr>
<tr>
<td>Ratio of active MMP-9/pro-MMP-9</td>
<td>0.09 (0.08–0.10)</td>
<td>0.09 (0.08–0.10)</td>
</tr>
<tr>
<td>Elevated MMP-3 and -9 (Sanger, one/both)</td>
<td>6.2 (3.4–8.2)</td>
<td>6.2 (3.4–8.2)</td>
</tr>
<tr>
<td>Results are expressed as medians and (interquartile range) Elevated active MMP-3 &gt; 3 ng/mL, active MMP-9 &gt; 2ng/mL.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2 Statistical analysis

Table 1 was used to perform statistical analysis. The distribution of continuous variables (kurtosis and skewness) was assessed and compared with either the Mann-Whitney U or ANOVA with the Fisher protected least significant difference test. Multiple logistic regression was used to evaluate the interaction between variables and MMPs in correlation with in-stent restenosis. A stepwise entry procedure was applied to identify significant or suggestive (p < 0.15) confounders of either patient group or MMP level. Results were given as mean ± SD except for non-Gaussian variables, which were expressed as medians and interquartile ranges. Odds ratios were expressed with 95% confidence intervals. A p-value of less than 0.05 was considered statistically significant.

Table 2

<table>
<thead>
<tr>
<th>Odds ratios associating MMPs with ISRI</th>
<th>p-Value</th>
<th>Adjusted OR</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active MMP-1 (&gt; 2.5 ng/mL)</td>
<td>1.49 (0.92–2.36)</td>
<td>0.01 (0.51–2.00)</td>
<td>0.18</td>
</tr>
<tr>
<td>Active MMP-2 (&gt; 1.0 ng/mL)</td>
<td>0.97 (0.34–1.01)</td>
<td>0.97 (0.32–1.07)</td>
<td>0.20</td>
</tr>
<tr>
<td>Active MMP-3 (&gt; 1.0 ng/mL)</td>
<td>0.57 (1.65–4.47)</td>
<td>&lt;0.0001 (0.18–12.25)</td>
<td>0.17</td>
</tr>
<tr>
<td>Active MMP-9 (&gt; 1.0 ng/mL)</td>
<td>0.57 (2.42–6.51)</td>
<td>0.57 (2.21–10.44)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elevated MMP-1 and -9</td>
<td>0.97 (0.92–2.36)</td>
<td>0.01 (0.51–2.00)</td>
<td>0.18</td>
</tr>
<tr>
<td>elevated MMP-1 and -9</td>
<td>0.97 (0.34–1.01)</td>
<td>0.97 (0.32–1.07)</td>
<td>0.20</td>
</tr>
<tr>
<td>Elevated MMP-3 and -9</td>
<td>0.57 (1.65–4.47)</td>
<td>&lt;0.0001 (0.18–12.25)</td>
<td>0.17</td>
</tr>
<tr>
<td>Elevated MMP-9</td>
<td>0.57 (2.42–6.51)</td>
<td>0.57 (2.21–10.44)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Adjusted model includes confounding demographic, angiographic, biochemical variables (BMI, diabetes, waist circumference, HDL-C, CRP, CAD severity, ACC/AHA score stent characteristics, number of stents implanted per patient) as well as MMP plasma markers (Table 1). Elevated active MMP-3 > 3ng/mL, active MMP-9 > 2ng/mL.
difficult to determine the role of MPP-3 and -9 in the development of ISRs. The ROC curves for these variables are shown in Figure 4 (A) and (B). A significant association was found between MPP-3 and -9 levels and the presence of ISRs (p < 0.05). The ROC curves indicate that MPP-3 and -9 levels have a high discriminatory power for the prediction of ISRs. A cutoff value of 0.7 has been established, with a sensitivity of 80% and a specificity of 85%

4. Discussion
This study evaluated circulating levels of multiple MPP-related markers in patients who had undergone coronary artery stenting and compared them with those of healthy controls. The results showed that MPP-3 and -9 levels were significantly higher in ISR-positive patients compared to ISR-negative controls. The ROC curves for MPP-3 and -9 demonstrated excellent discriminatory power, with areas under the curve (AUC) of 0.9 and 0.8, respectively. These findings suggest that MPP-3 and -9 may be useful biomarkers for the prediction and monitoring of ISR.

Table 3: Unadjusted and adjusted odds ratios associating MPPs with ISR.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Non-ISR, n=151</th>
<th>ISR, n=124</th>
<th>Two or more ISRs, n=28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP-3 (ng/mL)</td>
<td>2.2 (1.0-4.4)</td>
<td>2.6 (1.2-4.3)</td>
<td>3.5 (2.1-5.5)</td>
</tr>
<tr>
<td>MPP-4 (ng/mL)</td>
<td>10.2 (1.4-11.8)</td>
<td>13.5 (6.8-27.7)</td>
<td>12.8 (9.1-18.4)</td>
</tr>
<tr>
<td>MPP-5 (ng/mL)</td>
<td>2.4 (1.6-4.0)</td>
<td>3.0 (1.6-6.0)</td>
<td>6.0 (3.2-11.4)</td>
</tr>
<tr>
<td>MPP-6 (ng/mL)</td>
<td>0.4 (0.3-1.5)</td>
<td>2.1 (1.4-2.7)</td>
<td>2.5 (1.5-3.3)</td>
</tr>
<tr>
<td>ProMMP-9 (ng/mL)</td>
<td>242 (177-377)</td>
<td>216 (149-395)</td>
<td>227 (145-397)</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>2072 (185-256)</td>
<td>2642 (265-280)</td>
<td>2225 (199-3244)</td>
</tr>
<tr>
<td>Elevated MPP-3 and -9</td>
<td>48 (41, 112)</td>
<td>293.482, 16.99</td>
<td>141.222, 630</td>
</tr>
</tbody>
</table>

Results are expressed as median and (interquartile range). Elevated active MPP-3 > 3 ng/mL, active MPP-9 > 2 ng/mL.

Table 4: Summary of Associations Between MPPs and ISR.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Non-ISR, n=151</th>
<th>ISR, n=124</th>
<th>Two or more ISRs, n=28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP-3 (ng/mL)</td>
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<td>2.6 (1.2-4.3)</td>
<td>3.5 (2.1-5.5)</td>
</tr>
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<td>MPP-4 (ng/mL)</td>
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<td>13.5 (6.8-27.7)</td>
<td>12.8 (9.1-18.4)</td>
</tr>
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<td>MPP-5 (ng/mL)</td>
<td>2.4 (1.6-4.0)</td>
<td>3.0 (1.6-6.0)</td>
<td>6.0 (3.2-11.4)</td>
</tr>
<tr>
<td>MPP-6 (ng/mL)</td>
<td>0.4 (0.3-1.5)</td>
<td>2.1 (1.4-2.7)</td>
<td>2.5 (1.5-3.3)</td>
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<td>293.482, 16.99</td>
<td>141.222, 630</td>
</tr>
</tbody>
</table>

Results are expressed as median and (interquartile range). Elevated active MPP-3 > 3 ng/mL, active MPP-9 > 2 ng/mL.

The ROC curves for MPP-3 and -9 showed significant differences between ISR-positive versus ISR-negative controls (p < 0.05). The AUC for MPP-3 was 0.9, and for MPP-9, 0.8. These findings suggest that MPP-3 and -9 may be useful biomarkers for the prediction and monitoring of ISR.

4. Discussion
This study evaluated circulating levels of multiple MPP-related markers in patients who had undergone coronary artery stenting and compared them with those of healthy controls. The results showed that MPP-3 and -9 levels were significantly higher in ISR-positive patients compared to ISR-negative controls. The ROC curves for MPP-3 and -9 demonstrated excellent discriminatory power, with areas under the curve (AUC) of 0.9 and 0.8, respectively. These findings suggest that MPP-3 and -9 may be useful biomarkers for the prediction and monitoring of ISR.
MMP-9 present in the active form was significantly greater in patients with a history of MI suggesting increased MMP activation in these individuals. TIMPs are endogenous inhibitors of MMPs and bind in 1:1 stoichiometry [26]. Although ISR patients also had increased TIMP-1 levels, the substrate bias-assayed in this study only detects the active isoforms, which are not bound with a TIMP. Nevertheless, TIMP-1 levels were included in multiple logistic regression models to confirm that the active MMP associations with ISR were indeed independent of the levels of this endoprotease inhibitor.

Multiple logistic regression showed that both active plasma MMP-9 levels and the level of the ISR group. While some over-fitting may have existed in the mROC classifiers since active MMP-3 and -9 were moderately correlated (r=0.2, p=0.0003), the inclusion of both MMPs appeared to significantly increase the AUC for ISR compared with conventional demographic and clinical risk factors. Sole reliance on ROC curves has been questioned as a reliable strategy for assessing potential markers, as large associations are needed to provide significant increases in area under the curve [27]. Therefore, in our analysis we included both odds ratios and ROC curves. Nevertheless regardless of the mode of analysis employed the combination of active MMP-1 and -9 levels was significantly and independently associated with coronary-in-stenosis restenosis.

Although plasma levels of hs-CRP was correlated with pre-MMP-9 levels there was no significant association with active levels of these enzymes, suggesting that the increased MMP activation was not directly due to inflammation. Plasmin leads to stepwise activation of MMP-1 and -9 via a 'coagulum metaphor'. However, MMP-2 lacks this activation site [28]. Given such shared activation pathways and the ability to activate off members of the MMP family it was also important to include all MMP measures in the multiple logistic regression analysis in order to confirm the independence of any plasma MMP/ISR associations.

Other potential influences or circulating levels of MMPs, such as cigarette smoking, ethnicity, age, pharmaceutical intervention and gender have been investigated in specially designed studies [29]. However, the majority of participants in this study were New Zealand-European and an extensive range of potential confounders were included within the adjusted logistic regression model.

Recent concerns about an excess risk of late stent thrombosis with drug-eluting stents have not been confirmed in subsequent reports [30]. Systematic reviews and large-scale registry studies have demonstrated similar rates of overall mortality and myocardial infarction in patients treated with either drug-eluting or bare-metal stents [31]. There are, however, concerns about the risk of bleeding on long term dual antiplatelet therapy particularly those treated with anticoagulants and in elderly patients [32]. Previous studies have shown drug eluting stents to be more cost-effective in elderly high risk patients who have the highest risk of bleeding with prolonged dual platelet therapy [33]. The ability to risk stratify such patients in terms of risk of restenosis would provide a significant opportunity to selectively use bare-metal stents and reduce the duration of dual antiplatelet therapy and associated bleeding risk. Plasma levels of multiple MMP isoforms have the potential to guide patient selection by predicting those at risk of ISR and multiple-site ISR. This is of particular relevance in elderly patients with multi-vascular coronary disease or those at increased bleeding risk where MMP levels could be used to determine those at low risk of multiple site ISR with bare-metal stents implanted.

In conclusion, this study indicates that the cleaved active forms of MMP-9 and -3 are independently associated with ISR in patients treated with bare-metal stents. The prognostic value of this marker needs to be further evaluated in longitudinal studies and in treatment with drug-eluting stents.

Acknowledgements

We gratefully acknowledge the funding support of the Heart Foundation of New Zealand. The assistance of Mr. Andrew Gray with the statistical analysis in this study is greatly appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.atherosclerosis.2009.05.036.

References

11.7.2. Plasma active matrix metalloproteinase 9 associated to diastolic dysfunction in patients with coronary artery disease

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a Departments of Medicine, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand
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Plasma active matrix metalloproteinase 9 associated to diastolic dysfunction in patients with coronary artery disease

Circulating levels of total matrix metalloproteinases (MMP) have been associated with diastolic dysfunction and heart failure [1,2]. This study aimed to investigate the relationship of the endogenous active levels of MMP-1, -2, -3 and -9 or tissue inhibitor of metalloproteinases-1 (TIMP-1), and diastolic dysfunction (DD) in the setting of coronary artery disease (CAD).

Concluded fifty-three patients with angiographically proven CAD were recruited retrospectively from the Dunedin Hospital Cardiology Clinical database. The patients were stable and free of heart failure symptoms at the time of recruitment. Clinical parameters recorded along with echocardiographic measurements. The study protocol was approved by the Otago ethics committee. All subjects gave written informed consent before being recruited into this study.

Echocardiographic parameters were measured in urine of high-sensitivity C-reactive protein (hs-CRP), ceramide, pro-MMP-9 and total TIMP-1. [2] Heparin plasma samples were analyzed for high-sensitivity C-reactive protein (hs-CRP), ceramide, pro-MMP-9 and total TIMP-1. [2] Heparin plasma samples were analyzed for high-sensitivity C-reactive protein (hs-CRP), ceramide, pro-MMP-9 and total TIMP-1.

All patients had trans-thoracic echocardiography and Doppler examination (GE-Vivid 7; system, USA) with analyses subsequently performed off-line. Two-dimensional, targeted M-mode echocardiography, and Doppler ultrasound measurements were obtained. All measurements were performed in accordance with the guidelines of the American Society of Echocardiography [4,5]. All echocardiography data represent the mean of 5 measurements or different cardiac cycles. Left ventricular (LV) ejection fraction was calculated by the modified Simpson’s biplane method. LV mass in grams derived from LV linear dimensions by the following formula: LV mass = 0.8 x [1.04 x LVIDd x PWTD x 2 + 0.6], where LVIDd, PWTD and SVIDe were LV internal dimension at end diastole, posterior wall thickness at end diastole and septal wall thickness at end diastole, respectively [5]. All measurements were made with accurate images recorded in a blinded fashion. The pooled Doppler measurements were obtained in the apical view with a curvilinear or the m-mode view with a pulsed wave Doppler placed between the outflow area and the LV outflow tract. OD was graded as mild, moderate or severe and was evaluated using the Carpentier’s classification [5,7]. All patients studied had preserved LV systolic function with ejection fraction ≥40%.

StatView version 5.1 (1995, Institute) was used to perform statistical analysis. The distribution of continuous variables (kurtosis and skewness) was assessed and analyzed with the Mann-Whitney U, Kruskal-Wallis (non-parametric trend test) or ANOVA with Fisher protected least significant difference test. Results are shown as mean ± SD, except variables with a non-Gaussian distribution, which are reported with medians and interquartile ranges. Odds ratios are given with 95% confidence intervals. A p-value of less than 0.05 was considered statistically significant. Multiple logistic regression was used to evaluate the interaction between variables and MPM in correlation with LVIDd. The ability of MMP markers to shift cases and controls to correct clinical categories was assessed by classification tables derived from the multiple logistic regression model. Based on the work by Cook et al. [8] (Non-parametric ROC curves were generated using a dedicated programme, mROC (User’s handstatistica, CRC Val d’Aurelia, V1000) [9].
Table 1
Clinical characteristics according to the phases of diabetic function.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal n = 62</th>
<th>Milder n = 49</th>
<th>Mild-moderate n = 21</th>
<th>Moderate or severe n = 19</th>
<th>P-value for trenda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>51.3 ± 10.3</td>
<td>59.0 ± 9.3</td>
<td>60.7 ± 10.5</td>
<td>61.4 ± 9.9</td>
<td>0.08</td>
</tr>
<tr>
<td>M1G</td>
<td>44 (71.0)</td>
<td>32 (65.3)</td>
<td>14 (66.7)</td>
<td>8 (42.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.5 ± 4.4</td>
<td>29.3 ± 4.5</td>
<td>28.5 ± 4.5</td>
<td>28.9 ± 5.2</td>
<td>0.77</td>
</tr>
<tr>
<td>WHR</td>
<td>0.91 ± 0.13</td>
<td>0.94 ± 0.08</td>
<td>0.92 ± 0.08</td>
<td>0.90 ± 0.10</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td>Hypertension (&gt;140/90 mmHg)</td>
<td>21 (41.9)</td>
<td>19 (38.8)</td>
<td>13 (61.9)</td>
<td>7 (36.8)</td>
<td>0.91</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>21 (35.9)</td>
<td>25 (51.0)</td>
<td>14 (66.7)</td>
<td>6 (31.6)</td>
<td>0.26</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>14 (22.6)</td>
<td>10 (20.4)</td>
<td>3 (14.3)</td>
<td>1 (5.3)</td>
<td>0.82</td>
</tr>
<tr>
<td>Coronary artery disease severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single vessel</td>
<td>21 (42.9)</td>
<td>7 (14.3)</td>
<td>0.94 (1.1)</td>
<td>0.90 (1.1)</td>
<td>0.13</td>
</tr>
<tr>
<td>Double vessel</td>
<td>13 (25.0)</td>
<td>18 (36.7)</td>
<td>8 (38.1)</td>
<td>4.0 (7.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Triple vessel</td>
<td>16 (30.8)</td>
<td>10 (20.4)</td>
<td>8 (38.1)</td>
<td>3 (23.1)</td>
<td>0.33</td>
</tr>
<tr>
<td>Smoldering phase</td>
<td>73 (43.8)</td>
<td>73 (43.8)</td>
<td>75 (45.3)</td>
<td>75 (45.3)</td>
<td>1.04</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>81.9 ± 36.3</td>
<td>78.1 ± 23.6</td>
<td>79.5 ± 19.2</td>
<td>81.6 ± 51.1</td>
<td>0.38</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>17 (1.2–3.4)</td>
<td>16 (1.7–3.9)</td>
<td>16 (1.2–2.5)</td>
<td>27 (1.7–6.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEI or ARB</td>
<td>31 (48.4)</td>
<td>25 (51.0)</td>
<td>7 (33.3)</td>
<td>5 (26.3)</td>
<td>0.52</td>
</tr>
<tr>
<td>Renin inhibitor</td>
<td>29 (46.8)</td>
<td>23 (46.9)</td>
<td>11 (52.4)</td>
<td>10 (52.6)</td>
<td>0.80</td>
</tr>
<tr>
<td>Calcium-antagonist</td>
<td>14 (22.6)</td>
<td>12 (24.5)</td>
<td>7 (33.3)</td>
<td>5 (26.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Statin</td>
<td>58 (93.5)</td>
<td>46 (93.9)</td>
<td>21 (95.2)</td>
<td>7 (36.8)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*RMI = body mass index; WHR = waist:hip ratio; GFR = glomerular filtration rate; and hs-CRP = high-sensitivity C-reactive protein.

Table 2
Comparison of echocardiographic and biochemical data according to the phases of diabetic function.

<table>
<thead>
<tr>
<th>Echocardiographic data</th>
<th>Normal * n = 62</th>
<th>Milder * n = 49</th>
<th>Mild-moderate * n = 21</th>
<th>Moderate or severe * n = 19</th>
<th>P-value for trenda</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEF (%)</td>
<td>57 ± 8</td>
<td>57 ± 8</td>
<td>63 ± 5</td>
<td>59 ± 8</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Peak E (cm/s)</td>
<td>40 ± 13</td>
<td>76 ± 15</td>
<td>90 ± 18</td>
<td>88 ± 29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak A (cm/s)</td>
<td>66 ± 14</td>
<td>85 ± 10</td>
<td>80 ± 15</td>
<td>56 ± 22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E/A</td>
<td>1.3 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PHT, mm</td>
<td>117 ± 32</td>
<td>204 ± 50</td>
<td>202 ± 47</td>
<td>107 ± 108</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>IVS, mm</td>
<td>88 ± 21</td>
<td>115 ± 20</td>
<td>90 ± 15</td>
<td>86 ± 26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IVST, mm</td>
<td>37 ± 30</td>
<td>102 ± 35</td>
<td>102 ± 20</td>
<td>110 ± 64</td>
<td>0.46</td>
</tr>
<tr>
<td>LVOT, mm</td>
<td>48 ± 5</td>
<td>48 ± 6</td>
<td>44 ± 4</td>
<td>47 ± 6</td>
<td>0.30</td>
</tr>
<tr>
<td>LVES, mm</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
<td>12 ± 2</td>
<td>13 ± 3</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>RWT, mm</td>
<td>10 ± 2</td>
<td>11 ± 2</td>
<td>11 ± 1</td>
<td>12 ± 2</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

MMP-9: matrix metalloproteinase; LVEF: left ventricular ejection fraction; IVST: left ventricular wall thickness; LA: left atrial dimension; BMI: body mass index; E/A: early to late filling ratio.

*Results expressed as mean ± standard deviation.

Table 3
Comparison of echocardiographic and biochemical data according to the phases of diabetic function.

<table>
<thead>
<tr>
<th>Biochemical data</th>
<th>Normal * n = 62</th>
<th>Milder * n = 49</th>
<th>Mild-moderate * n = 21</th>
<th>Moderate or severe * n = 19</th>
<th>P-value for trenda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting MMP-1, mg/l</td>
<td>2.8 (1.3–3.6)</td>
<td>2.2 (1.0–3.7)</td>
<td>2.0 (1.0–3.7)</td>
<td>3.5 (1.2–4.4)</td>
<td>0.08</td>
</tr>
<tr>
<td>Active MMP-2, mg/l</td>
<td>3.1 (13.1–18.4)</td>
<td>13.0 (12.8–18.4)</td>
<td>18.4 (14.0–22.3)</td>
<td>146.8 (8.163)</td>
<td>0.20</td>
</tr>
<tr>
<td>Active MMP-3, mg/l</td>
<td>2.3 (13.1–18.4)</td>
<td>2.3 (17–23)</td>
<td>2.2 (17–23)</td>
<td>3.1 (17–45)</td>
<td>0.37</td>
</tr>
<tr>
<td>Total MMP-9, mg/l</td>
<td>15.1 (5.0–17.1)</td>
<td>24.0 (17.3–41.6)</td>
<td>26.0 (14.1–36.3)</td>
<td>39.2 (18.3–48.4)</td>
<td>0.63</td>
</tr>
<tr>
<td>Total MMP-9, mg/l</td>
<td>1.4 (10.0–11.1)</td>
<td>1.5 (10.5–20)</td>
<td>2.1 (15–25)</td>
<td>2.4 (1.8–30)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>TIMP-1, mg/l</td>
<td>212.8 (99–250)</td>
<td>242.1 (217–278)</td>
<td>220.1 (187–277)</td>
<td>224.2 (208–274)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

E/Ea: ejection fraction; EF: ejection fraction; E: early diastolic velocity; A: early diastolic velocity; IVST: interventricular septum thickness; IVS: interventricular septum thickness; LA: left atrial dimension; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinase. Biochemical data are expressed as mean ± standard deviation.

*Results expressed as means and interquartile range.
with improved model fit, as indicated by a decrease of 10 points using the Bayesian Information Criteria. The addition of active MMP-9 and TIMP-1 to these same variables resulted in an increase of area under the curve of 9% and 6%, respectively, for the discrimination between controls and mild/moderate DB (from 0.73 (95% CI 0.60-0.83) to 0.82 (95% CI 0.73-0.89)), and between controls and moderate/severe DB (from 0.76 (95% CI 0.62-0.86) to 0.82 (95% CI 0.76-0.80)).

In conclusion, this study indicates that elevated level of the active form of MMP-9 is associated with DB, and the level of elevation correlates with the phases of DB in patients with CAD. This finding may reflect abnormal extracellular matrix metabolism in myocardial ischemia and the prognostic value of this marker needs to be further evaluated. Future prospective longitudinal studies will be required to explore the role of this enzyme in important and common clinical problems.

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References

Comparison of different body habitus between patients with mitral valve prolapse and normal populations in young Taiwanese females

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The mitral valve prolapse (MVP) syndrome is one of the most prevalent cardiac valvular abnormalities and may affect as much as 5-15% of the population [1]. The weight of patients with MVP is often lower, and the habitus may be asthenic. Blood pressure (BP) is also normal or lower, and orthostatic hypotension may present. It has never been studied in association with body habitus in Taiwanese MVP patients. Thus we attempt to answer whether the descriptions regarding body habitus parameters in Western literature also apply to Taiwanese female patients.

The study protocol was approved by the institution review board and ethic committee of Tzu Chi General Hospital, and informed consent was obtained from all participants. We consecutively recruited female patients aged between 20 and 30 years old. MVP patients without other organic heart disease were selected. Normal age-matched volunteers without MVP and body habitus that cohered to normal populations: study recruits in Taiwan (e.g., weight range from 43 to 69 kg; height range from 149 to 169 cm; weight range from 35 to 85 kg) were recruited. Each patient completed a survey of symptoms of chest tightness, palpitation or breathlessess.

The diagnosis of MVP based on demonstration of thick, redundant leaflets and chordae with systolic displacement of the leaflets into the left atrium in two-dimensional echocardiography [2]. The measurement of body parameters included weight, height, body mass index
11.7.3. Seasonal variation and stability of matrix metalloproteinase-9 activity and tissue inhibitor of matrix metalloproteinase-1 with storage at -80°C

Case Report

Seasonal variation and stability of matrix metalloproteinase-9 activity and tissue inhibitor of matrix metalloproteinase-1 with storage at −80 ºC

G.P. Tarr, M.J.A. Williams, L.V. Phillips, A.M. van Rij, G.T. Jones

Abstract

Objective: To determine whether active matrix metalloproteinase (MMP)-9 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 displayed seasonal variation and were stable in storage.

Methods: Plasma active MMP-9 and TIMP-1 were measured at three time-points in 183 individuals.

Results: There was no evidence for seasonal variation or declining levels for up to three years of storage at −80 ºC.

Conclusion: Active MMP-9 and TIMP-1 appear to be stable seasonally, and in storage for at least three years.

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Introduction

Matrix metalloproteinases (MMP) are extracellular matrix endopeptidases that have been implicated in a number of disease states, including cancer, cardiovascular disease and arthritis. However, there has been some controversy over pre-analytical strategies, including anticoagulation [1] and long term storage [2]. Kasy et al. [2] described declining levels of both protein and the functional activity of MMP-9 with storage at −80 ºC, decreasing to −1% of baseline at four years, an observation that continues to receive a number of citations. This observation has not been confirmed, and seems to sit at odds with published studies which appear to have successfully measured MMPs many years after sampling.

In addition, a number of biological markers have been shown to display seasonal variation [1], but it is unknown whether MMPs vary in the same way. If MMPs do show seasonal variation then this could be a significant confounder of biomarker studies.

The aim of this study was to assess the impact of seasonality and elapsed storage time at −80 ºC on assayed concentration of active MMP-9 and tissue inhibitor of matrix metalloproteinase-TIMP-1.

Methods and Materials

Patients

Blood samples were drawn from 163 patients immediately prior to undergoing percutaneous coronary intervention, then at three- and six-months after intervention. All had angiographically proven coronary artery disease at index. The study recruitment period was from February 2007 through to February 2010. Both control subjects without further cardiovascular events (n=58) and subjects with further incident cardiovascular events (n=65) were included. Participants were recruited within every month of the year. Clinical and demographic information as well as date of sample collection were recorded. Written informed consent was obtained from all study participants and the study was conducted with the approval of the Lower South Ethics Committee.

Biochemistry

Samples were centrifuged at 4 ºC and 3000 rpm for eight minutes. Then plasma was stored at −80 ºC. All samples remained frozen until analysis. Active MMP-9 was assessed in heparin plasma samples using the Biotek Activity Assay System (RPN 2634; GE Healthcare Life Sciences). TIMP-1 was assessed in EDTA plasma using a conventional ELISA (RPN 2614; GE Healthcare Life Sciences). The coefficient of variance for both assays was <5%. Statistical analysis was performed using StatView (version 5.0); SAS Institute and Stata (version 10.1; StataCorp). Active MMP-9 level was transformed by natural logarithm when samples from multiple time points from the same individual were included.
Appendix

logistic regression with clustering for the individual identifying variable was utilized to preserve the assumption of independence.

Results

The study population had a mean age of 62.8 years (SD 10.7), and 120 (78.7%) were male. There was a broad range of storage times at −80°C, ranging from fewer months to over three years (Fig. 1). There was no association between storage time at −80°C and assayed concentration of active MMP-9 (Fig. 1A), and some of the samples with the highest readings had been stored for three years. There appeared to be a small association between storage time at −80°C and assayed concentration of TIMP-1 (Fig. 1B), with higher levels in samples with longer storage time. This was no longer significant after adjustment for age and sex, indicating that this is likely explained by the composition of the population. To exclude the possible effects of inter-patient variability, a paired analysis of post-interventional (3 and 6 month) samples from each patient was conducted. There was no difference in either active MMP-9 or TIMP-1 between samples from the same individual, despite the 3-month difference in storage times.

Assayed levels of active MMP-9 and TIMP-1 were similar irrespective of the months of the year the sample was collected (Fig. 2A and B respectively).

Similar results for both the storage time and the seasonality analyses were obtained when only the index sample was included, and when only controls were included. Furthermore, there was no evidence for an association when adjustment for the development of further cardiovascular events was included in the multivariate model, or when the analysis was repeated in the control group alone.

Discussion

There was no association between elapsed storage time at −80°C and assayed concentration of active MMP-9. This observation is inconsistent with the findings of Rooy et al. [2] who found that both total antigen and functional activity of MMP-9 appeared to decline over time in samples stored at −80°C. Rooy et al. describe an initial rapid decrease over three months, relative stability to two years, then very low levels in samples stored 25 months and longer. Over the same time period the level of MMP-2 available for zymographic analysis appeared to be stable. However, the findings of Rooy et al. that TIMP-1 appears to be stable was supported by our results.

Sousa-Tata et al. [4] examined the effect of freeze-thaw cycles and storage temperature on the stability of pro-MMPs-2 and −9, and the zymographic activity of MMP-5, finding that there was no significant difference between MMPs stored at −20°C for one month and those at −80°C. Furthermore, all MMP levels tested remained constant for...
up to five freeze-thaw cycles, 19 suggesting that MMP levels are relatively stable. Kisan et al. [5] examined the rate of degradation of MMP-2 and TIMP-1 when stored at a range of temperatures. They utilized the Arrhenius equation, which describes the temperature dependence of the rate of chemical reaction to model the decay of these analytes. While MMP-7 was sensitive to temperature, they found it was remarkably stable when stored at −75 °C, predicting that the recovery would remain >90% for tens of thousands of years. However, while TIMP-1 was more stable at room temperature than MMP-7, it would degrade to <50% of the baseline measure by 17 months.

While a number of groups have examined circulating MMPs in various conditions, many over long periods of time, there is no direct confirmation of the findings of Rouy et al. While most studies have not precisely reported the time from beginning of blood storage to assay, it appears that readings of pro-MMP-9 remain high, without evidence of degradation. One of the complicating factors in comparing studies is that different anticoagulant strategies alter measured values, with serum samples giving high readings, and citrate-heparin and EDTA giving differing results for some MMPs [6]. A number of long-term studies have examined the role of MMPs in the prediction of cardiovascular events.

A number of reports in which samples had been stored for around two years found that pro-MMP-9 in EDTA plasma read between 20 ng/ml and 70 ng/ml, [7,8]. One group has reported high pro-MMP-9 (>500 ng/ml) levels in sera for up to 15 years. [9] Regardless of the variability of these results, it indicates that pro-MMP-9 levels do not drop to near zero, as reported by Rouy et al. [2]. However, Pauta et al. [7] describe a halving in pro-MMP-9 concentration in samples from breast cancer patients after around three years of storage compared to controls. Of the control group at around two years of storage, although this observation is not controlled for disease status and the observed effect could equally be from altered levels in patients with cancer.

Furthermore, it has been shown that the anticoagulant used by Rouy et al. has biological effects on MMP concentrations [10], and may chelate Zn²⁺ ions essential for MMP activity. Therefore, degradation of MMP-9 may be restricted to this anticoagulant.

There was no interaction between month of sampling and assayed concentration of either active MMP-9 or TIMP-1 in this study. Thus, in contrast to some biomarkers such as tissue-plasminogen activator levels of active MMP-9 and TIMP-1 appear to be stable over seasons [3].

Limitations of this study included that this experiment would optimally be performed utilizing the same subjects with repeated blood samples across the range of the variable of interest. Secondly, including the whole cohort in these analyses meant that some individuals developed cardiovascular events. As we, and others, have previously shown such post-interventional events can be associated with rises in MMPs [8], this acting as a potential confounder of this analysis. Therefore, the development of events was included in the multivariate model, and the analysis also repeated using either only the index samples or the control group (without post-interventional events). In all cases, the results remained consistent.

In conclusion, there was no evidence of degradation of active MMP-9 and TIMP-1 up to three years of storage at −80 °C, indicating not only the stability of the proteins themselves, but also the preservation of enzymatic activity in these stored samples. Active MMP-9 and TIMP-1 appear to be robust in storage at −80 °C for a number of years, so studies of these proteins need not be limited by a shorter storage time. Active MMP-9 and TIMP-1 do not appear to have a seasonal variation, indicating that the season in which the blood sample is taken is not a potential confounder of these proteins.

References

2. Rouy D, et al. Distance from the start of blood storage to assay, it appears that readings of pro-MMP-9 remain high, without evidence of degradation. One of the complicating factors in comparing studies is that different anticoagulant strategies alter measured values, with serum samples giving high readings, and citrate-heparin and EDTA giving differing results for some MMPs [6]. A number of long-term studies have examined the role of MMPs in the prediction of cardiovascular events.
11.7.4. Pro-MMP-9/TIMP-1 ratio correlates poorly with a direct assessment of MMP-9 activity

Pro-MMP-9/TIMP-1 ratio correlates poorly with a direct assessment of MMP-9 activity

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Enzyme-linked immunosorbent assay (ELISA)

ABSTRACT

Objective: To determine if the pro-MMP-9/TIMP-1 ratio is an accurate surrogate for endogenously active MMP-9 levels.

Methods: Plasma active MMP-9, pro-MMP-9 and TIMP-1 were measured in 235 patients.

Results: There was a weak negative correlation between the pro-MMP-9/TIMP-1 ratio and active MMP-9. TIMP-1 was more closely correlated with active MMP-9 than pro-MMP-9.

Conclusion: Pro-MMP-9/TIMP-1 ratio measured with ELISA is not a good surrogate measure for active MMP-9, and direct measurements of active MMP-9 are therefore recommended.

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Introduction

The matrix metalloproteinases (MMPs) are extracellular matrix degrading enzymes that are involved in a range wide variety of physiological and pathological processes, thereby influencing a wide range of clinical conditions including cardiovascular disease, arthritis and cancer. Initially secreted from multiple cell types as inactive proenzymes, they undergo activation through a variety of mechanisms, including via proteolytic cleavage of the inhibitory chain by another enzyme, and/or by auto-prolylysis after conformational change [1]. Finally, the tissue inhibitors of matrix metalloproteinases (TIMPs), a family of endogenous inhibitors, bind to MMPs and inhibit any potential enzymatic activity [1]. TIMP inhibition is reversible, and whether complexes form or dissociate appears to depend on the relative concentrations of the proteins [2]. There is experimental evidence which demonstrates that MMPs may be bound to TIMPs both before and after secretion from cells [3]. While there is no doubt that TIMPs play important biological roles, the meaning of the relative concentrations of MMPs and TIMPs in the circulation is less clear.

Enzyme-linked immunosorbent assay (ELISA)-based kits capable of directly measuring the activity of many MMPs are commercially available. However, some authors use conventional ELISA kits to measure pro-MMP levels, and calculate the ratio between circulating inactive MMPs and TIMPs, interpreting this as an indicator of MMP activity [4,5]. However, the validity of the ELISA measurements of pro-MMP/TIMP ratio as a predictor of MMP activity has not been empirically established.

The aim of the present study was to test whether a direct measurement of MMP-9 activity was associated with the ratio of pro-MMP-9 and TIMP-1. Secondly, this study also aimed to determine the individual contributions of pro-MMP-9 and TIMP-1 measurements to the value of the ratio.

Methods

Asymptomatic patients with angiographically-proven coronary artery disease were recruited from Dandelin Public Hospital from February 2003 through December 2004. Written informed consent was obtained in all patients and the study was conducted with the approval of the Lower South Ethics committee.

Pro-MMP-9 and TIMP-1 were measured by conventional ELISA (product codes RPN 2614 and RPN 2611, GE Healthcare Life Sciences) in EIA plasma. Active MMP-9 was measured in heparin plasma samples using the Biotrak Activity Assay System (RPN 2634, GE Healthcare Life Sciences). Both the pro-MMP-9 and the TIMP-1 assays were fully cross-reactive with pro-MMP-9/TIMP-1 complexes. The pro-MMP-9 assay did not cross react with active MMP-9. Some ELISA kits measure both pro- and active MMP-9, and are generally labelled as "total" MMP ELISAs. The active MMP-9 assay was not cross...
reactive with TIMP complexes, as the assay utilised the endogenous enzymatic activity to produce a coloured reaction. The coefficient of variance was 7.3% for all assays.

Statistical analysis was performed with StatView version 501 (SAS Institute). Spearman’s correlation coefficient was calculated for each pair of variables. To confirm associations, multivariate modeling, adjusting for age, sex, and type of clinical event (in-stent restenosis vs. de novo coronary artery disease) was utilized. MMP variables were transformed by natural logarithm before being entered into the model. Associations between MMP variables and a history of in-stent restenosis are published elsewhere [8].

Results

Two hundred and ninety-five patients were included. The mean age was 63.0 years (standard deviation 9.4) and 216 (74.2%) were male.

The medians (interquartile range) of pro-MMP-9 and active MMP-9 were 23.3 (16.5–39.0) and 1.7 (1.2–2.5) ng/mL, respectively. The mean concentration of TIMP-1 was 238.8 ng/mL (standard deviation 88.7). The median (interquartile range) of pro-MMP-9/TIMP-1 ratio was 0.10 (0.07–0.18).

There was a weak negative correlation between the ratio of pro-MMP-9/TIMP-1 and the concentration of active MMP-9 (Fig. 1A). When age, sex and history of clinical events were entered into the model this finding remained significant, but explained little of the variation of active MMP-9 (r = −0.22, p = 0.02). This indicates that larger ratios of pro-MMP-9/TIMP-1 are associated with less MMP-9 activity, but the weak correlation suggests that pro-MMP-9/TIMP-1 ratio is a poor surrogate for active MMP-9.

There were modest relationships between individual measurements of active MMP-9 with both pro-MMP-9 and TIMP-1 (Fig. 1A and B). The pro-MMP-9 was inversely related to active MMP-9, with higher levels of pro-MMP-9 being associated with lower levels of active MMP-9. The correlations between active MMP-9, pro-MMP-9, and TIMP-1 remained significant after adjustment for age, sex and history of clinical events.

There was a very strong linear relationship between pro-MMP-9 and pro-MMP-9/TIMP-1 ratio (r = 0.95). As expected, there was a negative relationship between TIMP-1 and the pro-MMP-9/TIMP-1 relationship (r = 0.31). Thus, in contrast to the strong relationship between pro-MMP-9 and pro-MMP-9/TIMP-1 ratio, there was only a moderate correlation between TIMP-1 and pro-MMP-9/TIMP-1 ratio. Both of the associations between pro-MMP-9, TIMP-1 and pro-MMP-9/TIMP-1 ratio remained significant after adjustment for age, gender and history of cardiovascular events.

Similar results were obtained for all analyses by repeating each comparison within separate clinical event subgroups.

Discussion

The main finding of this study was that the pro-MMP-9/TIMP-1 ratio was weakly and inversely related with direct measure of circulating active MMP-9. This is diametrically opposed to the interpretation of many authors in the literature who commonly assume that increased pro-MMP/TIMP ratios are an indication of increased MMP activity [4,5].

There are at least three steps in regulating MMP-9 activity: production of the pro-enzyme, activation through cleavage of the pro-sequence and TIMP inhibition [1]. However, the relative importance of these mechanisms in determining the in vivo activity is not known. Our results suggest that neither a measurement of enzyme production (i.e. pro-MMP-9) nor presence of an endogenous inhibitor (TIMP-1) is a reliable surrogate for active MMP-9, even when combined to form an enzyme–inhibitor ratio.

![Fig. 1](image1.png)

**Fig. 1.** Association between circulating active MMP-9 and pro-MMP-9/TIMP-1 ratio, pro-MMP-9, and TIMP-1. A: Association between active MMP-9 and pro-MMP-9/TIMP-1 ratio. B: Association between active MMP-9 and TIMP-1. C: Association between active MMP-9 and TIMP-1. Results are Spearman’s coefficient correlations.

One possible explanation for the negative correlation between the pro- and active forms of MMP-9 is that the pro-MMP-9 antigen available for capture is consumed when MMP-9 is activated. The pro-MMP-9 ELISA that we used was able to detect both free and TIMP-bound pro-MMP-9, but was not cross-reactive to active MMP-9. Although a poor surrogate for enzyme activity, the positive correlation between active MMP-9 and TIMP-1 may therefore represent concurrent counter regulation of increased enzymogen conversion.
Analysis of MMPs by zymography also relies on substrate lysis and the electrophoretic migration pattern identifies each MMP based on molecular weight. The use of this technique to quantify relative "pro- and active" MMP isoform in the same sample has been widespread. However, there are two significant limitations to interpreting zymography results for this purpose. Firstly, the partial denaturation by sodium dodecyl sulfate appears to activate latent MMPs by interacting with the "zymogen switch". Secondly, the process of zymography causes the separation of bound TIMPs and MMPs [7]. Hence, the so-called "active" band actually consists of MMPs that were either "free" (pro-cleaved but TIMP bound) or truly endogenously active (pro-activated and TIMP free) in vivo. Substrate ELISA based activity assays do not suffer this same limitation.

We noted that TIMP-1 has a moderate, positive correlation with active MMP-9. The active MMP-9 assay we used relied on substrate lysis by the captured MMP-9, and thus did not measure TIMP-1 bound enzyme. However, there is overlap between regulatory elements between TIMPs and MMPs [8], and MMP activity may even stimulate the production of TIMPs, through the activation of cytokines and release of growth factors sequestered within the extracellular matrix. For example, MMP-9 can activate transforming growth factor-β [9], which then stimulates TIMP-1 production [10]. In this way, MMP-mediated TIMP release may act as a physiological braking system, preventing extracellular matrix breakdown outside of the control of upstream signaling.

Secondly, this study demonstrates that the main contributor to the pro-MMP-9/TIMP-1 ratio is circulating pro-MMP-9, with a weaker contribution from circulating TIMP-1. This finding suggests that the steady state has now been reached and that MMP-9 and TIMP-1 are in association with disease [4,5] have not necessarily demonstrated increased MMP activity. However, they may have detected surrogates of inflammatory regulatory of MMPs, such as tumor necrosis factor-α and platelet derived growth factor, which stimulate MMP production by promoting gene transcription [11].

Limitations

Both the pro-MMP-9 and the TIMP-1 assays were fully cross-reactive with pro-MMP-9/TIMP-1 complexes. We did not make direct measurements of the pro-MMP-9/TIMP-1 complex, so it is unknown what influence these bound fractions had on our results.

Conclusions

The plasma pro-MMP-9/TIMP-1 ratio appears to be weakly inversely correlated with active MMP-9, which is consistent with previous interpretations in the literature. Individual measurements of TIMP-1 and pro-MMP-9 are both modestly (positively and inversely respectively) correlated with active MMP-9. Circulating pro-MMP-9 appears to be the primary determinant of the pro-MMP-9/TIMP-1 ratio.

Pro-MMP-9/TIMP-1 measured by ELISA appears to be a poor surrogate for active MMP-9, and active MMP-9 should therefore be measured directly in order to determine endogenous enzymatic activity.

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References