VENTRICULAR REMODELLING FOLLOWING ISCHAEMIC EVENTS: A BIOCHEMICAL, NEUROHORMONAL & FUNCTIONAL STUDY

A thesis submitted for the degree of Doctor of Medicine

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by

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CONTENTS

INDEX OF FIGURES AND TABLES

Figure **Page**

Figure **Page**

CHAPTER ONE – INTRODUCTION

1.1 Coronary Heart Disease

1.1.1 Historical Review

(a) The Heart and Cardiovascular System

Man's understanding of the heart and the mechanisms of its diseases are increasing at an exponential rate, with much of our knowledge being attained in the last few decades. However, each period in history has provided new insights and added to the collective understanding of the heart, its functions and afflictions.

Cave paintings drawn more than 10,000 years ago illustrate that even primitive man recognised the importance of the heart as a life sustaining force. One such mural, drawn by Aurignacian Man in Northern Spain, depicts an outline of a mammoth with a heart shaped spot in the middle of the body and is generally assumed to depict where a hunter should aim (Pollak and Ashworth, 1968). The first recorded rudimentary description of the circulation can be found in ancient Chinese texts, dating from around 2,600 BC, where the Physician Huang Ti declared "the blood current flows continuously in a circle without a beginning or end and never stops". However, Huang Ti lived in a quasi-religious and superstitious time and this theory was never accepted, with traditional belief that the heart was the seat of happiness and a witness to the body's behaviour reigning supreme.

Two millennia later, the Classical Period of the Greek philosophical movement brought major advances in the medical arts. The advent of animal dissection allowed direct observation of the heart and vasculature. However, although these observations were more empirical and rational than previous times, they were still tainted by the religious and semi-spiritual beliefs of the period. For example, Alcmaeon of Greece noted empty arteries after death in 600 BC and deduced that they normally contained air. A few centuries later, Eransistratus of Egypt, claimed that in life a substance called Pneuma (air or spirit) circulated in the arteries, replaced with every breath, and that when an artery was cut, blood rushed in to fill the void left by escaping Pneuma. Therefore, it was loss of the life sustaining Pneuma rather than exsanguination that was the cause of death when arterial injury was sustained (Osler, 1921). However, this theory was disproved by direct observational evidence by Claudius Galenus of Alexandria (131 – 201 AD) who described the heart and movement of blood in the arteries. However, he did not realise that blood was circulated and taught of two distinct types of blood – "nutritive" which was made by the liver and carried by veins to the organs where it was consumed and "vital" which contained the spirit and was made by the heart and carried by arteries (Osler, 1921). These teachings remained largely unchallenged until the European Renaissance period of the 15th and 16th centuries.

The anatomical drawings, based on human dissection, of Leonardo da Vinci from the 15th century are famous for their detail and illustrate a greater understanding of the circulation than was evident previously. However, he still described the liver as the centre of circulation and drew holes in the interventricular septum. The

English court physician, William Harvey (1578 – 1657 AD) is often regarded as the father of cardiovascular medicine and is credited with the first accurate description of the circulation as a closed system in 1628 (Harvey, 1628). He even hypothesised the presence of tiny vessels which linked the arterial and venous systems. The advent of the microscope allowed the likes of Marcello Malpighi (1628 – 1694 AD) and Antoni van Leeuwenhoek (1632 – 1723 AD) to confirm the presence of capillaries, and the teachings of Harvey became widely accepted.

(b) Coronary Artery Disease

Although the coronary circulation had been described many years previously, it was not until 1768 that the term angina pectoris was coined by the English Physician, William Heberden in a lecture to the College of Physicians of London, posthumously published in 1802 (Heberden, 1802) -

> *"There is a disorder of the breast marked with strong and peculiar symptoms, considerable for the kind of danger belonging to it, and not extremely rare, which deserves to be mentioned more at length. The seat of it, and sense of strangling, and anxiety with which is attended, may make it not improperly be called angina pectoris. They who are afflicted with it, are seized while they are walking, more especially if it be uphill, and soon after eating, with a painful and most disagreeable sensation in the breast, which seems as if it would extinguish life, if it were to increase or to continue; but the moment they stand still, all this uneasiness vanishes……In all other*

respects, patients are, at the beginning of this disorder, perfectly well.....Males are most liable to this disease, especially such as have past their fiftieth year."

Greater understanding of the pathophysiology of coronary artery disease and coronary death was provided over the next few decades. In 1793 the Scottish pathologist John Hunter described the presence of disease of the coronary arteries at autopsy in a man who had died following a fit of anger. A few years later, another Scot, Edward Jenner described changes in the coronary arteries which he described as "bony canals". In 1808, Matthew Baillie concluded that "ossification of the coronary arteries would seem to produce, or to be intimately connected with, the symptoms which could constitute angina pectoris (Baillie, 1808)." However, it was not until 1867 that the first drug for the symptomatic relief of angina was introduced by T. Lauder Brunton in 1867. This drug was amyl nitrate and its related compounds remain a cornerstone in the modern management of angina.

Diagnostic cardiology was revolutionised by the invention of the electrocardiogram by Willem Einthoven in the early 20th century. The first description of the electrocardiographical changes associated with myocardial infarction came from the American physician James Bryan Herrick (1861 – 1954 AD). In a presentation to the American Association of Physicians in 1912 he postulated that myocardial infarction was caused by coronary thrombosis (Herrick, 1912) and following publication of the ECG changes occurring in dogs after coronary artery ligation by his colleague Fred Smith in 1918 (Smith, 1918), he

demonstrated the ability of the ECG to diagnose the presence of acute myocardial infarction in a living patient (Herrick, 1918). In the same year, Bousfield described the ECG changes during angina (Bousfield, 1918), ushering in the era of diagnostic electrocardiography.

In the last 80 years, there have been great advances in all aspects of cardiology. New diagnostic methods for the detection of coronary artery disease have been developed and refined, notably the introduction of staged exercise testing by Bruce and colleagues in 1963, the advent of cardiac catheterisation first reported by Forssmann in 1929, refined and popularised by Judkins and Sones in the 1970s, the development of nuclear and ultrasonic imaging and the recent explosion in magnetic resonance angiography. Increased understanding of the pathophysiological processes involved in coronary disease and resultant changes to the heart have widened our therapeutic targets and improved our ability to modulate the process and further advances are occurring in an exponential fashion.

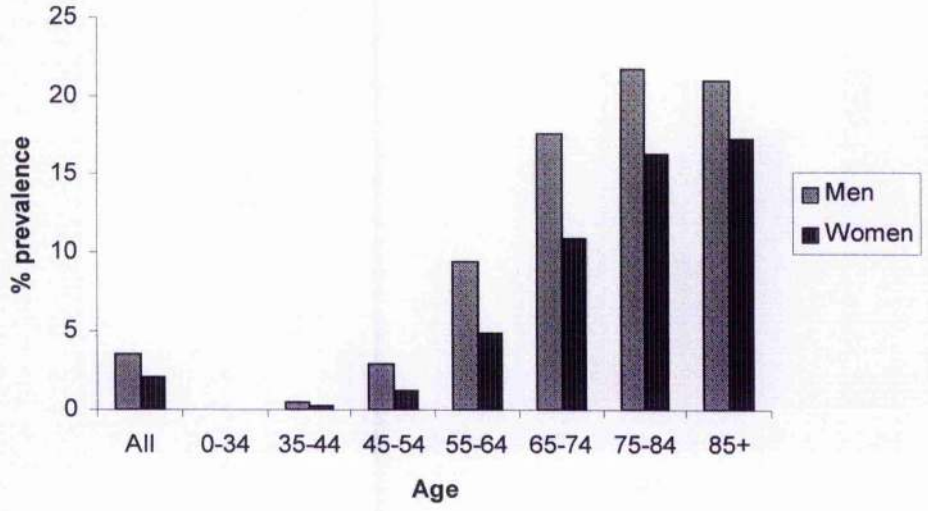
1.1.2 Epidemiology

In the 19th century, heart disease was the fourth leading cause of death, with infectious diseases accounting for the majority of mortality (Anonymous1993). Improvement in sanitation and the advent of antibiotic therapy has markedly reduced the incidence of many infections and their related mortality, especially in the Western world. This, coupled with a longer lifespan, along with environmental and dietary changes, has lead to the emergence of ischaemic heart disease (IHD) as the leading cause of death in the modern world. The Global Burden of Diseases

Study showed that ischaemic heart disease was directly responsible for 6.26 million deaths of the 50.6 million deaths worldwide in 1990, and that 29% of all deaths could be attributable to a cardiovascular cause (Murray and Lopez, 1997b). This figure is set to increase to 36% of all deaths by 2020 with IHD also being projected as the leading cause of disability (Murray and Lopez, 1997a).

These statistics are mirrored in the UK, with an overall prevalence of treated coronary heart disease (CHD) of around 3% of the population (Office for National Statistics, 2000; Ghandi, 1997). This figure is an underestimate, as those with asymptomatic or never treated disease are not included, and the true prevalence must therefore be much higher. Age, sex and socio-economic status are all powerful predictors of CHD. There is a marked incremental rise in CHD prevalence with advancing age, rising from 0.5% in men aged 35-44 to 9.3% aged 55-64 and 21.7% at age 75 and older. Overall, men are 2 to 3 times more likely to develop coronary disease than women but this age related increase in prevalence is mirrored (figure 1.1). Increasing life expectancy and reduced birth rate has meant that the population is ageing and the burden of disease will increase in the next few decades. Socio-economic deprivation is also associated with increasing frequency of CHD (table 1.1).

Figure 1.1



Legend - Prevalence of CHD by age and sex in the UK 1994/8. Adapted from data obtained from the Office of National Statistics (Office for National Statistics, 2000).

Table 1.1

Deprivation Category	Men (all ages)	Women (all ages)
Q1: least deprived	3.13%	1.74%
Q2	3.38	1.90
Q3	3.56	2.13
Q4	3.63	2.17
Q5: most deprived	4.09	2.60

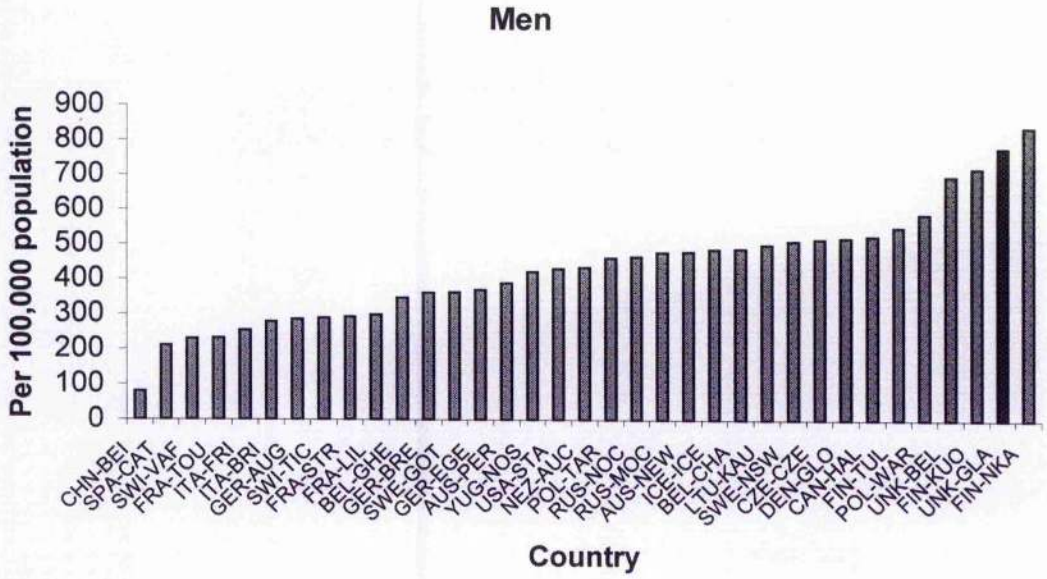
Legend - Prevalence of CHD by deprivation category 1986/92. Adapted from data obtained from the Office of National Statistics (Office for National Statistics, 1997).

The WHO MONICA Project has provided information on the geographical differences in prevalence of CHD over a 10 year period, starting in the mid-1980s (Tunstall-Pedoe et al. 1999). Scotland has the unenviable record for the highest incidence of coronary events per 100,000 population in women and the second highest event rate for men out of the 35 populations studied in 21 countries (table 1.2). The event rates (standardised for age) were 777 per 100,000 population for men and 265 per 100,000 for women, compared to 431 and 134 respectively in the USA and 233 and 36 in Toulouse, France. Some of these regional variations may be attributable to differing genetic predisposition in the populations studied, but dietary factors and other risk factors must play a role.

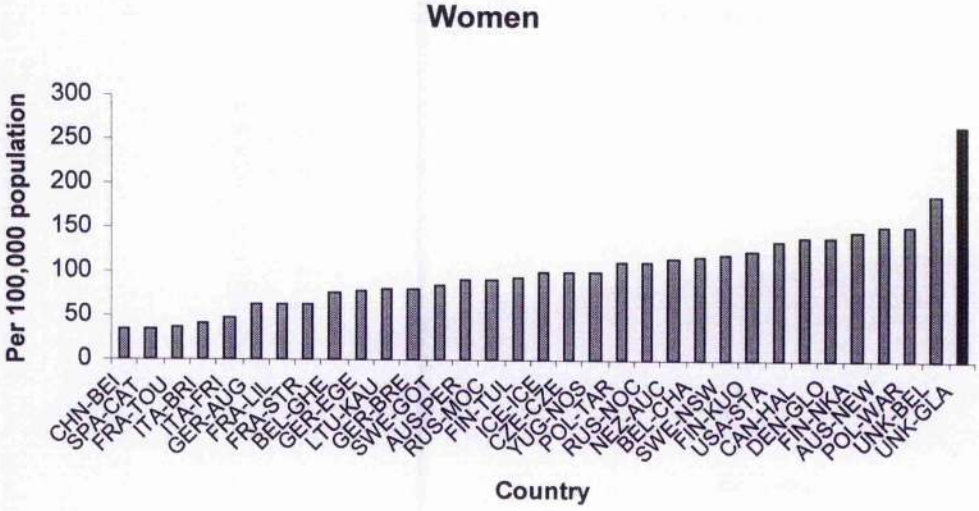
It is therefore clear that CHD is a common disease process and is the leading cause of death in the UK. In Scotland, there were 12,500 deaths from coronary heart disease in the year 2000 (data from Scottish Executive website). The sequelae of angina and myocardial infarction cause disability and premature death and are undoubtedly a major public health problem.

Figure 1.2

(a)



(b)



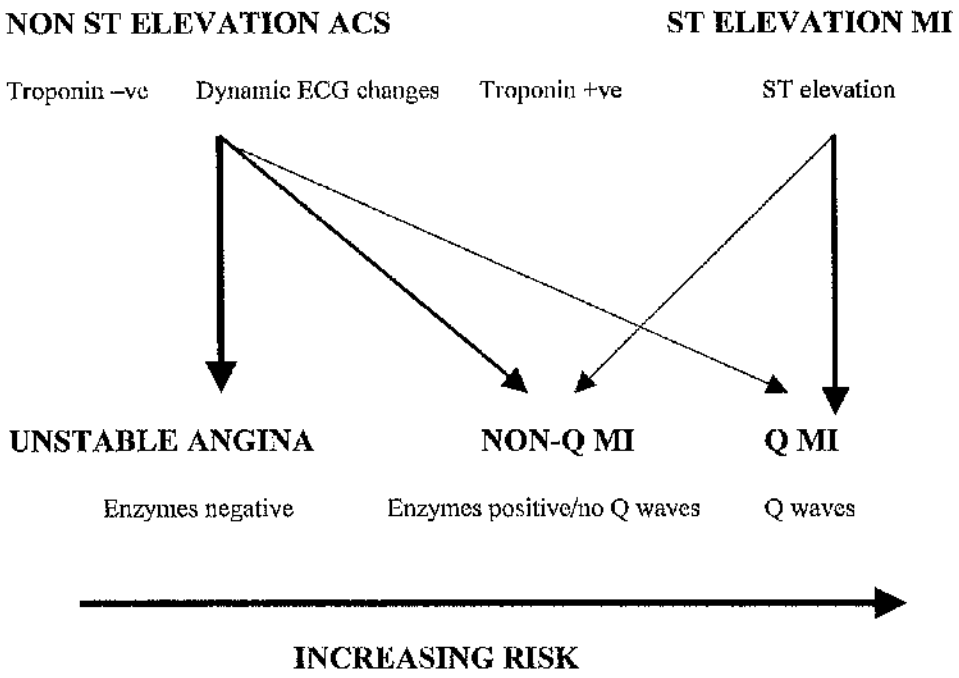
Legend - **Coronary event rates per country from the WHO MONICA Project for (a) men and (b) women. Adapted from data obtained from Tunstall-Pedoe *et al* 1999 (Tunstall-Pedoe et al. 1999).**

1.2 Acute Coronary Syndromes

1.2.1 Classification

Over the last few years, the classification of presentation with ischaemic heart disease has shifted from an ECG and enzyme-centric standpoint to a risk stratification and treatment orientated approach. The traditional classification of ischaemic coronary syndromes of unstable angina, non-Q wave and Q wave myocardial infarction was developed in the pre-thrombolytic era and was based on the progression of ECG changes and rise in standard cardiac enzymes (creatine kinase, lactate dehydrogenase and aspartate transaminase). However, improved treatment and better diagnostic tools have made this classification obsolete. For example, a patient presenting with chest pain and ST elevation on an ECG would be expected to develop a Q wave MI, but reperfusion, be it spontaneous or by intervention with thrombolytic therapy or primary angioplasty, can prevent the development of Q waves on the ECG. Similarly, patients without ST elevation on ECG can develop subsequent Q waves (figure 1.3) (Zaacks et al. 1999). In addition, more sensitive biochemical markers such as the troponin complexes can detect evidence of myocardial damage when traditional cardiac enzymes are negative.

Figure 1.3



Legend - Modern classification of Acute Coronary Syndromes and how they relate to the traditional classification

These observations, coupled with an increased understanding of the pathophysiology of acute ischaemia have lead to the development of a new classification under the broad umbrella term of acute coronary syndromes (ACS) (figure 1.3) (Joint European Society of Cardiology/American College of Cardiology Committee, 2000; Braunwald E et al. 2000). It is now recognised that acute ischaemic syndromes represent a continuum from the traditional diagnostic categories of unstable angina through to acute myocardial infarction. The key to the new classification is risk stratification and treatment approach. Occlusive

thrombus causing ST elevation on the ECG presents with the highest risk and is usually associated with cardiac enzyme rise. The treatment is with reperfusion therapy, pharmacological or catheter based, and is therefore classified independently – ST elevation MI. The term non-ST elevation acute coronary syndrome is applied to the remainder of unstable cardiac ischaemic syndromes – pain occurring at rest or on minimal exertion. These patients are risk stratified according to clinical, ECG and enzyme (including troponin) criteria which influence prognosis and allow targeting of therapy dependent on risk. They range from low risk with normal ECG and negative troponin to high risk with dynamic ECG changes and enzyme rise (figure 1.3). The terms ST elevation MI and non-ST elevation ACS will be employed in the rest of this thesis.

1.2.2 Epidemiology

As discussed in section 1.1.2, coronary heart disease is widespread in Scotland and the rest of the UK. There are 274,000 new myocardial infarctions per year in the UK. There are currently more than 1.3 million people in the UK who have had a heart attack, two thirds of whom are men (Office for National Statistics, 2000). The number hospitalised per annum with non-ST elevation acute coronary syndrome is more difficult to ascertain due to the changing diagnostic classification and the overlap with non-cardiac chest pain, but the number is conservatively estimated at more than 500,000. There are more than 2.1 million people in the UK with a diagnosis of angina pectoris (Office for National Statistics, 2000).

1.2.3 Pathophysiology

Stable angina is caused by fixed, flow-limiting, slowly developing, plaque stenoses of one or more of the epicardial arteries. Acute coronary syndromes are characterised by rupture of an unstable plaque with resultant thrombus formation (Davies, 2000). There are many factors which may be responsible for the transformation from stable to unstable plaque and this is an area of intense research (Davies, 2000; Davies, 1996; Lee and Libby, 1997; Fuster et al. 1999).

Fissuring of the vulnerable unstable plaque exposes the lipid rich core, promoting thrombin formation, fibrin generation and platelet activation and aggregation, further rupturing the plaque. Triggers for fissuring include recruitment of inflammatory mediators and influx of macrophages, activation of matrix metalloproteinases, haemodynamic sheer stress and increased blood viscosity (Ross, 1999; Ardissino et al. 1997; Davies, 1996; Malek et al. 1999; Rosenberg and Aird, 1999). This pathophysiology is common to both ST elevation MI and non-ST elevation ACS. The key difference is in whether there the resultant platelet, thrombin and fibrin rich thrombus is occlusive or non-occlusive. Occlusive thrombus tends to occur suddenly, producing pain, ST segment elevation on the ECG and myocardial necrosis with resultant enzyme rise due to complete absence of antegrade flow in an epicardial coronary artery. Partial or complete antegrade flow occurs in non-occlusive thrombi and patients present with a more stuttering course and have no ST elevation on ECG. The thrombus composition is also a little different, being platelet rich rather than fibrin rich, like the thrombi of ST elevation MI. Myocardial necrosis can occur due to distal

embolisation of platelet rich thrombi, occluding the microcirculation. Often this is at a level undetectable by standard cardiac enzymes, but the advent of troponin assays has allowed identification of minor degrees of myocardial damage. This forms the basis of the different therapeutic strategies employed in the two groups – reperfusion therapy in ST elevation MI and anti-thrombotic and anti-platelet therapy in non-ST elevation ACS.

1.2.4 Non-ST Elevation Acute Coronary Syndromes

(a) Prognosis

As previously discussed, non-ST elevation acute coronary syndrome is an umbrella term for presentations with cardiac chest pain but without ST elevation on the ECG. There is therefore a wide range of risk within this population and consequently, risk stratification is paramount. High-risk groups can have mortality rates as high as 9% (Antman et al. 1996) and combined rate of death, MI or urgent revascularisation of 40.9% (Antman et al. 2000). Table 1.2 outlines indicators of higher risk, and there are published scoring systems (Antman et al. 2000). Historical factors such as older age, diabetes, previous MI, and other vascular disease increase absolute mortality by up to 50% (Boersma et al. 2000). Clinical features such as presence of heart failure or hypotension also confer a higher risk (Boersma et al. 2000) as does prior use of aspirin (Alexander et al. 1999).

According to registry data, the presence of ST depression on the admission 12 lead ECG is a powerful adverse prognostic indicator (Nyman et al. 1993). ST segment depression of >0.05mm in two or more contiguous leads is associated with more than a two-fold risk of death at 1 year (Cannon et al. 1997), and depression >2mm with a 6 fold increase (Kaul et al. 2001), compared to those with no ST segment deviation. Sequential ECG changes are also important with new T wave inversion also increasing risk (Cannon et al. 1997).

More recently, the development of sensitive assays for the troponin complexes, has allowed further risk stratification. The troponin complex is formed by three proteins – troponin I, C and T. It regulates calcium dependent myosin-actin interaction and is present in both skeletal and cardiac muscle. The cardiac isoforms of troponin T and I are exclusively expressed in cardiomyocytes. Troponin should normally be undetectable in the blood, and therefore, the presence of cardiac troponin T or I in blood is specific for myocardial muscle damage. In the last decade or so, numerous clinical trials have clearly demonstrated the powerful predictive value of a positive troponin (T and I) with respect to predicting mortality and recurrent events in patients presenting with unstable angina (Hamm et al. 1992; Ohman et al. 1996; Antman et al. 1996; Lindahl et al. 1996; Galvani et al. 1997). In the TIMI 11A trial, Troponin positivity was associated with an early mortality of 4.7% compared to 0.4% in the troponin negative group (Antman et al. 1996). These data are supported by other trials and have clearly shown that troponin T and I predict events at 30 days. However, data from the FRISC trial show that admission troponin continues to predict events even at 5 months (Lindahl et al. 1996). Furthermore, it is not just

positivity for troponin that is predictive of outcome, the absolute level of troponin is also important, with higher levels associated with increased mortality rates (Antman et al. 1996).

Table 1.2

- **Historical Characteristics**
  - Age >65 years
  - Diabetes Mellitus
  - Previous Myocardial Infarction
  - Other Vascular Disease
  - Previous aspirin therapy
- **Clinical Characteristics**
  - Pulmonary crepitations
  - Pulmonary oedema on CXR
  - Hypotension
  - Recurrent pain
- **ECG**
  - ST depression
  - T inversion
- **Biochemistry**
  - Positive troponin
  - Raised CK-MB

Legend - High risk features in Non-ST elevation ACS

(b) Overview of Acute Treatment

As discussed previously, acute coronary syndromes are characterised by the unstable plaque with associated non-occlusive, platelet rich thrombus and distal embolisation of micro-thrombi. The cornerstone of treatment is therefore anti-platelet and anti-thrombotic therapy. Vasodilators such as nitrates and anti-ischaemics such as beta-blockers are often used to ameliorate symptoms, but have little or no effect on outcome. There has also been considerable debate in the literature regarding whether an early interventional approach in the catheterisation laboratory or an early conservative approach is optimal, and this is discussed briefly later. Most studies have used a combined endpoint of death and myocardial infarction, or death, myocardial infarction and urgent revascularistaion (MACE – Myocardial Adverse Clinical Events), and this terminology will be employed in the next few paragraphs.

There are three principle groups of antiplatelet agents currently available – aspirin (a cyclo-oxygenase inhibitor), clopidogrel (a thienopyridine inhibitor of the platelet ADP receptor) and the glycoprotein IIbIIIa inhibitors. Aspirin has been extensively investigated in the setting of non-ST elevation ACS and has been shown to reduce MACE by up to 70% (Lewis et al. 1983; Theroux et al. 1988; The RISC Group, 1990). The other classes of anti-platelet therapy have been studied against placebo *in addition* to aspirin. There are no head to head randomised control trials comparing classes in this population. The recent CURE study compared the early addition of clopidogrel to standard aspirin therapy vs. aspirin alone in patients presenting with ACS and demonstrated a relative risk

reduction of the primary endpoint of death/MI/stroke of 20% (9.3% vs. 11.4%) (Yusuf et al. 2001). Glycoprotein IIbIIIa inhibitors have been extensively studied in this patient population, both as medical management, and as an adjunct to early intervention. The results of medical management with these agents are conflicting. A meta-analysis of data from three large trials (PURSUIT, PRISM-PLUS and CAPTURE) shows an early 34% reduction in death or MI (predominantly MI) using these agents (Boersma et al. 1999), but the GUSTO-V ACS trial showed no benefit (Simoons ML and GUSTO V-ACS investigators, 2001). However, this trial recruited a low risk group, and the benefit seen in CAPTURE and PRISM-PLUS were all limited to the group who were troponin positive. These key differences may explain the apparent disparity.

Anti-thrombotic therapy with unfractionated heparin (UFH), in addition to aspirin, reduces death and MI by 33% compared to aspirin alone (Oler et al. 1996). More recently, there is evidence that low molecular weight heparin may be more beneficial in reducing events than UFH. Enoxaparin reduces MACE rates to 15.6% compared with 18.7% for UFH (Antman et al. 1999a). Again, benefit seems to be greatest in those at higher risk. For example, patients with positive troponin achieved the greatest benefit with enoxaparin in the TIMI 11B trial.

A large proportion of the cardiovascular literature in recent years has concerned the merits of both the early invasive and early conservative approaches to the treatment of non-ST elevation ACS (Table 1.3). Early studies such as TIMI IIIB and VANQWISH showed no benefit of an invasive strategy (The TIMI IIIB Investigators, 1994; Boden et al. 1998), and indeed, there were more early deaths

and myocardial infarctions in the invasive arm in the VANQWISH study (24% vs. 19%, p=0.05) (Boden et al. 1998). However, TACTICS-TIMI 18 and FRISC II have both demonstrated greater than 20% reduction in MACE in the invasive strategy compared to conservative (Cannon et al. 2001; Fragmin and Fast Revascularisation during InStability in Coronary artery disease Investigators., 1999), especially in high risk groups with dynamic ECG changes or positive troponin. The key differences in these studies were the concomitant use of glycoprotein IIbIIIa inhibition in TACTICS and prolonged treatment with low molecular weight heparin in FRISC II.

There are many therapeutic options in the treatment of ACS, and the key lies in defining risk so as best enable us to choose our strategy and to make best use of resources.

Table 1.3

TRIAL	Follow up	Invasive arm MACE	Conservative arm MACE	OR	P value
TIMI IIIB	6 weeks	16.2%	18.1%	0.90	0.33
VANQWISH	1 year	24.0%	18.5%	1.3	0.05
FRISC II	6 months	9.4%	12.1%	0.78	0.03
TACTICS	30 days	7.4%	10.5%	0.71	0.009
TACTICS	6 months	15.9%	19.4%	0.82	0.025

Legend - Rates of Myocardial Adverse Clinical Events (MACE) and Odds Ratio in trials of early invasive versus early conservative strategy in non-ST elevation ACS

1.2.5 ST Elevation Myocardial Infarction

(a) Prognosis

The early mortality associated with acute myocardial infarction (AMI) has been falling over the last two or three decades, primarily due to better acute treatment and secondary prevention (De Vreede et al. 1991; McGovern et al. 1996). Mortality rates in clinical trials can be as low as 2.5% (Grines et al. 1993), but these populations are highly selected, and probably do not reflect the true extent of case-fatality rates. Data from the American National Centre for Health Statistics show a fall in case-fatality rate from 37% in 1975 to 13% in patients aged 45-64 years (Levy, 1998). Further registry data suggest the current figure for in-hospital mortality may be higher, at around 18%, if all age groups are included (Mahon et al. 1999). However, it is important to remember that this figure represents the mortality rate for those reaching hospital, and if those dying prior to admission are included, the true fatality rate for AMI may be closer to 40-50%. Again, historical factors such as older age, diabetes, previous MI, and other vascular disease increase mortality. Clinical features such as presence of heart failure or hypotension also confer a higher risk (Hammermeister KE et al. 1979). Echocardiographic features of LV dilatation, degree of wall motion abnormality and ejection fraction are powerful adverse prognostic indicators (White HD et al. 1987; Sutton et al. 1997; Dujardin et al. 1997).

(b) Overview of Acute Treatment

The last 20 years have brought great advances in the treatment of ST elevation MI. Therapies in the acute phase shown to confer a survival benefit include antiplatelet therapy, reperfusion therapy (thrombolytic and mechanical), beta-blockers and ACE inhibitors.

With regard to antiplatelet therapy, aspirin has been studied in a huge cohort of patients, reducing mortality by more than 20% when used alone, increasing to 53% when given in combination with thrombolysis (Antiplatelet Trialists' Collaboration, 1994; ISIS 2 (Second international Study of Infarct Survival), 1988). There is some evidence that more powerful antiplatelet agents, such as the glycoprotein IIbIIIa receptor antagonists may also have a role in the treatment of AMI (Antman et al. 1999b).

The underlying pathophysiology of ST elevation MI is occlusive thrombus. Intravenous thrombolytic therapy has also been extensively studied, and coupled with aspirin, is the cornerstone of treatment of ST elevation MI in most countries, including the UK. There are no data to support the use of thrombolysis in patients without ST elevation (Fibrinolytic Therapy Trialists, 1994). Angiographic studies have clearly shown the importance of achieving patency and restoring blood flow in occluded infarct related arteries both in short and long term reduction of mortality (Lenderlink et al. 1995). Patency rates at 90 minutes vary from 50-75% depending on the thrombolytic agent used (The GUSTO Angiographic Investigators, 1993). The effect on mortality is striking, with a meta-analysis of

nearly 60,000 patients from 9 studies showing an 18% overall reduction in fatality rate, at the expense of a 1% stroke risk (Fibrinolytic Therapy Trialists, 1994). The benefits are time dependent with a larger survival benefit if given early (Boersma et al. 1996). However, there is still some benefit up to 12 hours from the onset of symptoms (LATE (Late Assessment of Thrombolytic Efficacy) Study Group, 1993). This benefit continues long after the acute event (Franzosi et al. 1998). It is important to remember that the largest gains are to be found in those patients who are at highest risk – anterior myocardial infarction, concomitant heart failure, older age and diabetics (Fibrinolytic Therapy Trialists, 1994).

The same is true of mechanical reperfusion strategies, and there is increasing evidence of its superiority to thrombolytic therapy, especially in high risk patients (Grines et al. 1999b). Patency rates of >90% can be achieved with primary angioplasty (Grines, 1996; Grines et al. 1993) and improvements in balloon and stent design, coupled with the newer antiplatelet agents may improve this figure further. This translates into a superior survival benefit and does not seem to be so temporally dependent as thrombolysis (Weaver et al. 1997; Grines et al. 1999a).

Other, non-reperfusion based, interventions include beta-blockers and ACE inhibitors. Acute administration of beta-blockade have been shown to reduce early and long term mortality in ST elevation MI, but their effects on mortality are probably not additive to thrombolytic therapy (Roberts et al. 1991). However, their use in secondary prevention is well established. There is a wealth of evidence to support the use of ACE inhibitors in the early phase of AMI (24-48 hours) and as secondary prevention and that these results are additive to reperfusion therapy

(Pfeffer MA et al. 1992; AIRE study investigators, 1993; Gruppo Italiono per lo Studio della Sopravvivenza nell'Infarcto Miocardio (GISSI) -3, 1994; ISIS-4 Collaborative Group, 1995). CONSENSUS II studied acute administration of intravenous ACE inhibition in ST elevation MI and gave a neutral result, primarily due to the increased incidence of hypotension in the ACE inhibitor group (Swedberg et al. 1992). However, some high risk subgroups showed a trend towards improved survival and it may be that this strategy would be of benefit in a more selected high risk population. Indeed, the HEART trial demonstrated the safety and efficacy of early administration of ramipril following AMI, although this was by the oral rather than the intravenous route (Pfeffer MA et al. 1997). Further studies would be required to investigate this in more detail.

There are numerous interventions at our disposal for the treatment of ST elevation MI, all with proven benefits, albeit at the expense of potential side effects. It is clear from most studies that high-risk groups have the most potential benefit from each of the strategies outlined. The future may lie in the selection of patients suitable for each intervention, based on the risk:benefit ratio of the individual rather than the population risk:benefit apparent from the mega-trials. This would allow better targeting of therapy, reducing the risk of potentially life threatening side effects in those at low risk of an adverse outcome and better use of limited resources. For example, prediction of those likely to have an adverse outcome, may influence the choice between thrombolytic or mechanical based reperfusion strategies and perhaps determine the timing of ACE inhibition or the use of beta-blockers and statins. Perhaps we should attempt to risk stratify all patients at

presentation and choose our management strategy based on available clinical, electrocardiographic and biochemical data.

1.3 Post-infarction Ventricular Remodelling

1.3.1 Historical Review

One of the most powerful predictors of adverse outcome following myocardial infarction is post-infarction ventricular remodelling which is associated with increased early and late mortality and late morbidity (White HD et al. 1987; Pfeffer MA and Braunwald E, 1990; Mahon et al. 1999). Alterations to ventricular size, shape and function are the hallmarks of remodelling, caused by complex changes within the ventricular architecture. These changes were first described by the German pathologist Wiegert in the late 19th century (Weigert, 1880), followed by the classic description of regional wall motion changes and systolic expansion following acute coronary artery ligation by Tennant in 1935 (Tennant R and Wiggers CJ, 1935). In 1965, Hort demonstrated that the duration of survival was related to left ventricular (LV) volume in the coronary ligation model of myocardial infarction in rats, and that infarct size was dependent on the duration of ligation. He also noted a relationship between infarct size, wall motion abnormalities and LV volume (Hort, 1965).

Since that time, there has been an enormous body of evidence, both in the experimental and clinical settings, that changes in LV function, structure and volume are important prognostic variables. Jan and Marc Pfeffer's group elegantly

demonstrated progressive LV dilatation in the days and weeks following experimental infarction in rats in 1979 and that this was related to infarct size (Pfeffer MA et al. 1979). A few years later, Hochman and Bulkley coined the "remodelling" as a description of the early thinning of the infarct site with subsequent LV dilatation seen in the rat model of coronary artery ligation (Hochman and Bulkley, 1982). Subsequent work recognised that the remodelling process is not confined to the infarct site, but occurs at sites remote from the ischaemic area (Weisman et al. 1985; McKay RG et al. 1986), and that survival was inversely proportional to the degree of remodelling (Pfeffer MA and Braunwald E, 1990).

At this point, it is important to remember that changes within the infarct site and alterations to LV structure are necessary for healing of the infarcted region, thereby producing a scar. Although these changes could be referred to as remodelling, the term really refers to a lack of control of these "healing" processes, causing inappropriate alterations to LV structure and function with the development of wall motion abnormalities and LV dilatation, through excessive changes within the myocyte and collagenous components of the heart, and would probably more appropriately be termed "adverse remodelling". However, in keeping with convention, the term "remodelling" will be used in the rest of this thesis and should be regarded as being synonymous with "adverse remodelling".

In man, progressive dilatation following AMI has been studied with serial chest X-rays, contrast ventriculography and echocardiography and all have confirmed the adverse prognostic importance of the remodelling process (Shanoff HM et al.

1969; Kostuk WJ et al. 1973; McKay RG et al. 1986; White HD et al. 1987). Dilatation is detectable within three hours of the onset of symptoms, and progresses over time (Korup et al. 1997). Early echocardiographic parameters such as end-systolic volume index, ejection fraction and extent of wall motion abnormalities all predict subsequent mortality (Migrino et al. 1997; Sutton et al. 1997; Galasko et al. 2001).

1.3.2 Pathophysiology

The last 2 or 3 decades has brought increased understanding of the pathological mechanisms responsible for the phenomenon of ventricular remodelling. Myocardial cell necrosis occurs within minutes following acute coronary artery occlusion, causing cessation of contractility in that area. This triggers an inflammatory cascade with an influx of macrophages, neutrophils and monocytes into the infarct zone followed by interstitial oedema and cell death (Fishbein MC et al. 1978; Schoen, 1999). Much of the cell death in AMI is due to apoptosis (Kajstura et al. 1996), which can also be found in the non-infarcted regions of the heart, as well as in the infarct zone (Olivetti G et al. 1996). Additionally, severe ischaemia can cause cellular changes without overt infarction, producing myocardial vacuolisation and oedema, which can progress to programmed or necrotic cell death (Zhao M et al. 1987; Charney et al. 1992; Schoen, 1999). Important changes also occur in the extracellular matrix and collagen scaffolding of the heart, and this is discussed in detail in Section 1.4.

Cell death and changes to the extracellular matrix make the infarct zone distensible by mechanical forces and abnormal loading conditions, allowing myocyte slippage, with thinning of the infarct site and distension and dilatation of the LV cavity (Olivetti G et al. 1990). This serves to increase the area occupied by the infarct zone and is termed infarct expansion and is independent of any further ischaemic stimuli (Hutchins and Bulkley, 1978; Weisman et al. 1988). Extreme expansion can lead to aneurysm formation or even cardiac rupture (Jugdutt and Michorowski, 1987; Schuster and Bulkley, 1979), and is more common in anterior infarction (Pirolo et al. 1986). Following this initial period of expansion, healing is characterised by fibroblast proliferation and collagen deposition resulting in scar formation (Fishbein MC et al. 1978). In addition to myocyte hypertrophy and interstitial fibrosis, further changes to LV geometry occur with progressive dilatation (Anversa P et al. 1985; Pfeffer MA and Braunwald E, 1990). These changes are known as ventricular remodelling.

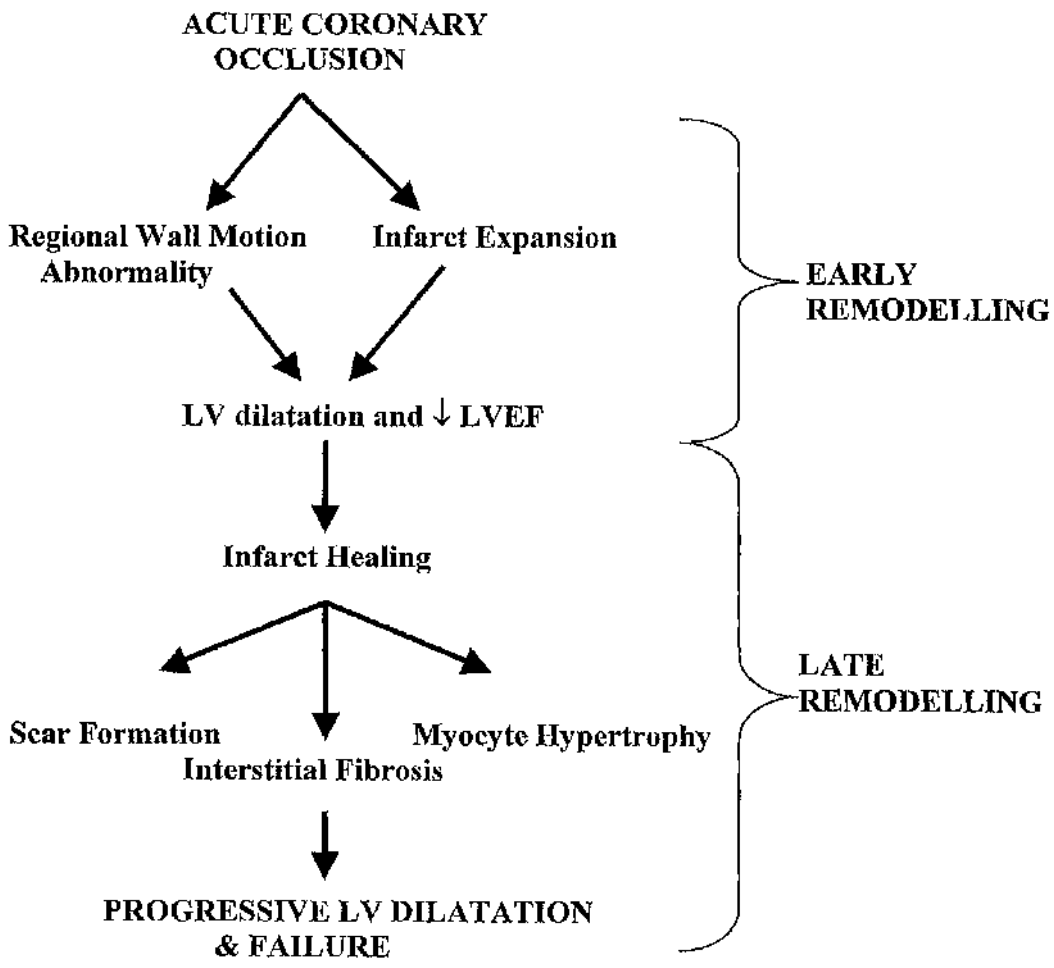
1.3.3 Early vs. Late Ventricular Remodelling

Previously, there was a clear distinction made between these early and late changes in ventricular architecture and function following ischaemia and infarction - early infarct expansion, and the later, adaptive, or probably more accurately, maladaptive, process of remodelling, occurring weeks to months to years following the acute event. However, it is increasingly recognised that remodelling is a continuum, starting during the acute event and progressing thereafter, and they are part of the same process, albeit with some key differences (Figure 1.4). This has prompted the use of the terms early and late ventricular

remodelling (Sutton and Sharpe, 2000). Early ventricular remodelling, arbitrarily defined as occurring within 72 hours (Sutton and Sharpe, 2000), should be regarded as synonymous with infarct expansion and is characterised by expansion and thinning of the infarct zone, development of wall motion abnormalities, LV dilatation and reduced ejection fraction. This occurs within hours of myocyte injury, causing changes both in the infarct zone and in the non-infarcted portion of the heart. This process is related to the degree of ischaemic insult and is dependent on the marked changes within the collagen framework and extracellular matrix, predominantly in the infarct border zone, manifested principally as collagen degradation (Section 1.4) (Cleutjens JPM et al. 1995).

Late remodelling follows with further changes to ventricular architecture, volume and function. Scar formation and fibrosis of the border zone and areas of the ventricle distal to the infarct are also dependent on the dynamics of collagen metabolism, this time with synthesis predominating. Infarct scars are healed at 4-6 weeks, but the process of late remodelling can continue for many months and years, often leading to progressive LV dilatation and failure.

Figure 1.4



Legend - The continuum of ventricular remodelling

1.3.4 Therapeutic Intervention

(a) Patency of the Infarct Related Artery

The open artery hypothesis states that LV remodelling may be prevented, or at least attenuated, by achieving or maintaining patency of the infarct related artery. Early evidence comes from a study in the pre-thrombolytic era, which showed that patency of the infarct related artery is a more important predictor of subsequent LV dilatation than infarct size (Jeremy et al. 1987). Further evidence is provided by the well established survival benefits of thrombolytic therapy, where survival is directly related to the extent of restoration of antegrade blood flow (Fibrinolytic Therapy Trialists, 1994; The GUSTO Angiographic Investigators, 1993; Lenderlink et al. 1995). Mechanical catheter based reperfusion strategies achieve higher patency rates and may account for the associated reduced mortality and event rate compared to thrombolysis (Grines et al. 1999b). There is also direct evidence of attenuation of the remodelling process with reperfusion therapy, limiting infarct size and LV dilatation, reducing wall motion abnormalities and improving left ventricular function and ejection fraction (Touchstone et al. 1989; Marino et al. 1989). However, the beneficial effects of an open infarct related artery is not solely due to prevention of damage, it seems that patency *per se* is associated with reduced remodelling and improved LV function (Jeremy et al. 1987), and that this benefit persists even when the infarct related artery is opened late and therefore, little or no myocardium subtended by that artery would be viable (Horie et al. 1998).

(b) Inhibition of the Renin-Angiotensin-Aldosterone System

There is clear evidence of systemic activation of the Renin-Angiotensin-Aldosterone System (RAAS) following AMI (Roulcau et al. 1993). In patients who develop LV failure following AMI, plasma renin activity and angiotensin II levels are elevated on admission, peaking a few days later, and remaining high for more than one week (McAlpine HM et al. 1988). In addition to this systemic activation, there is some evidence of de novo generation of the RAAS effectors at a tissue level (Dzau, 1987) and that tissue levels of cardiac ACE are increased in myocytes immediately following myocardial infarction, especially in the infarct zone (Sakharov et al. 1987). There is also upregulation of angiotensinogen gene expression in the infarcted heart (Lindpaintner et al. 1993). Both systemic and local RAAS activation are likely to have intense influence on remodelling and healing in AMI. However, given the delay in mounting a systemic RAAS response following infarction, coupled with the rapidity of increased activity of ACE at a tissue level and increased gene expression at a cellular level, it would be reasonable to hypothesise that paracrine regulation of local tissue levels of RAAS effectors, independent of the systemic renin-angiotensin axis, plays an important role in the remodelling process, especially in the early stages. The effectors of the RAAS, angiotensin II and aldosterone, influence remodelling through their profound effects on myocyte growth (Yamazaki et al. 1995), fibroblast function and the extracellular matrix (Brilla CG et al. 1995). This is discussed in more detail in the next section.

Animal studies have clearly demonstrated that ACE inhibitors limit LV dilatation following infarction and that this attenuation of the remodelling process confers a survival benefit (Pfeffer JM et al. 1985; Pfeffer MA et al. 1985). Early administration, within 2 days, of captopril to rats is associated with greater attenuation of LV dilatation compared to late administration at 21 days, even at 1 year (Pfeffer JM et al. 1985). Most of the large scale clinical trials of ACE inhibitors in humans have administered the drug within 48 hours and all have shown clear survival benefits (Pfeffer MA et al. 1992; AIRE study investigators, 1993; Gruppo Italiono per lo Studio della Sopravvivenza nell'Infarcto Miocardio (GISSI) -3, 1994; ISIS-4 Collaborative Group, 1995).

More specific antagonism of the AII receptor following myocardial infarction was studied in OPTIMAAL, which compared losartan vs. captopril (Dickstein et al. 2002). This showed no benefit of specific AII antagonism over ACE inhibition in this setting. Whether more extensive antagonism of the RAAS may have a more profound effect on mortality is being tested in the ongoing VALIANT trial, which compares combination ACE inhibition and AII receptor antagonism with ACEI or AII receptor blocker alone (Pfeffer MA and et al, 2000).

The results of a large study utilising the aldosterone inhibitor, eplerenone, in the context of heart failure complicating a recent myocardial infarction, have recently been presented showing an overall survival benefit with the addition of the aldosterone antagonist (Pitt B et al. 2003). A recent paper by Hayashi *et* al provides a possible mechanistic explanation (Hayashi M et al. 2003). They found that spironolactone administration following MI was associated with an improved

ejection fraction and reduced LV end-diastolic index at one month when compared to a control group. They also demonstrated suppression of cardiac extraction of aldosterone and evidence of reduced collagen synthesis in the spironolactone group, suggesting that inhibition of aldosterone at a local level and changes to collagen homeostasis are key in attenuating the remodelling process.

(c) Inhibition of the Sympathetic Nervous System

Plasma levels of the catecholamines, adrenaline and noradrenaline, rise quickly following myocardial infarction, peaking at 3 days following the event (McAlpine HM et al. 1988; Rouleau et al. 1993). Elevated levels promote arrhythmogenesis and increase myocardial oxygen demand. Absolute levels correlate with mortality (Sutton and Sharpe, 2000), providing sound rationale for the use of pharmacological inhibitors of this system. However, although there have been numerous studies demonstrating survival benefit in their acute use (Yusuf et al. 1985), only one study supports their use in the post-thrombolytic era (The CAPRICORN Investigators, 2001).

Nevertheless, super-activation of the sympathetic nervous system has deleterious effects on myocardial structure and function, causing myocyte hypertrophy and alterations in the extracellular matrix (Ju et al. 1998; Brilla CG et al. 1995). Additionally, acute administration of intravenous beta-blockade reduces the incidence of ventricular rupture (ISIS-1 (First International Study of Infarct Survival), 1988) and the recent beta-blocker in heart failure trials have

demonstrated the efficacy in reducing, and even reversing, remodelling and improving survival (Tendera and Ochala, 2001).

1.3.5 Identification of early remodelling

There are several interventions available to limit the remodelling process in AMI. Large-scale clinical trials have convincingly demonstrated the efficacy of reperfusion therapy and ACE inhibition in limiting remodelling and improving survival. However, most of these studies have been unselected, and although there is an overall benefit in the populations studied, it is clear that the greatest benefits are seen in those at highest risk. For example, reperfusion of anterior MI has a higher absolute benefit than reperfusion of inferior infarcts and ACE inhibitor therapy confers the greatest benefit on those with the poorest ejection fraction and greatest abnormality of wall motion. As discussed previously, it is therefore important to risk stratify patients using the available historical, clinical, biochemical and echocardiographical information available.

Older age and diabetes confer a higher risk of remodelling as does anterior infarction, failed reperfusion, peak enzyme rise, hypotension and presence of heart failure (Hammermeister KE et al. 1979; Mahon et al. 1999). However, echocardiography within a few days of infarction can also provide important predictive information. In addition to information regarding the presence of infarct expansion and wall motion abnormalities, the cornerstone of early remodelling, end systolic LV volume assessed within a few days of infarction is one of the most important predictors of subsequent remodelling and mortality (White HD et al.

1987). Echo also allows estimation of the extent of myocardial damage by measurement of wall motion abnormalities, the extent of which are directly related to subsequent dilatation and mortality (Kitamura et al. 1973; Kan et al. 1986; Sutton et al. 1994). Indeed, echocardiographic sub-studies of both SAVE and GUSTO-1 clearly showed that the degree of wall motion abnormality is the most powerful echocardiographic predictor of both short and long-term mortality (Sutton et al. 1997; Migrino et al. 1997). This was also true in the TRACE echo sub-study, where one year mortality was inversely related to degree of wall motion abnormality (Kober L et al. 1994).

More recently, Doppler indices of LV filling have been shown to be a powerful prognostic determinant. In the days following AMI, the heart becomes "stiffer", displaying a restrictive filling pattern, representing an impairment of diastolic function (Williamson et al. 1990; Chenzbraun et al. 1992), the degree of which can be assessed by measurement of the mitral deceleration time by pulsed wave Doppler through the mitral valve (Little et al. 1995). Decreased deceleration time, or restrictive filling, has been documented in patients with larger infarcts (Popovic et al. 1996), and recently, has been shown to correlate with subsequent remodelling (Cerisano G et al. 1999). Echocardiography is therefore a powerful tool in the assessment of early remodelling and the prediction of late remodelling and associated mortality.

Other non-invasive imaging techniques include radionuclide ventriculography and cine-magnetic resonance imaging, both of which can be used to detect serial changes of remodelling with a high degree of reproducibility (Cohn PF et al.

1974; Marshall RC et al. 1978; Longmore DB et al. 1985; Van Rossum AC et al. 1988; Kramer CM et al. 1993). However, their use is limited by their lack of portability and echocardiography is the modality most extensively studied and remains the mainstay of non-invasive assessment of LV size and function.

As discussed in sections 1.3.4 (b) and (c), there is neurohormonal activation following AMI and that increased levels of the effectors of the RAAS and sympathetic nervous systems may be a reflection of early remodelling in addition to abnormal haemodynamic loading conditions (McAlpine HM et al. 1988; Rouleau et al. 1993; Sutton and Sharpe, 2000; White M et al. 2001). Similarly, it is well documented that atrial and brain natriuretic peptides are elevated immediately following acute myocardial infarction (Morita A et al. 1993; White M et al. 2001), and correlate with increased LV dimensions at 1 and three months (Yoshitomi Y et al. 1998), which may suggest that they have some use in identifying those at risk of subsequent remodelling. Furthermore, infusion of atrial natriuretic peptide reduces remodelling, probably through a reduction in circulating plasma aldosterone levels(Hayashi M et al. 2001a).

1.4 Myocardial Collagen and the Extra-cellular Matrix

Historically, remodelling was thought to be due primarily to phenotypic changes in myocytes. However, it is increasingly recognised that changes in the connective tissue compartment (extracellular matrix and non-myocyte cells) account for a large degree of the remodelling process. It is therefore important to understand the

normal structure and function of collagen and the extracellular matrix in health and disease.

1.4.1 Myocardial Collagen Structure and Function

The earliest description of collagen was in skeletal muscle in 1907 (Holmgren, 1907), with the first three sub-types identified in 1971 (Miller et al. 1971), with new additions to the class being described each year. There are now 19 identified collagen subtypes and they are the most abundant protein form in man, comprising nearly one third of total body protein and, to date, more than 30 genes have been identified in their production (Brown and Timpl, 1995). Collagen is comprised of varying sizes of polypepetide chains. Its final configuration, and subsequent strength, is due to repeated folding. Recurring triplets of glycine, prolyl and hydroxyprolyl facilitate cross-linking within the chain, allowing formation of a triple helical configuration of alpha-chains, which weave around each other forming a rope-like structure (Nimni ME, 1993).

Although all types share this commonality with regard to structure, changes in extracellular aggregates provide a great diversity in properties of subtypes, ranging from the high tensile strength of Type I collagen through to the more distensible and elastic type III collagen. Type I collagen predominates within the heart, constituting 75% of the total, with the rest comprising mainly of type III, with lesser amounts of types IV, V and VI also being present (Medugorac and Jacob, 1983; Weber KT et al. 1988a).

Collagen provides several fundamental functions in the healthy heart: the epimysium surrounds myocytes, forming a three dimensional scaffold, preventing distending forces and maintaining myocardial shape and function in both systole and diastole; the perimysium weaves between myocytes, maintaining myocyte alignment and forming struts facilitating contraction of the heart as a syncitium; and the endomysium providing a vehicle for capillary networks and maintaining their patency (Robinson TF et al. 1983; Robinson TF et al. 1988; Rossi MA et al. 1998).

1.4.2 Changes to Myocardial Collagen in Disease States

Given the ubiquitous nature of collagen within the tissues of the body, changes to content and type have been extensively studied, especially in the field of wound healing, tissue repair and metabolic and inflammatory bone disease (Mutsaers SE et al. 1997). In the sphere of cardiology, most work has focussed on hypertensive heart disease, heart failure and myocardial infarction. Many cardiac disease processes are ultimately fibrogenic, with replacement fibrosis compensating for myocyte loss in end-stage heart disease of whatever aetiology. However, fibrosis can also be a response to an acute or chronic stimulus, such as tissue injury, haemodynamic changes and neurohormonal activation. All these factors play a role in the development of hypertensive heart disease and clinical and laboratory studies in this condition have provided insights into the pathophysiology and sequelae of fibrotic heart disease.

(a) Hypertensive Heart Disease

Although collagen constitutes almost 30% of the body's total protein, it's light weight means it only accounts for 2-4% of wet LV mass in health (Weber KT, 1989; Nimni ME, 1993). Myocardial fibrosis has been defined as an increase in collagen content (collagen concentration X ventricular weight) above normal levels (Weber KT, 1989). This simple definition of fibrosis as an absolute increase in fibrillar collagen content has allowed investigation of the myocardial response to disease in both human and animal studies in hypertension. Tissue obtained by endomyocardial biopsy or at autopsy of hypertensive hearts show increased collagen accumulation, predominantly of type I collagen (Weber KT et al. 1988a; Bishop JE et al. 1990; Weber KT et al. 1990). Indeed, studies in the hypertensive rat heart have shown that collagen can occupy as much as 18% of the LV mass (Weber KT et al. 1990).

The absolute collagen content and ratio of collagen sub-types determine the viscoelasticity of the extracellular matrix and the overall distensibility or compliance of the heart (Mutsaers SE et al. 1997; Pauschinger et al. 1999). Therefore, in the hypertrophied heart, fibrosis causes impairment of ventricular relaxation, increasing preload and myocardial oxygen demand. This is seen when collagen content is increased by a factor of two (Thiedemann et al. 1983). The functional significance of this has been termed diastolic dysfunction and can produce heart failure even when LV systolic function is preserved (Vasan and Benjamin, 2001). Progressive fibrosis disrupts myocyte alignment, reducing

contractility and ultimately causing LV systolic failure and dilatation (Vasan et al. 1995).

At this point, it is important to emphasise that although left ventricular hypertrophy (predominantly due to myocyte hypertrophy) and myocardial fibrosis often co-exist, they are not inter-dependent on one another. It is clear that there are marked alterations in the distensibility and relaxation properties of the hypertrophied, fibrotic heart. This is due to fibrosis rather than as a consequence of LVH. This is supported by studies where LVH is present in the absence of significant increases in collagen content, such as anaemia, AV fistulae and hyperthyroidism. In these volume overloaded conditions, there is myocyte hypertrophy without fibrosis and relaxation properties are normal (Holubarsch et al. 1983).

In addition to the adverse haemodynamic effects of fibrosis, it can worsen myocardial ischaemia by causing sub-endocardial micro-perfusion abnormalities and contributing to a reduction in coronary flow reserve. Fibrosis also results in the formation of barriers to the ventricular excitation wave front, causing changes to the elctrophysiological milieu, facilitating the creation of re-entrant arrhythmias (Cameron et al. 1983; Strain et al. 1983). Indeed, the incidence of ventricular arrhythmias is proportional to the degree of fibrosis (McLenechan JM et al. 1987). This may partly explain the increased risk of sudden death in hypertensive heart disease (Levy et al. 1990).

(b) Dilated Cardiomyopathy

Changes to the collagen matrix can be quantitative or qualitative and often both will co-exist. Qualitative changes have been studied most extensively in heart failure due to dilated cardiomyopathy. Dilated cardiomyopathy can be associated with an overall increase or decrease in collagen content. However, in both situations, there are alterations in collagen type which effect the tensile properties of the extracellular matrix. This serves to reduce structural integrity, as does changes to cross-linking (Weber KT et al. 1988b; Gunja-Smith Z et al. 1996). In addition, there is also a phenotypic shift to increased collagen type I/III ratio, further altering structural dynamics (Marijianowski et al. 1995). These features cause distortion of ventricular architecture, or adverse ventricular remodelling, and subsequent LV dilatation and failure.

(c) Myocardial Infarction

As discussed in section 1.3, remodelling occurs following acute myocardial infarction and the rapidity of onset serves as a reminder of the dynamic nature of collagen metabolism. Early remodelling, within 72 hours, is characterised by infarct expansion and the development of wall motion abnormalities (Sutton and Sharpe, 2000). This is principally due to changes to collagen content and function rather than myocyte loss (Whittaker P et al. 1991). Early remodelling is dependent on both quantitative and qualitative changes to the collagen scaffolding. Influx of inflammatory cells and bio-active chemokines into the infarct zone alter the interstitial milieu, promoting net collagen loss due to immediate breakdown by

matrix metalloproteinases (section 1.4.4) (Cleutjens JPM et al. 1995). In the rat model, there is a 25% loss of collagen within the infarct zone within 24 hours (Cannon et al. 1983). Loss of supportive viscoelastic collagen and disruption of the intermyocyte struts means the ventricle is less able to cope with the abnormal haemodynamic loading conditions found following myocardial infarction. Myocyte slippage and infarct expansion occurs, adversely altering LV geometry and function (Whittaker P et al. 1991; Olivetti G et al. 1990; Kim et al. 2000). Similar changes can occur in ischaemic myocardium, when the ischaemic burden has been sufficient to disrupt systolic and diastolic function, but not to cause overt myocyte necrosis. Ultrastructural studies have clearly demonstrated changes within the extracellular matrix with loss of collagen and disruption to cross-linking in the ischaemic, yet viable, stunned myocardium (Zhao M et al. 1987; Charney et al. 1992).

In the days following infarction, phenotypically transformed fibroblasts, myofibroblasts, invade the infarct zone and collagen production is upregulated. Collagen deposition starts within the infarcted area by day 3 and further accumulation allows formation of a scar which is normally complete by 4-6 weeks following infarction (Cleutjens JPM et al. 1995). There is a phenotypic shift to increased type I collagen compared with type III, further altering structural dynamics (Wei et al. 1999). However, it is not just the infarct site which undergoes remodelling. Excessive accumulation of collagen in the non-infarcted regions increases myocardial stiffness, thereby reducing compliance and function. This late remodelling causes further collagen deposition and fibrosis, which can

continue for many months and years, often leading to progressive LV dilatation and failure (Fishbein MC et al. 1978; Pfeffer MA and Braunwald E, 1990).

A tissues response to injury is a time dependent phenomenon and this is elegantly demonstrated following myocardial infarction. Collagen metabolism is a dynamic process with ongoing synthesis and degradation. It is the balance of these two processes which determines the overall collagen content. The immediate response following myocardial infarction is one of net degradation of collagen, the balance of which changes in favour of net collagen accumulation over a period of a few days which often continues for many months. To assess the net collagen balance, one must therefore consider both synthesis and degradation, processes that are controlled by fibroblasts and the biologically active macromolecules of the extracellular matrix.

1.4.3 The Extracellular Matrix and Collagen Metabolism

Although cardio-myocytes constitute 80% of LV volume, they comprise less than one third of total cell number. All other cell types reside within the interstitial space and include fibroblasts, endothelial cells, vascular smooth muscle cells and macrophages. The non-cellular component of the interstitial space is called the extracellular matrix (ECM) and contains large quantities of collagen, predominantly type I, with smaller amounts of type III, elastin, non-collagenous glycoproteins and glycosaminoglycans (Frank and Langer, 1974; Weber KT, 1989). Previously, it was thought that the ECM was an inert substance, merely providing a passive framework for myocytes. However, it is increasingly

recognised that the ECM is a self regulating, extremely biologically active entity, containing metabolically active cells such as fibroblasts and activated leucocytes, and macro-molecules including matrix metalloproteinases and their tissue inhibitors, transforming growth factor-beta, and cytokines. Tight homeostasis of matrix components is therefore essential.

Fibrillar collagens are the main component of the ECM and they are produced by fibroblasts, which are the principal cell type in the heart, outnumbering myocytes by nearly three to one (Weber KT, 1989). They are pluripotent cells, present in vast numbers and are free to move through the interstitial space. They contain messenger RNA for the fibrillar collagens and are responsible for collagen transcription and production. They secrete procollagen for types I and III into the extracellular space. Aminoterminal and carboxyterminal propeptide moieties are cleaved, allowing integration of the collagen helix into the growing fibril (Nimni ME, 1993). In health, fibroblasts tightly regulate total collagen content, as they not only control collagen production, but also are responsible for the production of the degradative matrix metalloproteinases (section 1.4.4).

However, tissue injury, either acute or chronic, causes recruitment of inflammatory mediators from the blood and interstitial space, including interleukins, Tumour Necrosis Factors, adhesion molecules and Transforming Growth Factor-B. These chemokines cause phenotypic change to the fibroblast, producing myo-fibroblasts, and stimulate collagen mRNA transcription, resulting in increased collagen production, and ultimately altering the balance towards a fibrogenic state in the longer term (Brilla CG et al. 1995; Nicoletti A and Michel

JB, 1999). The effectors of the Renin-Angiotensin-Aldosterone System (RAAS) also have a marked role in control of collagen metabolism and this is discussed in section 1.4.6. Another peptidergic system exhibiting pro-fibrotic attributes is the endothelin system. Overexpression of the endothelin 1 gene in the transgenic murine model produces marked interstitial fibrosis in many tissues including the kidney, independent of blood pressure changes (Hocher et al. 1997). Additionally, in the rat model, antagonism of the endothelin system reduces myocardial fibrosis due to hypertension (Karam et al. 1996). It is perhaps not unsurprising that these peptidergic systems play an important role in regulating myo-fibroblast activity, as myo-fibroblasts express receptors for angiotensin II and endothelin, in addition to TGF-B and other cytokines (Weber KT et al. 1997; Sun Y et al. 1998). Osteopontin, an adhesive glycophosphoprotein also produced by fibroblasts, also plays a key role in matrix organisation through its effects on fibronectin and collagen and with its interaction with angiotensin-II, the integrins and other cytokines (Denhardt DT and Guo X, 1993; Mukherjee et al. 1995; Ashizawa N et al. 1996). In the murine knockout model, lack of osteopontin was associated with exaggerated LV dilatation and reduced collagen deposition following experimental MI (Trueblood NA et al. 2001), confirming its importance in matrix and collagen homeostasis.

Collagen production by fibroblasts is therefore tightly regulated. Inhibition of fibroblast production of collagen following AMI attenuates the remodelling process (Nwogo JI et al. 2001). However, it is important to remember that net myocardial collagen content is a product of a dynamic equilibrium between collagen synthesis and degradation. One must therefore also consider the

regulation of collagen breakdown when discussing collagen turnover and remodelling. Matrix metalloproteinases are produced by myo-fibroblasts and reside in the interstitial space. They are capable of degrading all components of the extracellular matrix, including collagen (Matrisian LM, 1990).

1.4.4 Matrix Metalloproteinases

(a) Structure and Function

Matrix Metalloproteinases (MMPs) are a family of zinc containing, calcium dependent endopeptidases with molecular masses ranging from 28-92 kDa. More than twenty have been identified, and all share three fundamental domains – pre, pro and catalytic domains. The functional significance has not been elucidated for all identified MMPs, but they can be classified by substrate specificity into the collagenases, gelatinises, stromelysins and membrane-type MMPs (MT-MMPs) (Dollery CM et al. 1995; McDonnell et al. 1999). MMP-1, or interstitial collagenase, is probably the best characterised. It is a 45kDA polypeptide with a haemopexin-binding domain linked to the catalytic domain allowing cleavage of fibrillar collagens. MMP-1 is the most abundant MMP within the heart, first described in 1975, and plays a key role in myocardial matrix remodelling (Montfort and Perez-Tamayo, 1975).

Given their powerful degradative ability, MMPs are produced as inactive zymogen precursors. Fibroblasts, and activated myo-fibroblasts, secrete MMPs as a proenzyme (pro-MMPs) which lie dormant in the interstitial space until activated.

Activated MMPs can be neutralised by naturally occurring inhibitors – the Tissue Inhibitors of MMPs (TIMPs). In addition to their proteolytic role, MMPs display a diversity of biological actions. They are involved in the regulation of Tumour Necrosis Factor alpha (TNF-alpha), interleukin 1 (IL-1) and Transforming Growth Factor-beta (TGF-beta) (Gearing et al. 1994; Schonbeck et al. 1998), and are also capable of increasing their own production by their ability to convert fibroblasts into the more active myo-fibroblasts. Clearly it is essential that MMP activity is tightly regulated, and this operates at both transcriptional and post-transcriptional levels, in addition to regulation through inhibition by TIMPs (figure 1.5) (Birkedal-Hansen H et al. 1993).

(b) Regulation of MMP Activity

(i) Transcriptional Regulation

There is low-level background gene expression of MMPs in all tissues. However, this expression can be modified at the transcriptional level by a large number of modulatory cytokines, hormones and growth factors (figure 1.5). Many of these serve to increase MMP gene expression and transcription, including IL-1 and –6, platelet-derived growth factor (PDGF) and TNF-alpha (Birkedal-Hansen H et al. 1993; Schonbeck et al. 1997). It is important to remember that MMPs are also involved in the regulation of IL-1, TNF and TGF-beta so the control mechanisms of levels of genetic expression are interlinked (Gearing et al. 1994; Schonbeck et al. 1998). More recently, a cell surface glycoprotein has been identified which serves to upregulate MMP-1 mRNA expression in a dose dependent manner (Guo

et al. 1997). This glycoprotein is called extracellular matrix metalloproteinase inducer, or EMMPRIN, and its own expression seems to be upregulated in disease states (Spinale FG et al. 2000).

Suppression of MMP gene expression also occurs. Transforming growth factor-beta (TGF-beta) suppresses MMP-1 transcription (and increases TIMP expression). Corticosteroids, heparin, Ang-II and IL-4 also inhibit MMP transcription (Birkedal-Hansen H et al. 1993; Mauviel, 1993; Brilla CG et al. 1994).

(ii) Pro-enzyme Activation

Pro-MMPs are zymogen precursors and are secreted and stored in the extracellular matrix. The active enzyme is liberated following cleavage of latent propetides attached to it, usually a 20 amino-acid sequence. This occurs in the ECM predominantly through plasmin-dependent stepwise activation, but activation by plasmin-independent stepwise activation and by cell surface bound MT-MMP occurs, as does intracellular activation, although at much smaller magnitude (figure 1.5) (Nagase H, 1997).

The stepwise sequence is so-called due to the stepped nature of activation (Nagase H et al. 1990). The first step is usually plasmin mediated, attacking the propeptide domain of pro-MMP leaving it susceptible to further proteolysis, usually by an active MMP. Plasmin levels and, consequently, the extent of the plasmin-mediated response, are regulated by urokinase plasminogen activator (uPA) and

plasminogen activator inhibitor (PAI), and these systems therefore influence MMP activation. Plasmin-independent pathways are also present, the first step being performed by other proteinases such as chymase and trypsin (Murphy et al. 1994; Nagase II, 1997).

The second step of this stepwise activation is dependent on a second proteinase, usually another MMP. This demonstrates the ability of active MMPs to auto-induce further activation of latent precursors. Obviously, the extent of this auto-induction must be tightly regulated. The rate limiting stage has yet to be established, but is probably the availability of plasmin which is controlled by the plasminogen activator-plasminogen system. MMP activation also occurs as a single stage via the cell surface bound, membrane type MMP (MT-MMP) (Knauper and Murphy, 1998). Finally, intracellular formation of active MMPs has been described, although the functional significance of this is unclear at present (Nguyen et al. 1998).

(iii) Tissue Inhibitors of Matrix Metalloproteinases

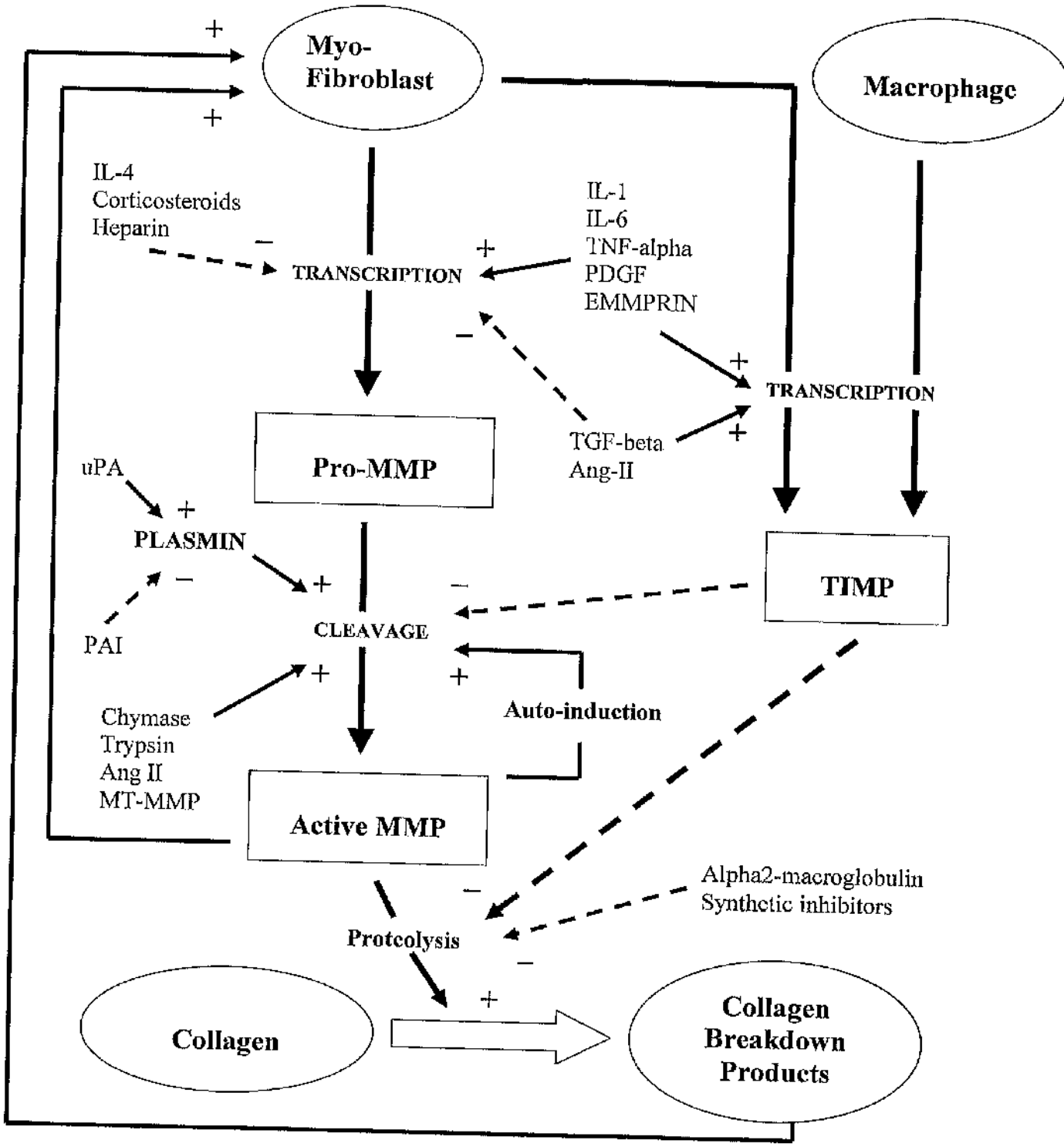
MMPs are specifically inhibited by a family of naturally occurring glycoproteins – the tissue inhibitors of metalloproteinases (TIMPs) (figure 1.5). At present four mammalian TIMPs have been identified with up to 40% sequence homology between them and all have six disulphide bridges. They are low molecular weight proteins (20-30 kDa) and exert their effects by the formation of irreversible non-covalent high affinity bonds in the catalytic domain, in a 1:1 stoichiometric form, effectively inhibiting MMP proteolytic activity (Matrisian LM, 1990; Woessner

JF, 1999). Macrophages, as well as most connective tissue cells including fibroblasts, produce TIMPs. TIMP gene expression is upregulated by IL-1 and –6, Ang-II, TGF-beta, PDGF and TNF-alpha, in addition to the previously discussed effects on MMP gene transcription by these substances (Mauviel, 1993; Chua et al. 1996).

The primary role for TIMPs would appear to be regulation of activated MMP activity by inhibition of their proteolytic properties. However, there are increasing data to suggest that TIMP-MMP interaction may be a little more complex and that TIMPs have other biologically active roles. For example, membrane type MMP can bind TIMP allowing activation of MMP precursors, TIMPs can inhibit pro-MMP activation, and TIMP-1 and –2 exhibit growth factor like activity and can inhibit angiogenesis (Kikuchi K et al. 1997; Nagase H, 1997; Hayakawa et al. 1994).

There are other inhibitors of MMPs, the principal one in-vivo being alpha2-macroglobulin, but the TIMP system is the key inhibitor of MMP activity, the regulation of which is crucial to maintain the ECM homeostasis in healthy tissues. Given the importance of MMP activity in remodelling, synthetic MMP inhibitors have been developed and studied in a variety of conditions including heart failure, myocardial infarction, coated stents to reduce in-stent restenosis and adjunctive treatment of cancers. The earliest compounds were modified tetracycline derivatives and most are still related, based on a hydroxamate structure (Skotnicki et al. 1999). This is discussed in more detail in the next section.

Figure 1.5



Legend - **Regulation of MMP activity. See text for full explanation**

IL	Interleukin	→ +	Positive effect
PDGF	Platelet Derived Growth Factor	⇢ -	Negative effect
TNF-alpha	Tumour Necrosis Factor-alpha	PAI	Plasminogen Activator Inhibitor
Ang II	Angiotensin II	MT-MMP	Membrane type matrix metalloproteinase
EMMPRIN	Extracellular matrix metallo-Proteinase inducer	Pro-MMP	pro-Matrix metalloproteinase
		MMP	Matrix metalloproteinase
uPA	Urokinase Plasminogen Activator	TIMP	Tissue inhibitor of matrix metalloproteinase

1.4.5 The Role of MMPs and TIMPs in Disease

Tight regulation of MMP and TIMP gene expression and activities are paramount for the maintenance of the dynamic turnover of collagen and other components of the extra-cellular matrix in healthy tissues. This maintains extracellular turnover to sustain the necessary milieu for normal tissue function, growth and remodelling. Although there are many physiological examples of supra-expression of one or more of the MMP-TIMP components, such as during trophoblast invasion and development, endometrial remodelling, ovulation and angiogenesis, disruption of the MMP-TIMP balance plays a pivotal role in many disease processes, including tumour growth and metastasis, arteriosclerosis, arthritis and heart disease (McDonnell et al. 1999). Of these, tumour growth and metastasis is probably the most studied. Indeed some of the MMPs were first localised in tumour tissue and many oncogenes express genes for them (Basset et al. 1990).

(a) Genetic Models and Cardiovascular Disease

With regard to MMPs and TIMPs within the cardiovascular arena, their roles are probably best illustrated through genetic studies. Gene-knockout models provide invaluable information regarding MMP and TIMP function. MMP-9 deficient mice display have attenuated left ventricular enlargement and collagen accumulation following experimental myocardial infarction and reduced cardiac rupture (Heymans et al. 1999; Ducharme et al. 2000). However, this beneficial effect was seen at the expense of impaired angiogenesis and in one study, the MMP-9 deficient mice had an increase incidence of subsequent heart failure

(Heymans et al. 1999). This illustrates the biological diversity of MMP function and they cannot just be thought of as proteolytic enzymes. Similarly, collagen turnover is a finely balanced dynamic equilibrium, and such dramatic changes to the equilibrium apparent in genetic knockout models can have untold effects on the extracellular matrix and subsequent phenotype.

However, similar protection with regard to cardiac aneurysm and rupture following experimental MI is seen in mice deficient in the gene for uPA. Diminished uPA activity reduces plasmin-mediated stepwise activation of pro-MMP and therefore levels of active MMP, and these animals display reduced LV dilatation (Heymans et al. 1999). Conversely, transgenic overexpression of MMP-1 in the mouse ventricle leads to LV dysfunction and dilatation (Kim et al. 1999). These studies illustrate the importance of active MMPs in collagen degradation during early ventricular remodelling (Matrisian LM, 1990). The significance of plasmin-mediated activation of MMP is also illustrated in plamsinogen deficient mice, where post transplant arteriosclerosis is ameliorated, again illustrating the role of active MMPs in the development of arterial remodelling and subsequent arteriosclerosis (Moons et al. 1998).

With regard to genetic models of TIMP expression, TIMP-1 gene knockout mice show increase arteriosclerosis, LV mass and volume and decreased collagen content (Roten et al. 1990). Transgenic over-expression of TIMP-1 inhibits vascular smooth muscle cell migration and neointimal proliferation during experimental vascular balloon injury (Dollery CM et al. 1999), leading to the assessment in the clinical setting of synthetic MMP inhibitors post angioplasty,

either systemically or by local delivery via drug eluting stents (Li et al. 2002; Cherr et al. 2002).

(b) Heart Failure

Although genetic studies illustrate the consequences of under and over expression of MMP and TIMPs, observational studies of natural expression of MMP and TIMP in disease states provide further evidence of the importance of MMP-TIMP balance in cardiovascular disease. There is a wealth of experimental evidence that dilated cardiomyopathy is associated with increased expression of MMPs, especially MMP-1. Studies utilising zymography demonstrate that this increased gene expression equates to increased MMP activity (Tyagi et al. 1996; Spinale FG et al. 1998; Coker et al. 1998; Spinale FG et al. 2000). Additionally, gene expression and production of TIMP-1 and –3 are reduced in both dilated and ischaemic cardiomyopathy (Li et al. 1998). There is therefore good evidence in a shift in the balance of MMP-TIMP genetic expression and activity in the failing heart, which correlates well with the documented reduction in collagen content and altered function seen in this condition (Weber KT et al. 1988b; Gunja-Smith Z et al. 1996). Furthermore, administration of a synthetic MMP inhibitor reduced LV dilatation and wall stress and improves ejection fraction in the experimental pacing induced model of heart failure in the pig. It also attenuates collagen loss (Spinale FG et al. 1999).

(c) Myocardial Infarction

Synthetic MMP inhibitors have also been used with some success in experimental models of myocardial infarction. This is perhaps unsurprising given the body of evidence surrounding MMP activity following MI. MMP expression, especially MMP-1, is upregulated following MI and again, zymography demonstrates that this is associated with increased gene product (Carlyle et al. 1997; Herzog et al. 1998; Danielsen et al. 1998). The time-scale for increased MMP production is diverse among the studies, and may be influenced by reperfusion and other factors. However, the earliest evidence of up-regulation of MMP expression appears to be within a few hours of infarction (Herzog et al. 1998). Similarly, there appears to be varying data on TIMP expression following MI. One study demonstrates suppression of TIMP-1 expression following myocardial ischaemia (Baghelai et al. 1998), but another showed increased TIMP-1 mRNA transcription 2 days following MI (Cleutjens JPM et al. 1995). Even accepting these apparent discrepancies, it is clear that the TIMP-MMP balance shifts towards increased MMP activity. Again this is in keeping with the pathological studies of early remodelling following acute myocardial infarction, the hallmarks of which are collagen loss, infarct expansion and myocyte slippage (Pfeffer MA and Braunwald E, 1990).

Only two studies have examined the use of synthetic MMP inhibitors in potential attenuation of post-infarction remodelling. The drugs were given 4 to 14 days following infarction. Both studies demonstrated decreased LV dilatation with improved fractional shortening (Rohde et al. 1999; Creemers et al. 1999). This

was most apparent when MMP inhibitor was given early (Creemers et al. 1999). Although these results are encouraging, animals given the broad spectrum MMP inhibitor ilomastat, in the study by Creemers *et al*, also demonstrated delayed infarct healing and loss of collagen (Creemers et al. 1999). This apparently paradoxical finding is probably explained by the fact that MMPs have diverse biological actions, and are not limited to their proteolytic effects on myocardial collagen. As previously discussed, MMPs, especially MMP-9, are involved in the regulation of biologically active growth factors such as TNF-alpha, TGF-beta and IL-1 (Gearing et al. 1994; Schonbeck et al. 1998). A broad spectrum MMP inhibitor may interfere with these pathways and have marked effects on the regulation and growth of other cellular and matrix components. Similarly, MMP inhibition may retard the activation of fibroblasts, thereby paradoxically reducing collagen synthesis, and therefore selective inhibition of MMP sub-types may be of theoretical benefit. This hypothesis was tested recently in a study in the pig model of pacing-induced heart failure(King MK et al. 2003). This group examined selective MMP inhibition, given before the development of heart failure, and demonstrated reduced wall stress, improved haemodynamics and LV function in the group given selective MMP inhibitor, adding support to the hypothesis.

However, all these studies illustrate the time-dependency of MMP-TIMP interaction following infarction, underpinning the relative importance of the processes of collagen synthesis and breakdown at any given time point in the continuum of remodelling. Therefore, the timing of initiation and duration of therapy is likely to be of critical importance if therapeutic intervention of the

MMP-TIMP system is to be considered. It is also important to remember that the time dependency may vary between species and even between individuals.

Even accepting these issues, manipulation of the MMP-TIMP system is an attractive concept. Phase III clinical trials in Hodgkin's and non-Hodgkin's lymphoma are ongoing. However, in the field of cardiovascular medicine, especially post infarct, there are many questions still to be addressed. These include selective vs. broad spectrum inhibition, timing of initiation and the safety parameters of these drugs. Like all therapeutic interventions, the risk:benefit ratio must be established, and it is likely that the key in potential utilisation of MMP inhibition following infarction will be the identification of those high-risk individuals who are most likely to benefit.

1.4.6 Evidence of Involvement of the Renin-Angiotensin-Aldosterone System

As previously discussed, there is good evidence of super-activation of the RAAS following myocardial infarction, both systemically and at a tissue level (Dzau, 1987; McAlpine HM et al. 1988; Lindpaintner et al. 1993; Roulcau et al. 1993). Furthermore, large-scale clinical trials have demonstrated the ability of ACE inhibitors to attenuate the remodelling process and reduce mortality (Pfeffer MA et al. 1992; AIRE study investigators, 1993; Gruppo Italiono per lo Studio della Sopravvivenza nell'Infarcto Miocardio (GISSI) -3, 1994; ISIS-4 Collaborative Group, 1995). Although the effectors of the RAAS, angiotensin II (Ang-II) and aldosterone, have modulatory effects on myocyte growth (Yamazaki et al. 1995),

they also have profound effects on the cells of the extracellular matrix and collagen metabolism.

There are many strands of evidence to support this. Firstly, at a genetic level, over-expression of the ACE gene has been demonstrated in hypertension and this high level expression induces the transformation of quiescent fibroblasts to active myo-fibroblasts (Challah et al. 1995). Furthermore, increased expression has also been demonstrated following myocardial infarction (Sun Y et al. 1994). This increase in genetic expression is associated with increased tissue levels of ACE and Ang-II, and this, coupled with the high AT-1 receptor density displayed by activated fibroblasts, means the effect of this system is enhanced following MI. The human heart also expresses mineralocorticoid receptors suggesting that the heart is a target organ of aldosterone (Lombes M et al. 1995; Kathleen M et al. 2000), and suppression by atrial natriuretic peptide reduces plasma aldosterone levels with subsequent reduction in LV remodelling (Hayashi M et al. 2001a). Furthermore, administration of the mineralocorticoid receptor antagonist spironolactone immediately following AMI reduces LV dilatation and improves ejection fraction (Hayashi M et al. 2003).

Evidence supports Ang II induction of fibroblast proliferation and up-regulation of collagen synthesis (Kato H et al. 1991; Schorb and et al, 1993). Indeed, in-vitro studies have shown Ang-II and aldosterone stimulate collagen synthesis in a dose dependent manner. The effects of Ang-II are not limited to collagen synthesis. In-vitro studies have shown Ang-II to have an inhibitory effect on MMP-1, a feature not seen with aldosterone (Brilla CG et al. 1994). It also has a stimulatory effect

on production of TIMP-1, further altering MMP-TIMP balance (Chua et al. 1996). Antagonism of the RAAS with ACE inhibitors or AT-1 receptor blockers is associated with reverse remodelling of myocyte hypertrophy (Tamura et al. 2000). It is also associated with increased collagen degradation and regression of fibrosis in the hypertensive model (Brilla CG et al. 1996; Varo et al. 2000). It is therefore clear that the RAAS has an important role in collagen metabolism and ECM turnover, partly mediated by its effects on fibroblast activation and MMP activity.

1.5 Biochemical Assessment of Collagen Turnover

The gold standard for assessing fibrosis is direct histological examination. However, in the field of cardiology, this involves invasive endomyocardial biopsy with all its attendant risks. Furthermore, changes to myocardial collagen content can be patchy and the biopsied sample may not be representative of the overall picture of collagen balance. This has prompted the search for a reliable non-invasive method for assessing the degree of fibrosis and overall collagen turnover. This may aid in the diagnosis of diseases involving collagen and perhaps allow monitoring of therapy.

Historically, determination of urinary hydroxyproline was used as a non-invasive indicator of total body collagen content and turnover. Proline residues in collagen undergo hydroxylation and it was thought that measurement of its urinary excretion would allow monitoring of collagen flux. However, proline residues on other non-collagenous proteins, such as elastin, also undergo hydroxylation. There

is also a large degree of variation in the extent of hydroxylation between different collagen types. These factors make the use of urinary hydroxyproline as a measurement of collagen turnover misleading at best, and cannot be recommended in clinical practice or in the research setting (Miller and Gay, 1992). In the last decade or so, interest has moved to serological markers of collagen turnover and the available data supports their superior accuracy (Risteli L and Risteli J, 1990).

1.5.1 Available Serological Assays

Fibroblasts synthesise procollagen and two terminal propeptide moieties must be removed before the collagen molecule can be integrated into the growing fibril (Prockop et al. 1979; Nimni ME, 1993). These moieties are one small amino-terminal and a larger carboxy-terminal propeptide, both of which may be measured in serum or plasma by immunoserologic techniques when they are liberated from procollagen. There are two commercially available radio-immunoassays which are of use in monitoring collagen synthesis. Procollagen type I carboxy-terminal propeptide (PICP), also known as C-propeptide for type I collagen, is the larger carboxy-terminal propeptide cleaved during synthesis of type I collagen fibrils and reflects the degree of synthesis of this collagen type (Melkko JT et al. 1990). Similarly, procollagen type III amino-terminal propeptide (PIIINP) is the smaller amino-terminal propeptide and is cleaved during conversion of procollagen type III and is a marker of synthesis of type III collagen (Horslev-Petersen K et al. 1988). However, unlike PICP, PIIINP is incompletely cleaved during the production of type III collagen and is also released when type III collagen is degraded and, therefore, increased serum levels

may reflect synthesis or breakdown (Nimni ME, 1993; Risteli J et al. 1995a; Risteli J and Risteli L, 1995b).

When assessing collagen content and turnover, it is important to remember that net collagen content is a product of a dynamic equilibrium between synthesis and breakdown. It is therefore imperative that collagen degradation is also considered. Fortunately, there is a radio-immunoassay available for a telopeptide liberated during degradation of type I collagen, and serum or plasma levels reflect collagen breakdown. It is called C-telopeptide for type I collagen (CITP) and is released by endopeptidase cleavage during collagen degradation in a 1:1 fashion (Risteli J and Risteli L, 1995b). These propeptides are eliminated by hepatic and renal routes and therefore, significant hepatic or renal dysfunction also increase levels (Risteli L and Risteli J, 1990).

There are therefore serological tools available to assess both synthesis and breakdown of collagen. However, it is unclear as to the contribution of the normal heart to the plasma pool of these propeptides.

There are also serological assays (ELISA) to measure the levels of both MMPs and TIMPs in the blood, and as previously discussed, collagen breakdown is dependent on the balance of MMP and TIMP activity. It is important to note that serum levels are not elevated as part of an acute phase response and, therefore, these assays probably reflect levels at a tissue level and be useful in the assessment of collagen turnover (Timms et al. 1999). However, it is also important to remember that there is some cross reactivity between MMPs and the measurement

does not reflect active MMP but also includes inactive pro-MMP and MMP bound with TIMP. Similarly, assay for TIMP does not distinguish between bound and unbound TIMP. These features make interpretation of blood levels more difficult. Finally, it is well documented that analysis of serum samples is less reliable than measurement of these compounds in plasma, and assays of plasma levels is more accurate and is therefore to be recommended (Lein et al. 1997; Jung, 1997).

1.5.2 Changes in Heart Disease

The use of immunoserological testing of collagen markers has been extensively studied in a variety of clinical conditions. The most widely used marker is PIIINP and serum levels of PIIINP are elevated in many conditions involving fibrosis, such as rheumatoid arthritis, pulmonary fibrosis, hepatitis and wound healing. Indeed, the absolute level appears to correlate with the degree of fibrosis (Low RB et al. 1983; Horslev-Petersen K et al. 1986; Haukiporo et al. 1987; Trinchet JC et al. 1991). However, as mentioned previously, there are sound theoretical reasons for the superiority of PICP as a marker of collagen synthesis, and increased levels of PICP have also been demonstrated in liver fibrosis, wound healing and bone disease (Savolainen et al. 1984; Parfitt et al. 1987; Schuppan, 1991).

These two markers of collagen biosynthesis are also the most extensively studied in the field of cardiology, mainly in arterial hypertension. Serum concentrations of both PICP and PIIINP are elevated in patients with untreated hypertension (Laviades C et al. 1994; Diez J et al. 1995; Lindsay et al. 2002). There is also evidence that elevated levels are associated with increased fibrosis, LV mass and

incidence of arrhythmias (Diez J et al. 1995; Diez J et al. 1996). It is also well documented that the renin-angiotensin-aldosterone system is powerfully fibrogenic and antagonism of it with ACE inhibitors reduces LVH and reverses fibrous tissue accumulation in the spontaneously hypertensive murine model (Brilla CG et al. 1991; Brilla CG et al. 1995). The raised PIIINP levels found in hypertension normalise with treatment with lisinopril, providing further evidence that these elevated levels reflect collagen biosynthesis and tissue fibrosis (Laviades C et al. 1994). Similarly, antagonism of the RAAS by aldosterone in patients with heart failure also reduces serum levels of PIIINP and this is associated with improved heart rate variability (MacFadyen et al. 1997). Raised serum PIIINP is associated with increased fibrosis in heart failure patients and is an independent predictor of mortality (Klappacher et al. 1995). The RALES collagen marker substudy in congestive heart failure showed that high baseline levels of PIIINP were associated with increased risk of sudden death and that the benefit seen with spironolactone treatment was associated with a reduction in the serum levels of this marker of collagen turnover (Zannad et al. 2000). Indeed, the effect of spironolactone was only significant in patients with elevated baseline levels of PIIINP, suggesting that alterations to collagen turnover may be a possible mechanism of benefit of spironolactone in the treatment of heart failure.

Serum levels of CITP display a similar picture, with levels also correlating with fibrosis and predicting mortality in heart failure (Klappacher et al. 1995). The fact that markers of both synthesis and degradation are elevated reflects the complexity of collagen flux and matrix turnover. For true assessment of collagen flux, it is imperative that both processes are considered. As outlined above, CITP is a

marker of collagen breakdown and most work has been its use in bone disease (Hakala et al. 1993; Charles et al. 1994; Hosoya et al. 1997), with really very little being performed in the field of cardiovascular medicine. Indeed, the only published cardiovascular studies utilising serum CITP was in a cohort with heart failure, mentioned above, a small study in myocardial infarction (Klappacher et al. 1995; Murakami T et al. 1998), and a recent study in essential hypertension (Lindsay et al. 2002). Researchers have focused more on MMP activity as an indicator of collagen degradation. Most of these have looked at MMP expression in atheromatous plaques. Many in-vitro studies have demonstrated increased expression of MMP-1 (interstitial collagenase) and MMP-13 (a variant of interstitial collagenase) in atheromatous plaques, with resultant increased enzymatic activity (Henney AM et al. 1991; Galis et al. 1995; Sukhova et al. 1999). It is thought that increased MMP activity may play a role in the transition from stable to unstable plaque.

There are only two serological studies in cardiology examining levels of MMPs in the blood. One was by Kai and colleagues who looked at plasma levels of MMP-2 and –9 in a cohort of patients presenting with MI, unstable angina or stable angina pectoris (Kai H et al. 1998). They showed that plasma MMP-2 and –9 are elevated in patients presenting with an acute coronary syndrome (UAP or MI) compared with those with stable symptoms, and remained elevated for several days. They postulated that this represented increased MMP activity in atheromatous plaque and the increased levels came from plaque and not the myocardial ECM.

The other serological study looking at MMP levels in peripheral blood was performed in a small cohort (13 patients) presenting with acute ST elevation myocardial infarction (Hirohata S et al. 1997). They performed serial samples for MMP-1 and TIMP-1 and demonstrated initial MMP levels below the normal range for the first few days which then increased at day 4, peaking 2 weeks later. TIMP-1 levels also showed time dependent changes with a very similar picture. Day 5 levels of both MMP-1 and TIMP-1 positively correlated with LV ejection fraction and negatively correlated with LV end systolic volume index. There is no obvious mechanistic reason to explain theses findings and they would seem to be contradictory to the fact that early remodelling is probably more dependent on collagen degradation, and that MMP inhibition improves outcome in experimental MI (Creemers et al. 1999). One would therefore expect increased early MMP activity and reversal of the correlations seen in Hirohata's study. However, as previously discussed, assays for MMP-1 detects pro-MMP and MMP complexed with TIMP in addition to active MMP, and therefore blood levels may not reflect actual activity. The same is true of TIMP-1 assay.

Given that there are only limited data with respect to direct markers of collagen degradation following MI, it is difficult to know how to interpret this study. There is only one published study examining serum levels of CITP as a marker of collagen breakdown following MI (Murakami T et al. 1998). Murakami and colleagues examined a small cohort of 13 patients with myocardial infarction and found that serum PICP was reduced immediately following AMI and CITP was more than two standard deviations below a normal population mean on admission. Both serum PICP and CITP subsequently exhibited a time dependent rise, with

CITP rising beyond the normal range. PICP had a very weak correlation with indices of LV volume but no such correlation was found with CITP. Again it is difficult to know how to interpret the results. The numbers involved in this study were small and with large standard deviations, making statistical and functional interpretation difficult. In addition, the authors do not offer a hypothesis to explain the initial fall then rise of PICP or why the initial CITP was more than 2 standard deviations below the mean of their normal population. Furthermore, the use of serum for analysis of markers of collagen flux may be less reliable than plasma assays (Lein et al. 1997). However, even accepting these limitations it is the only study to date which has utilised markers of both processes of synthesis and degradation.

The other serological work in MI has mainly focused on PIIINP. A Danish group demonstrated elevated PIIINP from the third post-infarction day, peaking around day 7 and remained elevated for several months performed three studies, in a small cohort of patients presenting with ST elevation MI. There also found a weak correlation between peak levels and infarct size as assessed by cardiac enzyme release (Jensen LT et al. 1990). This was confirmed in a subsequent studies performed by the same group, where they also demonstrated that higher levels were associated with a restrictive filling pattern and were predictive of a poor outcome including death (Host NB et al. 1995; Poulsen SH et al. 2000). A similar picture was demonstrated by an Italian group and, more recently, the same group demonstrated reduced ventricular dilatation and serum PIIINP levels following AMI with treatment with an aldosterone antagonist started on discharge (Modena et al. 2001).

It is therefore clear that changes to PIIINP, a marker of mainly collagen synthesis, provide information on remodelling. However, as previously discussed, PIIINP can be also be increased during degradation as it is incompletely cleaved from pro-collagen during synthesis of collagen type III and some will therefore be liberated during breakdown (Risteli L and Risteli J, 1990). This makes it difficult to postulate a mechanistic hypothesis, and one must really consider both synthesis and degradation when attempting to assess collagen turnover by biochemical means.

1.6 Hypotheses

It is clear from the literature that coronary disease is common and that acute coronary events are associated with significant morbidity and mortality. Ventricular remodelling following myocardial infarction is a powerful adverse prognostic indicator, and there are strategies available to reduce the remodelling process. Risk stratification is the key and early intervention in patients at high-risk of remodelling may have an impact on morbidity and mortality. Echocardiography has a role to play in risk stratification, but will only identify early remodelling when it has already begun. Early post-infarction remodelling is characterised by marked changes to myocardial collagen content and the extracellular matrix, causing infarct expansion, myocyte slippage and LV dilatation. Biochemical markers of collagen turnover may be a useful tool in identifying those at risk of remodelling and allow us to monitor the process. They may also have some use in

the risk stratification of non ST elevation acute coronary syndromes, as ischaemia can cause alterations to the ECM in the absence of overt myocyte necrosis.

My hypotheses are:

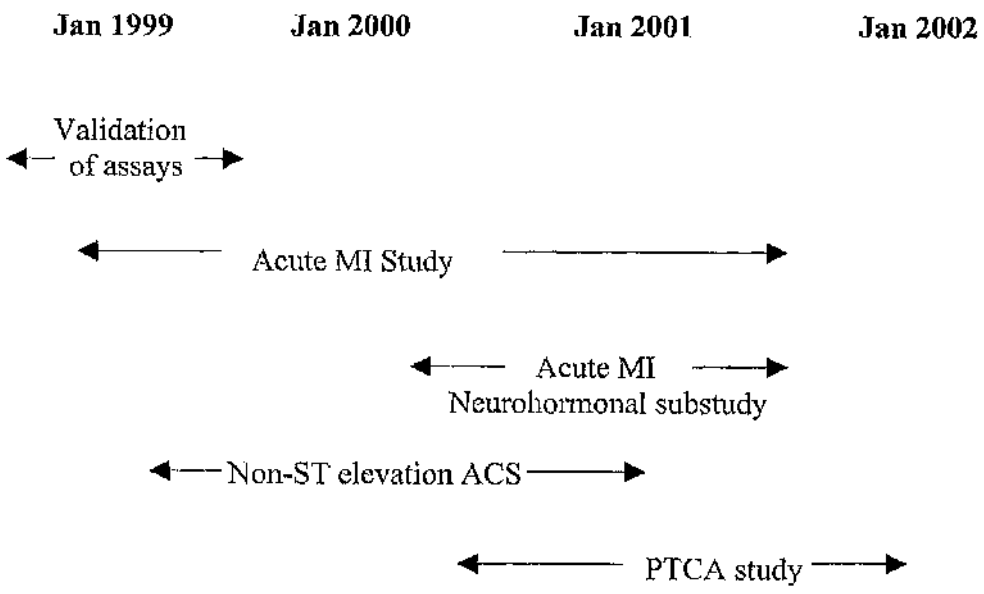
1) There will be time dependent alterations in plasma levels of markers of collagen synthesis and degradation (PICP, CITP and TIMP-1) following acute myocardial infarction, reflecting alterations to myocardial collagen content and structure.
2) Plasma CITP will be elevated early following AMI with a later rise in PICP reflecting collagen degradation as the principal finding during early remodelling and collagen synthesis as the principal finding in late remodelling.
3) Increased plasma CITP will reflect early ventricular remodelling as assessed by echocardiographic parameters.
4) The changes in plasma levels are not under systemic RAAS control.
5) In CK negative, non-ST elevation acute coronary syndromes, there will be time dependent changes in these markers in "high risk" patients as manifested by positive troponin I or dynamic ECG changes, and this may reflect a degree of remodelling.
6) The changes in plasma levels will be rapid in onset, reflecting rapidity of onset of remodelling, and will be further investigated using a human model of acute coronary occlusion.

CHAPTER TWO – MATERIALS & METHODS

2.1 Summary of Studies Performed

The time-line of studies is illustrated in figure 2.1.

Figure 2.1



Legend - Time-scale for studies

Following intra and inter-assay variability studies, alterations in plasma levels of markers of collagen turnover were examined in four distinct populations: healthy volunteers, ST elevation MI, non-ST elevation ACS and patients undergoing coronary angiography and angioplasty. A total of 30 healthy volunteers were studied for estimation of normal ranges and studies of variablitiy within individuals. In addition, 64 patients with ST elevation MI, 52 with non-ST

elevation ACS and 22 undergoing coronary angiography or angioplasty were also studied. I undertook study design and obtained ethical approval. I personally screened and recruited all participants, performed venesection, analysed ECGs and performed and analysed echocardiograms. I also collected and analysed all data.

2.2 Validation of Biochemical Assays of Collagen Turnover (Chapter 3)

2.2.1 Study Population

The study population for the assessment of assay variability was drawn from a mix of patients who agreed to participate in the ST elevation MI and non-ST elevation acute coronary syndrome studies, in addition to healthy volunteers and patients participating in other studies at Stobhill Hospital. These included patients with hypertension and liver disease. These studies were performed from January 1999 till September of the same year (figure 2.1).

To allow characterisation of normal ranges and intra-individual variability, healthy volunteers were recruited from the departments of Biochemistry and Cardiology and following advertisement in the local press. Exclusion criteria applicable to normal volunteers and patients enrolled in the subsequent studies of acute coronary syndromes (ST elevation and non ST elevation) and percutaneous transluminal coronary angioplasty are listed in table 2.1. They include factors influencing collagen metabolism and elimination of plasma propeptides (Risteli L and Risteli J, 1990).

Table 2.1

Patient Characteristics

- Unable to give informed consent
- Psychiatric illness
- Alcohol intake >50 U/week

Conditions Know To Alter Collagen Flux

- Surgery within last 6 months
- Admission for an acute coronary syndrome within last 6 months
- Percutaneous coronary intervention in last 6 months
- Active tumour
- Pulmonary fibrosis
- Rheumatoid disease / Connective Tissue Disease
- Treated hypertension
- Previous MI or heart failure

Conditions Know To Influence Metabolism Of Pro-peptides

- Concurrent steroid or NSAID use
- Concurrent ACE inhibitor, Angiotensin II Receptor Blocker or spironolactone use
- Liver dysfunction - ALT > 2x ULN
- Renal dysfunction – Creatinine > 130micromol/l

Legend - Exclusion criteria for all studies

2.2.2 Study Design

(a) Assay Variability

10mls of whole blood was venesected with the patient in the recumbent position and collected in Lithium Heparin tubes. It is well documented that analysis of plasma samples is more accurate and reliable than analysis of serum samples and therefore all samples were collected in Lithium Heparin tubes (Lein et al. 1997; Jung, 1997). Samples were then centrifuged at 3000rpm for 7 minutes and plasma decanted into crystal tubes and frozen at -70°C until use. Intra-assay variability was performed by assaying the same investigational sample twice using the same assay kit. Inter-assay variability was determined by assaying the same investigational sample twice using different kits.

(b) Normal Ranges

Following verbal consent, 10mls of whole blood was venesected from healthy volunteers in the recumbent position during the morning. Samples were collected in Lithium Heparin tubes and processed as above.

(c) Intra-individual Variability

Blood was collected from volunteers on two occasions - one in the morning, another in the afternoon. Again, samples were collected in Lithium Heparin tubes and spun and frozen till use. Verbal consent was obtained prior to venesection.

2.2.3 Biochemical Method for Assay of Markers of Collagen Turnover

(a) Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1)

Plasma TIMP-1 was measured using a modification of the method developed by Plumpton (Plumpton TA et al. 1995). This is a two-step sandwich enzyme linked immunosorbent assay (ELISA) assay using a commercially available kit – RPN2611 (Amersham Pharmaceuticals, Little Chalfont, UK). 100 microlitres of the investigational plasma sample is incubated in a microtitre well coated with anti-TIMP antibody following equilibration at room temperature. After incubation and washing with buffer solution, the bound TIMP-1 is measured by reaction with a second antibody labelled with horseradish peroxidase. The oxidised substrate is read in a photospectrometer at 450nm. A value for the amount of TIMP-1 in the investigational sample is read directly from a standard curve constructed by calculating percentage binding of five standard concentrations prepared in the same way.

(b) C-propeptide for Type I Collagen (PICP)

A radio-immunoassay (RIA) was used for the assessment of plasma PICP (Orion Diagnostica, Espoo, Finland). This assay utilises the standard principles of RIA with 100 microlitres of the investigational sample containing an unknown amount of PICP being mixed with 200 microlitres of radio-active PICP (labelled with ^{125}Iodine) (Melkko JT et al. 1990). 200 microlitres of PICP anti-serum (rabbit) is added to this mix resulting in competition for the limited number of high affinity

sites of the antibody (PICP anti-sera) between the labelled and unlabelled PICP. After 2 hours of incubation at 37°C, the proportion of radioactive PICP in the antigen-antibody complex in the reaction mixture is inversely proportional to the amount of unlabelled PICP in the investigational sample. Adding a separation agent and centrifuging removes free PICP. The supernatant is decanted and the sediment containing the precipitated antigen-antibody complex is counted in a gamma camera.

A standard curve is produced after calculation of the binding of five standards prepared in the same way. These values are expressed as a percentage of maximum possible binding. Bound radio-activity from investigational samples are plotted along this curve and PICP values read directly.

(c) C-telopeptide for Type I Collagen (CITP)

A radio-immuno assay (RIA) for CITP was used (Orion Diagnostica, Espoo, Finland). Method is identical to that for PICP with ^{125}Iodine labelled CITP and rabbit CITP antiserum replacing labelled PICP and anti-PICP (Risteli J et al. 1993). Standard curve for bound radioactivity is constructed after assaying five standard concentrations of CITP and results of investigational samples read directly.

2.2.4 Analysis of Results

(a) Variability Studies

Two values for each sample were obtained in the intra and inter-assay variability studies and in the study of intra-individual variability. A mean of the two values was calculated for each sample and differences expressed as absolute and percentage differences of the mean. Correlation between the values was calculated and expressed as a co-efficient. The distribution of the collagen markers was tested for normality using the Anderson Darling method. Results from the group were not normally distributed for PICP and TIMP-1, so log transformation was performed prior to testing of statistical significance. Statistical comparison was performed using Student's t test. All statistical analyses were performed using Minitab statistical software (Minitab Inc, Pennsylvania, USA). A p value of <0.05 was considered significant.

(b) Normal Ranges

Values for the group were analysed and expressed as a mean +/- standard deviation, including 95% confidence intervals.

2.3 Acute Myocardial Infarction – Biochemical, Neurohormonal and Functional Studies (Chapters 4 & 5)

2.3.1 Study Population

(a) Recruitment

Patients were recruited from the Coronary Care Unit (CCU) at Stobhill Hospital in the North of Glasgow over a two and a half year period, from February 1999 till August 2001 (figure 2.1). Those presenting with a history suggestive of an acute coronary syndrome with ST elevation on a 12 lead ECG were eligible for entry into the study. Entry criteria consisted of chest pain for more than 20 minutes and presentation within 6 hours from the onset of pain. The CCU at Stobhill hospital operates a "back-door" policy, allowing General Practitioners and ambulance personnel to refer directly to CCU, bypassing the busy Accident & Emergency department. Patients are brought directly to Coronary care by ambulance and are immediately assessed by senior nursing and medical staff following a 12 lead electrocardiogram. The efficacy of such a system in reducing time to thrombolysis has been demonstrated previously (Burns et al. 1989; Prasad N et al. 1997) and the unit currently has a median time to thrombolysis of 28 mins from admission and 22 mins from diagnostic ECG. 64% of patients receive thrombolysis within the audit standard of 30 minutes. This CCU therefore provided the ideal setting for the early identification of patients suitable for inclusion in this study.

Twenty healthy volunteers were recruited following advertisements in the local press. These individuals were on no cardiac medication. This group had a similar age and sex distribution to the investigational group.

(b) Inclusion and Exclusion Criteria

All patients presenting within 6 hours of chest pain with an ECG fitting the criteria for thrombolysis were considered for inclusion into the study. Standard electrocardiographic criteria were adopted regarding eligibility for thrombolysis - ST elevation in 2 or more contiguous leads of >1mm in the limb leads or >2mm in the chest leads or new left bundle branch block (LBBB) (Fibrinolytic Therapy Trialists, 1994). Exclusion criteria are listed in Table 2.1.

2.3.2 Study Design

All studies were performed in accordance with the Declaration of Helsinki. The Local Research & Ethics Committee at Stobhill Hospital agreed study design and protocol in December 1998. Written, informed consent was obtained from all patients after the study had been explained verbally and by a study information sheet. A protocol of investigation is outlined in table 2.2.

Table 2.2

	Blood for markers of collagen turnover	Blood for RAAS effectors	Trans-thoracic echocardiogram	12 lead ECG
On admission	Yes	Yes		Yes
1 hour post thrombolysis				Yes
12 hours	Yes			
24 hours	Yes			Yes
Day 2	Yes			
Day 3	Yes	Yes		
Day 4	Yes		Yes	
Day 30	Yes			Yes

Legend - Summary of protocol of investigation for Acute Myocardial Infarction Study

A cannula was inserted and sequential blood samples drawn at serial time points – on admission, at 12 hours, 24 hours, days 2, 3, 4 and 30. Venesection was performed with the patient in the recumbent position. 10mls of blood for plasma markers of collagen turnover was collected in Lithium Heparin bottles and centrifuged at 3000rpm for 7 minutes. Plasma was distilled and decanted into

crystal tubes as three equal aliquots. Samples were labelled and frozen at –70 °C until use. Samples were not thawed and refrozen. A subgroup of patients had further samples collected in Lithium Heparin and EDTA tubes on admission and at day 3 for assessment of the effectors of the RAAS (additional 10mls of whole blood). These samples were prepared and stored as outlined above.

A 2 dimensional trans-thoracic echocardiogram with detailed Doppler studies was performed on day 4 or 5 following admission. Initially, the study design included an echo performed at day 30 to assess change in LV dimensions and function over this time period. However, this was abandoned for several reasons. Firstly, after seeking statistical advice, it was felt that in order to achieve adequate statistical power to detect *change* in measured parameters, the numbers of patients required would be so large as to make recruitment impractical. Secondly, many patients were reluctant to have a second echocardiogram performed at day 30, but were happy to have venesection at that time. Finally, although remodelling is defined as a *change* in LV shape, structure and function, the process can be detected early. It is well documented that a *single* echocardiographic study performed within a few days of infarction examining wall motion, LV volume and ejection fraction and Doppler interrogation of the mitral inflow identifies those in whom remodelling is already evident and therefore having the worst prognosis (Kitamura et al. 1973; Kan et al. 1986; White HD et al. 1987; Sutton et al. 1994; Kober L et al. 1994; Popovic et al. 1996; Migrino et al. 1997; Sutton et al. 1997; Cerisano G et al. 1999).

Patients were invited back 30 days following the index event for repeat blood sampling. 12 lead ECGs were performed on admission, 90 minutes following initiation of thrombolytic therapy, at 24 hours and on day 30.

2.3.3 Biochemical Method for Assay of Markers of Collagen Turnover

Methods for the assay of plasma TIMP-1, PICP and CITP are identical to those outlined in sections 2.2.3 a, b and c.

2.3.4 Assessment of Neurohormones

Patients recruited into the study from March 2000 till September 2001 were eligible for the neurohormonal sub-study. In addition to blood taken for assay of the above biochemical markers, a further 10mls of blood was venesected in these patients on admission and 72 hours post admission. Blood was collected in EDTA and Lithium heparin tubes, spun immediately at 3000rpm for 7 minutes and frozen at –70 °C until use. Assays for plasma renin and aldosterone estimation were performed by Dr Ian Morton at the Blood Pressure Unit, Western Infirmary, Glasgow, by standard ELISA technique.

2.3.5 Echocardiography

(a) Data Acquisition and Storage

Detailed transthoracic echocardiographic studies were performed with the patient in the left lateral position in a darkened room using a Vingmed System V Ultrasound Machine (General Electronics, Milwaukee, USA) connected to a PowerMacIntosh G3 computer with digital archiving facilities. Patient archive information was stored on both the hard drive of the PowerMacIntosh computer and optical disks. Unlike previous generations of ultrasound machines, which use a fixed output and receiving frequency in the mid-range, the System V incorporates secondary harmonic imaging technology using an output frequency of 1.7mHz, receiving at 2 octaves higher via a phased array probe. As lower ultrasonic frequencies give better tissue penetration and higher frequencies provide improved image resolution, secondary harmonics allow superior imaging with enhanced resolution of the endocardial and epicardial borders, increasing the accuracy of wall motion scoring, volume assessment and calculation of ejection fraction.

Each examination was recorded onto Super VHS tape and images stored on digital optical disks. Acquisition of images was gated to ECG telemetry allowing storage of whole cardiac cycles and accurate correlation of single frames to phases of the cardiac cycle such as end-systole and end-diastole. Off-line analysis was performed using system specific software (EchoPAC) incorporating software for wall motion scoring and the assessment of left ventricular volumes and ejection

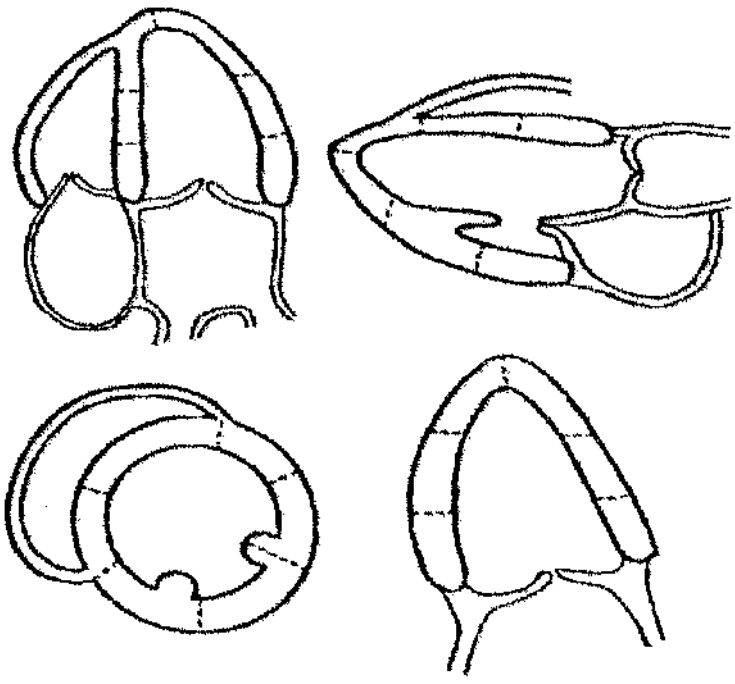
fraction. Date of birth was used to code all echo examinations to enable blinding to patient history and biochemical results (which were coded by patient name)

(b) Wall Motion Scoring

(i) Method

Images were acquired in four standard views with the patient in the left lateral position at end-expiration - parasternal long axis, parasternal short axis at the level of the papillary muscles, apical 4 chamber and apical 2 chamber. Analysis was performed offline by a single observer (AMcG) blinded to the results of biochemical analyses. A sixteen segmental scoring system was employed in accordance with the American Society of Echocardiography guidelines (figure 2.2) (Schiller et al. 1989). Each segment was visualised and given a score from 1 – 5 depending on its movement (table 2.3). Eight segments are visualised in more than one view and the overall score for these segments is the mean of both scores. The sum of the scores from all segments were totalled and divided by sixteen giving a wall motion index. A score of 1 indicates normal movement of all segments, i.e. normal wall motion with no regional wall motion abnormality. A score of greater than one represents the presence of abnormal wall motion of one or more segments. The higher the score above one, the greater the degree of regional wall motion abnormality.

Figure 2.2



Legend - Graphical representation of 16 segmental scoring system for calculation of wall motion index

Table 2.3

Score	Wall Motion
1	Normal wall motion
2	Hypokinesia
3	Akinesia
4	Dyskinesia
5	Aneurysmal

Legend - Scoring system for wall motion

(ii) Intra-operator Variability

Wall motion scoring was performed offline on twenty randomly selected studies on two separate occasion with the observer blinded to the previous score. Reproducibility was good with all segments in 17 studies being given the same wall motion score and a difference in scoring of one segment was seen in three studies, altering wall motion index by <10%. However, with regard to overall wall motion index, no patient with a normal index on one score was scored as abnormal, nor were any patients with abnormal index scored as normal.

(c) Left Ventricular Volume and Ejection Fraction

(i) Method

Standard apical two-dimensional 2 and 4 chamber images were obtained in the usual manner. A single operator blinded to biochemical results performed offline analysis. End diastole was identified from ECG gating, being defined as the onset of the R wave. End systole was identified from visual inspection, being defined as the smallest ventricular dimension in that cardiac cycle. Tracing of the endocardial contours in both diastole and systole was performed and LV volumes calculated from the disc summation method or Simpson's rule (Schiller et al. 1979; Erbel et al. 1982). The mean of three cycles were calculated (avoiding extrasystolic or immediate post-extrasystolic beats) and normalised for body surface area, giving values for left ventricular end diastolic volume index (LVEDVI) and left ventricular end systolic volume index (LVESVI).

Ejection fraction was calculated as follows:

$$\frac{\text{(LV end diastolic volume – LV end systolic volume)}}{\text{LV end diastolic volume}} \times 100$$

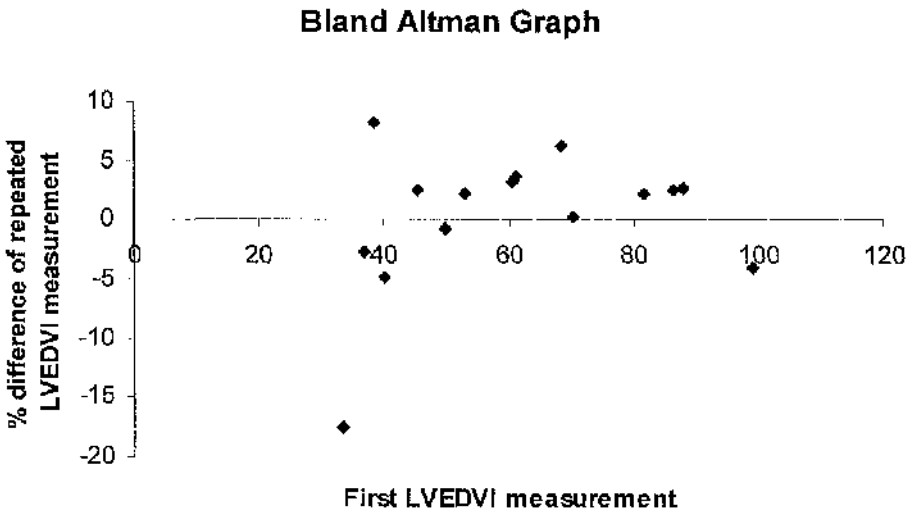
This is a well validated method of calculating LV ejection fraction, giving similar results to radionuclide ventriculography and cineventriculography at cardiac catheterisation (Folland et al. 1979; Starling et al. 1981; Erbel et al. 1983).

(ii) Intra-operator Variability

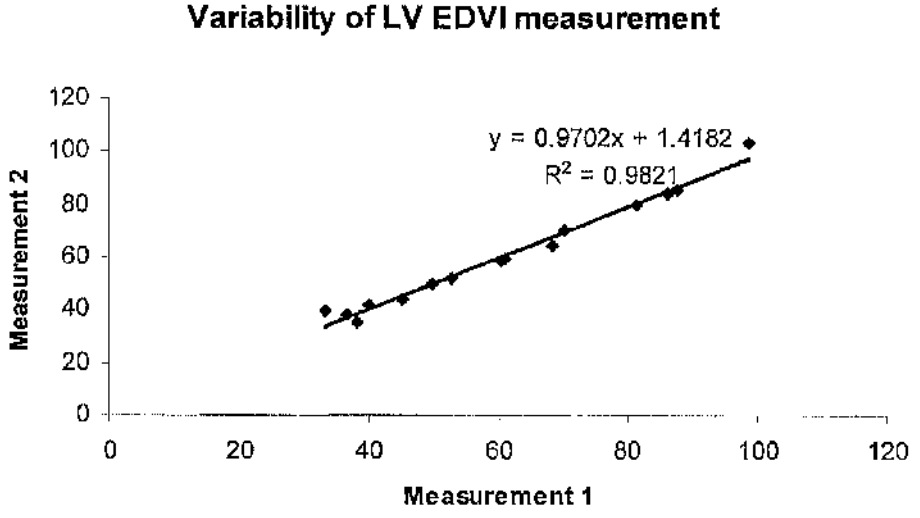
Analysis of fifteen studies was repeated with no knowledge of previous results. Percentage differences in repeated measurement of EVDI and ESVI were <5%, corresponding to absolute differences of 2.1 and 1.5 ml/m^2 respectively (correlation co-efficient 0.99 and 0.99). This is shown graphically in figures 2.3 and 2.4. Correlation of repeated measurement of LV ejection fraction was excellent, with a correlation coefficient of 0.98 (figure 2.5 (a) and (b)).

Figure 2.3

(a)



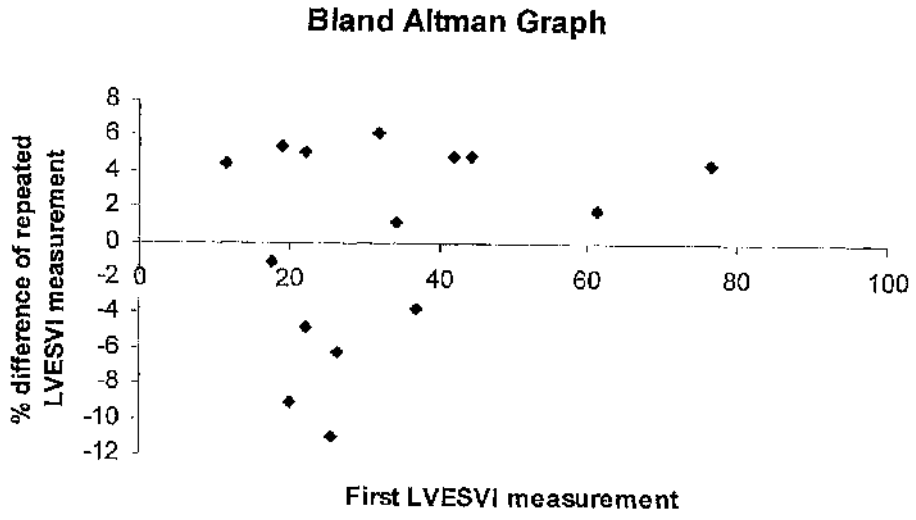
(b)



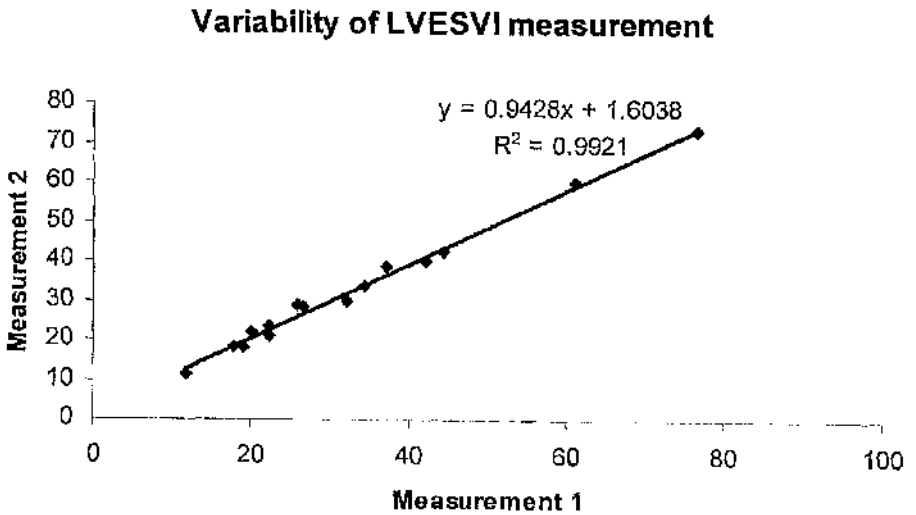
Legend - Variability of repeated measurement of LVEDVI in (a) Bland-Altman and (b) Scatterplot form.

Figure 2.4

(a)



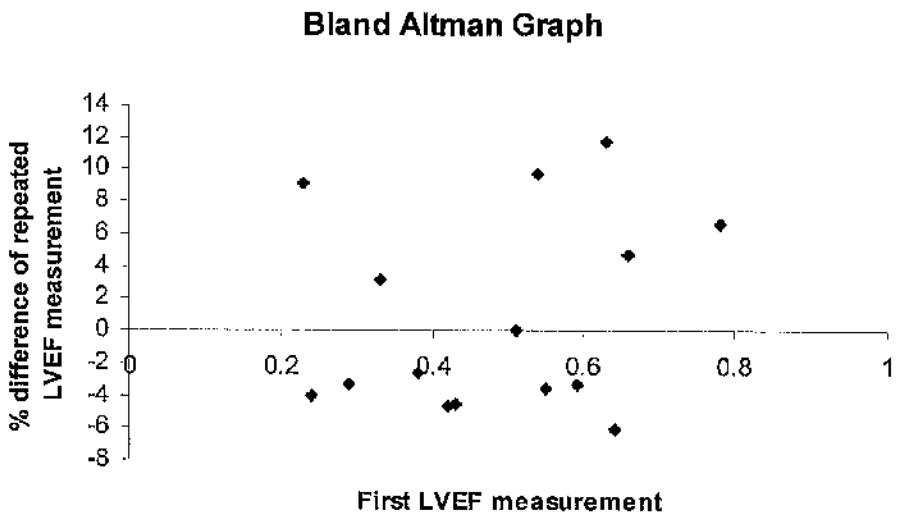
(b)



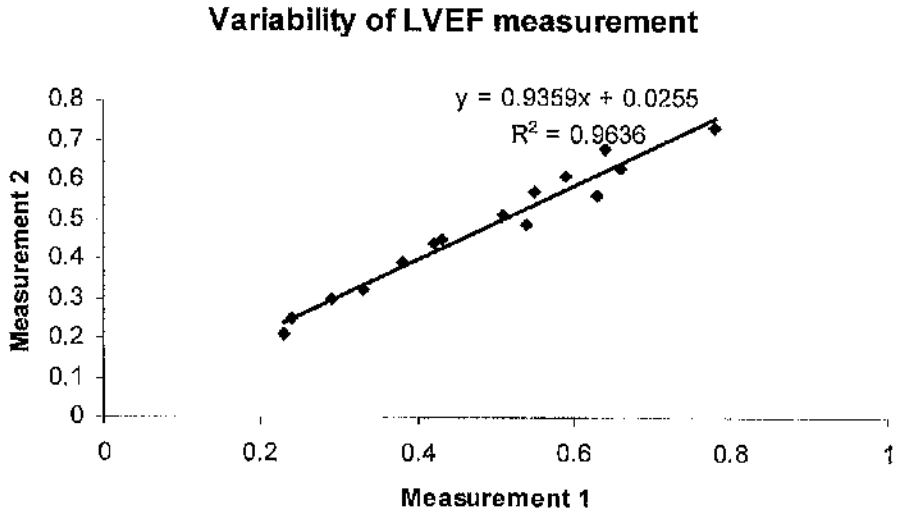
Legend - Variability of repeated measurement of LVESVI in (a) Bland-Altman and (b) Scatterplot form.

Figure 2.5

(a)



(b)



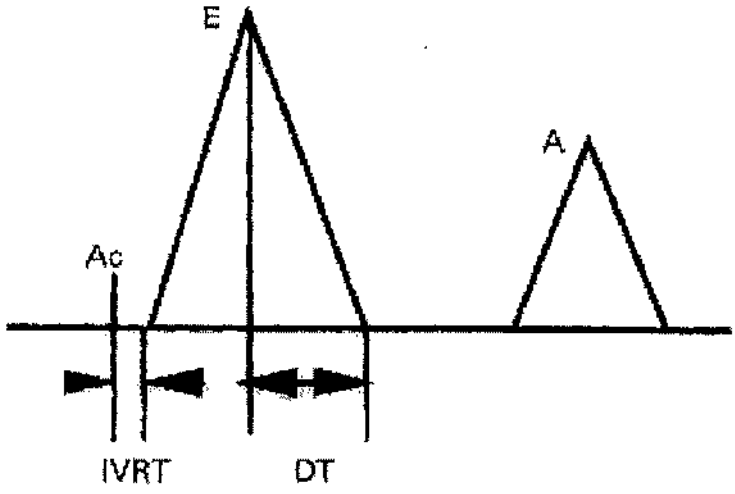
Legend - Variability of repeated measurement of LVEF in (a) Bland-Altman and (b) Scatterplot form.

(d) Mitral Deceleration Time

(i) Method

Pulsed wave Doppler signals were obtained through the tips of the mitral valve inflow in the apical four chamber view with the patient in the left lateral position at end-expiration. Three cardiac cycles per study were acquired from patients in sinus rhythm. Those with atrial fibrillation were excluded. Offline analysis was performed by a single observer blinded to biochemical results, with measurement of mitral deceleration time (Dt) being performed on each cycle. Dt was measured as the time from the peak of the early mitral inflow (E-wave) to baseline (figure 2.6). The mean of the three cycles was used for the purposes of analysis.

Figure 2.6



Legend - Diagrammatical representation of a Pulse Wave Doppler signal through the tips of the mitral valve leaflet.

(ii) Intra-operator Variability

Analysis of fifteen studies was repeated with no knowledge of previous results. Differences in repeated measurement of Dt was <4% with excellent correspondence between measurements (correlation co-efficient 0.98) (figure 2.7 (a) and (b)).

2.3.6 Electrocardiography

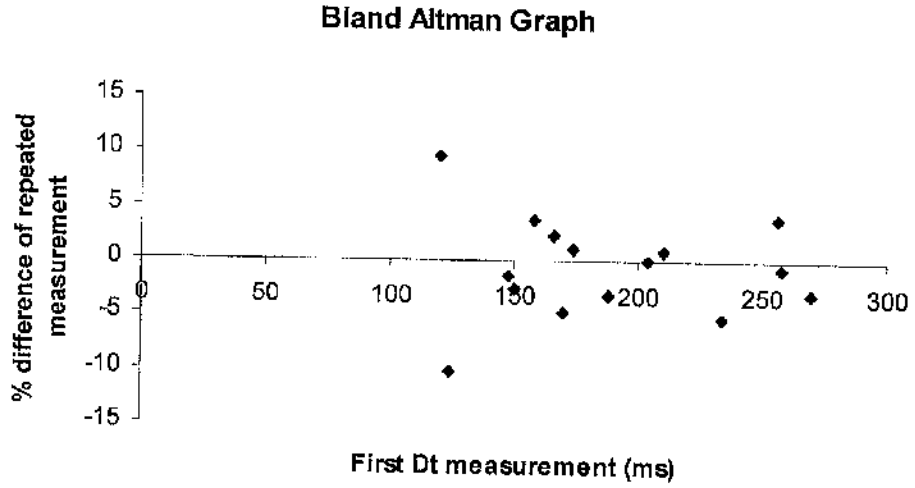
Standard 12 lead electrocardiograms were performed on admission on a Pagewriter XLA (Hewlett Packard, USA) to identify patients eligible for thrombolysis using the standard criteria (Fibrinolytic Therapy Trialists, 1994). A further 12 lead ECG was obtained at 90 minutes post thrombolytic therapy for non-invasive assessment of reperfusion, using the criteria developed by Hogg *et al* - >50% reduction in ST segment elevation in one lead (Hogg KJ et al. 1988).

2.3.7 Mortality Data

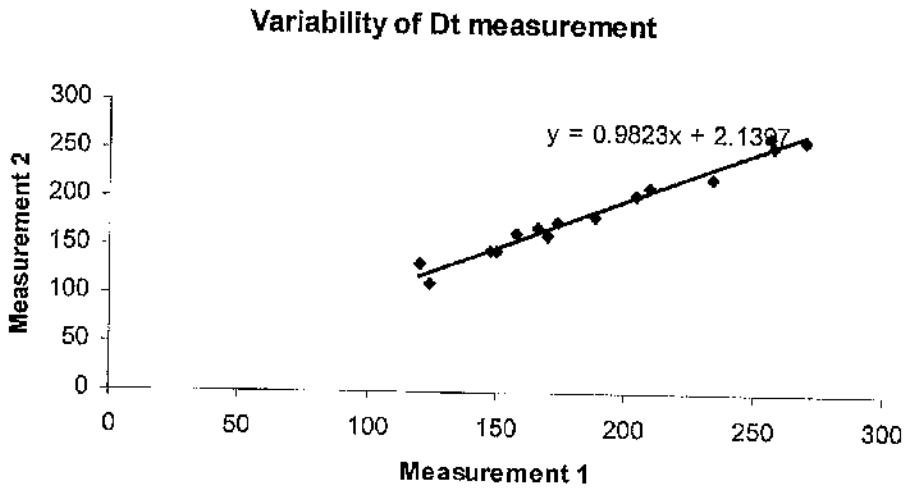
Mortality data at six months was obtained from The Common Services Agency, Edinburgh. Although the study was not formally powered to assess relationship of the investigational variables to mortality, these data allow us to better assess the risk of the overall study group and to examine trends in mortality. Cause of death was not ascertained.

Figure 2.7

(a)



(b)



Legend - **Variability of repeated measurement of Dt in (a) Bland-Altman and (b) Scatterplot form.**

2.3.8 Statistical Analysis

(a) Power Calculation

Dr David Young, Lecturer at the Robertson Centre for Biostatistics, University of Glasgow, provided a power calculation for this study. It was based on a pilot study of 18 patients with ST elevation MI and was calculated from the mean and standard deviation of plasma values of TIMP-1, PICP and CITP in this group compared to the mean and standard deviation in a normal population (table 2.4). The larger of the standard deviations was used as the common one for both groups, giving a more conservative sample size. Given the large differences between group means in the pilot study, recruitment of only 8 patients would give a 95% power to detect a difference in TIMP-1 of 974ng/ml using a two group t-test with a 0.05 two sided significance test. With regard to CITP, 24 patients would be required to have a similar power to detect a 2.09ng/ml difference. Given the marked heterogeneity in the levels of plasma PICP in the pilot group, the standard deviation for this group was high. As we had elected to use the higher of the standard deviations as the common one, 93 patients would be required to detect a difference of 44ng/ml with 95% power. It was therefore elected to reduce power to 90%, requiring 64 patients to be recruited.

No published data are available for correlation of these markers with echocardiographic parameters of remodelling. We wished to dichotomise patients into two groups – those with evidence of remodelling and those with no evidence

of remodelling. The power calculation was based on a pilot study of patients with echocardiographic evidence of remodelling and it was decided that recruitment should continue until at least this number had adequate echo studies indicative of remodelling, allowing adequate comparisons between the groups. However, it was felt that recruitment of this number of patients for this study was unrealistic, so recruitment continued until at least 24 patients (sufficient for comparison of TIMP-1 and CITP) had adequate echo studies demonstrating evidence of early remodelling.

(b) Analysis of Results

As previously stated, statistical analyses were performed using Minitab statistical software (Minitab Inc, Pennsylvania, USA). Methods of analysis were discussed with Dr Young. All continuous variables are expressed as mean +/- one standard error of the mean. Two of the three biochemical variables were not normally distributed so log transformation was performed to allow parametric statistical testing methods. Repeated measurements were tested by repeated measurement of analysis of variance. Student's t test was used for comparison of means. Multiple regression models were constructed using Minitab. A p value of <0.05 was considered significant.

Table 2.4

TIMP-1

Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
Group 1 mean	2150	2150	2150	2150
Group 2 mean	202	202	202	202
Difference in means	1948	1948	1948	1948
Common standard deviation	974	974	974	974
Effect size	2	2	2	2
Power (%)	80	90	95	99
Number required	6	7	8	11

CITP

Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
Group 1 mean	4.69	4.69	4.69	4.69
Group 2 mean	2.6	2.6	2.6	2.6
Difference in means	2.09	2.09	2.09	2.09
Common standard deviation	1.36	1.36	1.36	1.36
Effect size	1.54	1.54	1.54	1.54
Power (%)	80	90	95	99
Number required	14	19	24	34

PICP

Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
Group 1 mean	173	173	173	173
Group 2 mean	129	129	129	129
Difference in means	44	44	44	44
Common standard deviation	59.8	59.8	59.8	59.8
Effect size	0.736	0.736	0.736	0.736
Power (%)	80	90	95	99
Number required	54	64	93	133

Legend - **Power calculation for TIMP-1, CITP and PICP for Acute MI study.**

2.4 Non ST Elevation Acute Coronary Syndrome Study (Chapter 6)

2.4.1 Study Population

Once again, the Coronary Care Unit at Stobhill Hospital was used to identify patients appropriate for the study. Recruitment took place from August 1999 till December 2000 (figure 2.1). Those presenting with a history suggestive of an acute coronary syndrome without ST elevation on a 12 lead ECG were considered for the study. For inclusion, pain had to be typical of myocardial ischaemia and occur at rest or on minimal exercise and be present for at least 20 minutes. Furthermore, presentation had to be within 6 hours of the onset of pain. Patients with ECG fitting the criteria for reperfusion therapy were excluded. Exclusion criteria are listed in table 2.1 and include factors influencing collagen metabolism and elimination of plasma propeptides. In addition, patients with bundle branch block on ECG, concurrent digoxin therapy or permanent pacemaker in situ were excluded due to difficulty in interpreting the ST/T segments of a 12 lead electrocardiogram.

2.4.2 Study Design

The Local Research & Ethics Committee at Stobhill Hospital agreed study design and protocol in December 1998. Written, informed consent was obtained from all patients after the study had been explained verbally and by a study information sheet. A protocol of investigation is outlined in table 2.5.

A cannula was inserted and sequential blood samples drawn at serial time points with the patient in the recumbent position – on admission, at 12 hours, 24 hours, and 48 hours. 10mls of blood for plasma markers of collagen turnover was collected in Lithium Heparin bottles and centrifuged at 3000rpm for 7 minutes. A further 7mls of blood for assessment of troponin I was venesected at 12 hours following admission and collected in SST tubes. Plasma was distilled and decanted into crystal tubes and frozen at –70 °C until use.

12 lead electrocardiograms were performed on admission and at 24 hours. Further ECGs were performed if the patient developed further pain.

Table 2.5

	Blood for markers of collagen turnover	**Blood for troponin I**	**12 lead ECG**
On admission	Yes		Yes
12 hours	Yes	Yes	
24 hours	Yes		Yes
48 hours	Yes		
During pain			Yes

Legend - Summary of protocol of investigation for non ST elevation ACS study

2.4.3 Biochemical Analyses of Markers of Collagen Turnover

Plasma PICP, CITP and TIMP-1 were measured according to the methods outlined in sections 2.2.3a, 2.2.3b and 2.2.3c.

2.4.4 Risk Stratification

(a) Definition

Patients were dichotomised into high and low-risk groups based on ECG criteria and measurement of plasma Troponin I. High-risk was defined as the presence of dynamic ST depression/sequential T wave changes and/or Troponin I >0.4ng/ml at 12 hours post admission. Low-risk was defined as the absence of ECG changes and a negative Troponin.

(b) Electrocardiography

Standard 12 lead electrocardiograms performed on a Pagewriter XLA (Hewlett Packard, USA) were used to aid risk stratification in this group of patients with unstable coronary syndromes. ECGs were performed on admission, at 24 hours and during pain to assess ST segment deviation and T wave morphology. ST depression was defined as planar or down-sloping ST depression of 0.5mV, 80ms after the J point in two or more contiguous leads which was not there on prior ECGs (if available). Up-sloping ST depression was discounted. Sequential ECG

changes were defined as new biphasic T waves or T wave inversion in two or more contiguous leads on ECGs performed subsequent to admission.

(c) Troponin I

A Microparticle Enzyme Immunoassay technique (MEIA) was used for the quantitative determination of cardiac troponin I in the plasma using a commercially available reagent pack (troponin I No 3C29-66, Abbott Laboratories, Illinois, USA) and an automated assay system subject to external quality control (AxSYM System, Abbott Laboratories, Illinois, USA).

Standards and diluted plasma (100 microlitres) were combined with microparticles coated with murine monoclonal antibody to troponin I (anti-troponin I) at room temperature. The troponin I in the sample binds to the microparticles forming an antigen-antibody complex. An aliquot of the sample/microparticle mixture is transferred to a glass fibre matrix cell where the microparticles bind irreversibly. Conjugated goat anti-troponin antibody is then added, binding the troponin I of the antigen-antibody complex, forming an antibody-antigen-antibody complex. After incubation and washing with buffer solution, 4-Methylumbelliferyl phosphate is added and the fluorescent product formed measured by the optical assembly. The rate of the fluorescent product is directly proportional to the amount of troponin I in the investigational sample and is read from a standard calibration curve generated by analysis of six known troponin I concentrations.

Based on data from Abbott Laboratories and the 95th percentile values from population studies, the expected values for normal healthy individuals is <0.4ng/ml. In the study, values above 0.4ng/ml were considered to represent myocardial muscle damage.

2.4.5 Mortality Data

As with the ST elevation MI study, this study was not formally powered to assess relationship of the investigational variables to mortality, but the mortality data at six months obtained from The Common Services Agency, allows examination of trends. Cause of death was not ascertained.

2.4.6 Statistical Analysis

(a) Power Calculation

Dr Young assisted with a power calculation based on a pilot study of 15 patients with unstable angina – eight high risk, seven low risk (table 2.6). Recruitment of 52 patients would give a 90% power to detect a difference in plasma CITP between the groups of 0.95ng/ml assuming a common standard deviation of 1.5, using a two group t-test with a two sided significance test. With regard to PICP, 136 patients would be required to have a similar power to detect a 23.2ng/ml difference in means using the same analysis. Given the difficulty in recruiting such numbers, it was decided to recruit 52 patients for this study. Only 33 patients had

samples taken for TIMP-1 analysis, giving a 90% power to detect a difference between the groups of 91.4 ng/ml.

(b) Analysis of Results

Due to the lack of normality of distribution of the biochemical variables, log transformation was performed prior to parametric statistical analysis. All continuous variables are expressed as mean +/- one standard error of the mean. Repeated measurement of analysis of variance was employed for changes over time. Student's t test was used for comparison of means. A p value of <0.05 was considered significant.

Table 2.6

TIMP-1

Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
Group 1 mean	362	362	362	362
Group 2 mean	270.6	270.6	270.6	270.6
Difference in means	91.4	91.4	91.4	91.4
Common standard deviation	110	110	110	110
Effect size	0.831	0.831	0.831	0.831
Power (%)	80	90	95	99
Number required	24	32	39	55

CITP

Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
Group 1 mean	4.5	4.5	4.5	4.5
Group 2 mean	3.55	3.55	3.55	3.55
Difference in means	0.95	0.95	0.95	0.95
Common standard deviation	1.5	1.5	1.5	1.5
Effect size	0.633	0.633	0.633	0.633
Power (%)	80	90	95	99
Number required	41	52	66	93

PICP

Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
Group 1 mean	146.7	146.7	146.7	146.7
Group 2 mean	169.9	169.9	169.9	169.9
Difference in means	-23.2	-23.2	-23.2	-23.2
Common standard deviation	58.8	58.8	58.8	58.8
Effect size	0.395	0.395	0.395	0.395
Power (%)	80	90	95	99
Number required	102	136	168	238

Legend - Power calculation for CITP, PICP and TIMP-1 for non-ST elevation ACS study.

2.5 Percutaneous Transluminal Coronary Angioplasty Study (Chapter 7)

2.5.1 Study Population

Patients undergoing elective percutaneous transluminal coronary angioplasty (PTCA) at Glasgow Royal Infirmary were eligible for inclusion into this study which was performed from May 2000 till December 2001 (figure 2.1). Only those patients undergoing percutaneous coronary intervention to a single coronary artery were recruited to the study. Exclusion criteria included those listed in table 2.1 and patients in whom interpretation of ST/T segments of the 12 lead electrocardiogram would be difficult – those with permanent pacemaker systems, LBBB or concurrent digoxin therapy. All patients were ACE inhibitor naive. All studies were performed on a Phillips Integris H3000 system (Phillips, Rotterdam, Holland).

To correct for possible changes in biochemical markers seen due to either catheter manipulation within the vasculature and coronary ostia or to the effects of intra-coronary contrast injection, a cohort of patients undergoing elective diagnostic coronary angiography was recruited during the same time period as a control group.

2.5.2 Study Design

Study design and protocol was passed by The Local Research & Ethics Committee at Stobhill Hospital in September 1999. Written, informed consent was obtained

from all patients after the study had been explained verbally and by a study information sheet. A protocol of investigation is outlined in table 2.7.

Sequential blood samples were obtained at serial time points with the patient in the recumbent position. Patients undergoing PTCA had blood drawn at eight time points - prior to the procedure, 1 minute following first balloon inflation, at the end of the procedure, then 4, 8 and 16 hours post procedure. Due to the fact diagnostic coronary angiography is performed as a day case, the control group had samples taken on only three occasions – prior to angiogram, immediately at the end of the test and at 4 hours post procedure. A further 7mls of blood for assessment of troponin I was venesected at 16 hours following admission. All samples were collected in Lithium Heparin tubes and centrifuged at 3000rpm for 7 minutes. Plasma was distilled and decanted into crystal tubes and frozen at –70 °C until use.

Table 2.7

	PTCA			Angiography
	Bloods for plasma markers of collagen turnover	**Blood for troponin I**	**ECG**	**Bloods for plasma markers of collagen turnover**
Pre-procedure	Yes		Yes	Yes
During pain/ECG changes			Yes	
Post balloon inflation	Yes			
End of procedure	Yes			Yes
4hrs	Yes			Yes
8hrs	Yes			
16hrs	Yes	Yes		

Legend – Protocol of investigation for PTCA study.

2.5.3 Biochemical Analyses of Markers of Collagen Turnover

Plasma PICP and CITP were measured according to the methods outlined in sections 2.2.3b and 2.2.3c. TIMP-1 could not be measured due to problems with the TIMP assay as discussed in Chapter 3.

2.5.4 Assessment of Periprocedural Ischaemia

(a) Electrocardiography

All patients undergoing PTCA had 12 lead electrocardiograms performed prior to the procedure using a Pagewriter XLA (Hewlett Packard, USA). Continuous ECG monitoring was performed in the cardiac catheterisation laboratory and was analysed for ST segment fluctuation using a Siemens ECG monitoring system (Siemens, Stockholm, Sweden). Correlation with symptoms of chest pain was documented. Transient ST segment elevation or depression during balloon inflation was taken as electrocardiographic evidence of induced ischaemia.

(b) Troponin I

In 2001, a standardised assay for troponin I was introduced across all sites in North Glasgow University NHS Trust. Quantification of plasma troponin I in the PTCA study was performed using this new assay and the method is therefore different from that outlined in section 2.3.4(b). This assay, ADVIA Centaur cTnI (Bayer Corporation, New York, USA), is automated and used in routine clinical practice. It is subject to external quality control. It is a two-site sandwich immunoassay using direct chemiluminometric technology.

An aliquot of 100 microlitres of investigational agent is mixed with 100 microlitres of reagent containing polyclonal goat antibody to troponin I, labelled with acridinium ester. Following incubation at 37°C for 2.5minutes, the second

antibody is added (monoclonal murine anti-troponin I). Acid and base reagents are added initiating chemiluminescent reaction. A photometric system is employed to assess the amount of relative light units (RLUs), the amount of which is directly proportional to the amount of troponin I in the investigational sample. Calibration is carried out on a regular basis using known controlled concentrations of troponin I.

Based on data supplied the company, the minimum detectable concentration is 0.01ng/ml. The between run standard deviation was 0.02ng/ml on average with a 95% confidence interval of 0.07ng/ml. The 99th percentile for normal volunteers is 0.07ng/ml and levels above this in the study were considered positive for myocardial damage.

2.5.5 Statistical Analysis

(a) Power calculation

The main outcome for this study was change in plasma markers of collagen turnover following controlled occlusion of a coronary artery during balloon inflation. Therefore, power calculation was performed using the mean values in a normal population as a baseline, but using standard deviations observed in the pilot study from the non ST elevation acute coronary syndrome population. We felt it more appropriate to use the standard deviation from this group rather than that of a normal population as we hypothesised that these patients would behave more like the acute coronary syndrome group due to the ischaemic stimulus of

balloon inflation. Recruitment of 14 patients undergoing elective PTCA would give a 90% power to detect a rise in CITP from baseline of 25% and a 90% power to detect a 50% rise in PICP, assuming a standard deviation of 1.5 and 58.8 respectively, using a two group t-test with a 0.05 two sided significance test (table 2.8).

Table 2.8

CITP

	10%	**25%**	**50%**	**75%**
Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
First condition mean	3.2	3.2	3.2	3.2
Second condition mean	3.52	4.0	4.8	5.6
Mean difference	-0.32	-1.2	-1.6	-2.4
Standard deviation of differences	1.5	1.5	1.5	1.5
Effect size	0.21	0.8	1.1	1.6
Power (%)	90	90	90	90
N	88	14	6	4

PICP

	10%	**25%**	**50%**	**75%**
Test significance level	0.050	0.050	0.050	0.050
1 or 2 sided test?	2	2	2	2
First condition mean	193	193	193	193
Second condition mean	212.3	241.2	289.5	337.8
Mean difference	-19.3	-48.2	-96.5	-142.8
Standard deviation of differences	58.8	58.8	58.8	58.8
Effect size	0.33	0.82	1.64	2.43
Power (%)	90	90	90	90
N	165	69	13	3

Legend - Power calculation for CITP and PICP for PTCA study.

(b) Analysis of Results

All continuous variables are expressed as mean +/- one standard error of the mean. Log transformation allowed parametric testing. Results are expressed as absolute values and percentage change from baseline. Repeated measurement of analysis of variance was performed to detect a change of variables over time and, if significant, Student's t test was used for direct comparison of means between the two groups. A p value of <0.05 was considered significant.

CHAPTER THREE – VALIDATION OF BIOCHEMICAL ASSAYS OF COLLAGEN TURNOVER

3.1 Introduction

As discussed in chapter one, plasma markers of collagen turnover may be useful in the monitoring of the remodelling process following ischaemic events. Type I fibrillar collagen is the predominant collagen type within the heart and therefore, markers of synthesis and degradation of this collagen type were used in all of my studies (Frank and Langer, 1974; Weber KT, 1989). C-propeptide for type I collagen (PICP) is cleaved from pro-collagen allowing integration of the collagen helix into the growing fibril and is therefore a marker of synthesis (Melkko JT et al. 1990; Nimni ME, 1993). Similarly, C-telopeptide for type I collagen (CITP) is cleaved during collagen breakdown and is an indicator of degradation (Risteli J et al. 1993; Risteli J and Risteli I., 1995b). Tissue Inhibitor of Matrix Metalloproteinase-I (TIMP-I) is a marker of inhibition of collagen degradation due to its inhibitory action on the powerful family of proteolytic enzymes, the matrix metalloproteinases (Matrisian LM, 1990).

The radioimmunoassays for PICP and CITP have been used in the setting of metabolic and metastatic bone disease for more than 10 years, mainly at an experimental level (Azria, 1989; Charles et al. 1994; Hosoya et al. 1997). However, only limited data are available in other populations. In addition, given the longitudinal nature of my studies, repeated measurements of plasma PICP and CITP are required. It is therefore essential that the assay methods are reliable and

robust with accurate reproducibility and good quality control. Similar characteristics are necessary for the ELISA assay for plasma TIMP-1. Indeed, few studies using plasma TIMP-1 in any population exist and subsequently, less information on quality control and reproducibility is known regarding this assay.

We sought to determine the intra and inter assay variablities of the three assays to assess whether the quality control would be adequate for repeated measurement for each test in a longitudinal study. Additionally, we aimed to characterise plasma levels of TIMP-1, PICP and CITP in a normal population drawn from the surrounding district of a similar age and sex distribution to the study populations. From this, a normal range and mean can be calculated for a local normal population. Finally, we wished to examine the within individual variability of plasma level of all three markers, to exclude variation of a diurnal nature. All analyses were performed on plasma samples rather than on serum due to the increased accuracy and reliability of measurement in plasma (Lein et al. 1997; Jung, 1997).

However following the replacement of the antibody to TIMP-1 in assay RPN2611 by Amersham in December 1999, further studies were required for validation of the new Amersham TIMP-1 assay. Although the company provided data showing apparent similar curve shape and sensitivity and no change to assay recovery or lincarity, absolute values for TIMP-1 were several times higher with the new assay. Amersham stated a correlation between the assays of 0.88 on 16 samples, of which only four were from samples collected in Lithium Heparin tubes. The company provided a correction factor from a regression equation to allow

comparison between the assays. We therefore felt that additional experiments for quality control purposes were necessary. To this end, we wished to examine variability between the two assays for plasma TIMP-1. However, given the concerns regarding the apparent discrepancies between the two kits, alternative assays for TIMP-1 were considered. The original kit from Amersham was based on an assay developed by Professor Tim Cawston's group in Newcastle. He had the original cell line and supplied us with the same monoclonal antibody to TIMP-1 used in the kit from Amersham. We therefore decided to compare the new assay from Newcastle with the original Amersham kit.

3.2 Methods

3.2.1 Assay Variability

Samples were randomly selected from a mixed patient population for the assessment of intra-assay and inter-assay variability. This included samples drawn from patients with hypertension, post myocardial infarction and liver disease in addition to samples from a normal population to allow assessment of variability across a wide range of absolute values for each of plasma TIMP-1, PICP and CITP. Assay methods for the three substances are outlined in Chapter 2, section 2.2.3 (a), (b) and (c).

Intra-assay variability was performed by repeated measurement of samples using the same assay kit. 30 samples were used for plasma TIMP-1, 20 each for PICP and CITP. Inter-assay variability was performed by measurement of samples on

two separate occasions using different assay kits. 15 samples were used for TIMP-1, 20 for PICP and CITP. A mean of the two values was calculated for each sample and differences expressed as absolute and percentage differences of the mean. Results for PICP and TIMP-1 were not normally distributed, so were log transformed prior to statistical comparison between groups using Student's t test. Correlation co-efficient was also calculated. A p value of <0.05 was considered significant. Data are presented in the non-logarithmic format.

The two kits from Amersham were compared by measuring plasma TIMP-1 on 20 randomly selected plasma samples from a mixed population. The Newcastle assay for TIMP-1 was compared with the old Amersham kit on 40 samples across a range of patient groups and a range of levels. The methodology for the Newcastle assay for TIMP-1 is identical to that outlined in Chapter 2, section 2.1.3(a) with one exception. In the Amersham assay, horseradish peroxidase is directly bound to the second antibody, but in the Newcastle assay, the peroxidase is bound to streptavadin and is added to biotin bound second antibody. There is therefore one additional step in this method.

3.2.2 Normal Ranges

A local newspaper, the Kirkintilloch Herald, ran an article on cardiac research at Stobhill Hospital in early 1999 and repeated in early 2001 calling for help with ongoing studies from healthy volunteers in the surrounding area. The response from the public was excellent and 20 volunteers agreed to have blood venesected for assay of TIMP-1, PICP and CITP. These volunteers had no past medical

history of note and were not on any cardiac medication. They were of a similar age and sex distribution to the ST elevation MI study to reduce the influence of age and sex when comparing results.

3.2.3 Intra-individual Variability

The variability of plasma level of PICP and CITP within healthy individuals was examined by performing assays on blood drawn on two separate occasions. Ten people volunteered for this study from a mixture of staff from the Biochemistry and Cardiology Departments and members of the public. A mean of the two values was calculated for each sample and differences expressed as absolute and percentage differences of this mean. Statistical comparison was performed using Student's t-test following log transformation, and correlation co-efficient calculated.

3.3 Results

3.3.1 Assay Variability

Absolute and percentage differences between results were calculated for both the within and between assay studies and results expressed as percentage difference from mean with 95% confidence intervals. Each result is also shown in graphical form in a scatter plot for correlation and in the form of a Bland-Altman graph.

Intra-assay variability for plasma TIMP-1 was 4% (95% CI 3.23 – 4.87) with a correlation co-efficient of 0.99. Raw data are shown in table 3.1 and correlation demonstrated in figure 3.1 (a & b). Inter-assay variability was 4.4% (95% CI 3.36 – 5.46) with a correlation co-efficient of 0.99 and is demonstrated in figure 3.2 (a & b). Absolute values shown in table 3.2. Linearity was demonstrated to 900 ng/ml. No statistically significant differences in means were noted using Student's t-test.

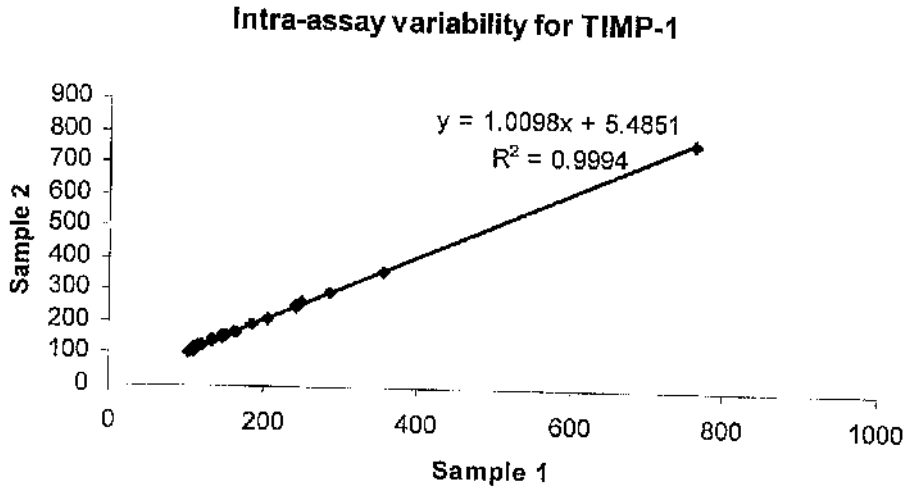
Table 3.1

TIMP-1 sample1	TIMP-1 sample2	Average	absolute difference	% difference
213	205	209	8	3.8
118	125	121	7	5.8
146	152	149	6	4.0
108	115	111.5	7	6.3
241	256	248	15	6.0
121	126	123.5	5	4.0
182	183	182.5	1	0.5
162	168	165	6	3.6
129	117	123	12	9.8
110	118	114	8	7.0
152	158	155	6	3.9
102	103	102.5	1	1.0
286	296	291	10	3.4
126	121	123.5	5	4.0
364	356	360	8	2.2
764	774	769	10	1.3
243	250	246.5	7	2.8
114	122	118	8	6.8
186	194	190	8	4.2
110	110	110	0	0.0
165	170	167.5	5	3.0
250	263	256.5	13	5.1
158	147	152.5	11	7.2
133	139	136	6	4.4
107	111	109	4	3.7
111	117	114	6	5.3
135	146	140.5	11	7.8
102	104	103	2	1.9
248	257	252.5	9	3.6
146	153	149.5	7	4.7
Mean			**7.3ng/ml**	**4%**

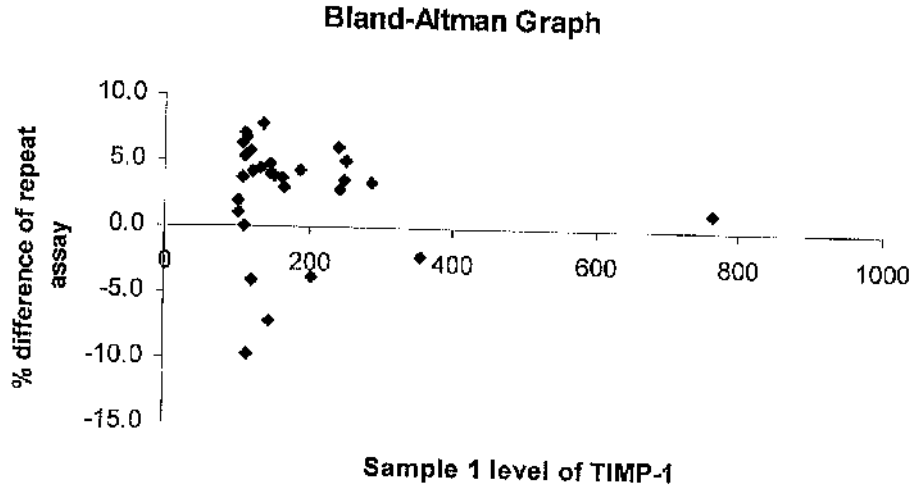
Legend - Intra-assay variability for plasma TIMP-1. Absolute and percentage differences between values are also shown.

Figure 3.1

(a)



(b)



Legend - Intra-assay variability for plasma TIMP-1 in (a) scatterplot and (b) Bland-Altman form.

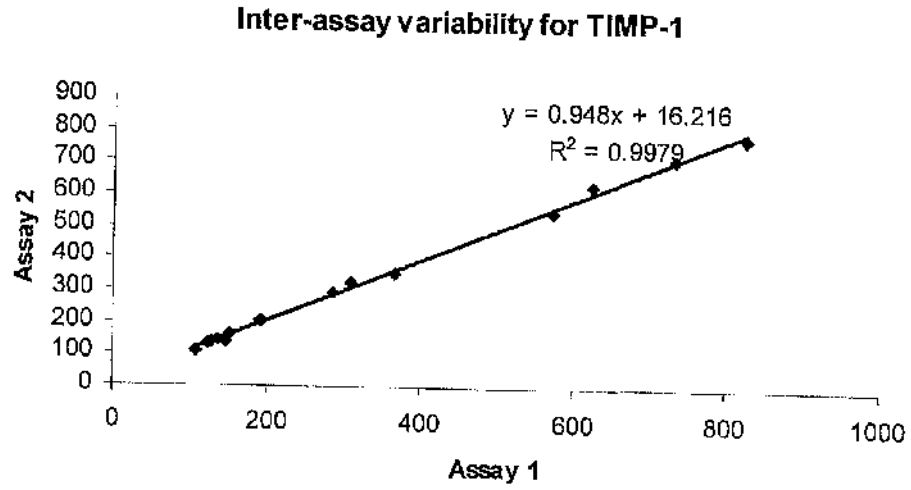
Table 3.2

TIMP-1 Assay1	TIMP-1 Assay2	Average	Absolute difference	% difference
731	714	722.5	17	2.4
623	629	626	6	1.0
572	547	559.5	25	4.5
826	783	804.5	43	5.3
153	165	159	12	7.5
191	203	197	12	6.1
128	136	132	8	6.1
107	109	108	2	1.9
194	202	198	8	4.0
136	145	140.5	9	6.4
123	131	127	8	6.3
146	141	143.5	5	3.5
367	356	361.5	11	3.0
308	323	315.5	15	4.8
Mean			12.7ng/ml	4.4%

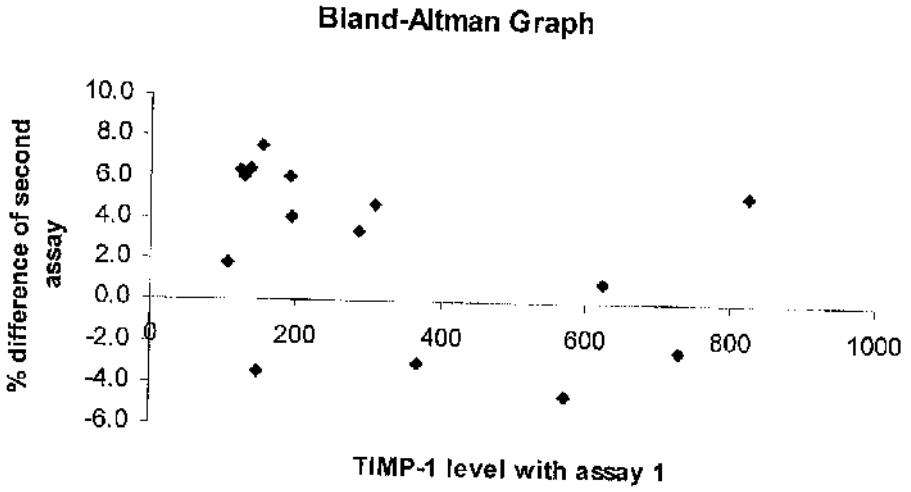
Legend - Inter-assay variability for plasma TIMP-1. Absolute and percentage differences between values are also shown.

Figure 3.2

(a)



(b)



Legend - Inter-assay variability for plasma TIMP-1 in (a) scatterplot and (b) Bland-Altman form.

With regard to PICP, we demonstrated linearity to 300 nanogrammes/ml with intra and inter assay variabilities of 3.1% (95% CI 1.9 – 4.38) and 6.9% (95% CI 4.82 – 9.14) respectively. The lower limit of detection was 12ng/ml. The correlation co-efficient was 0.99 for intra-assay variability (table 3.3 & figure 3.3 (a & b)). Correlation co-efficient was 0.94 for inter-assay variability and is demonstrated in figure 3.4 (a & b) with absolute values shown in table 3.4. There were no statistically significant differences noted using students t-test analysis of the means.

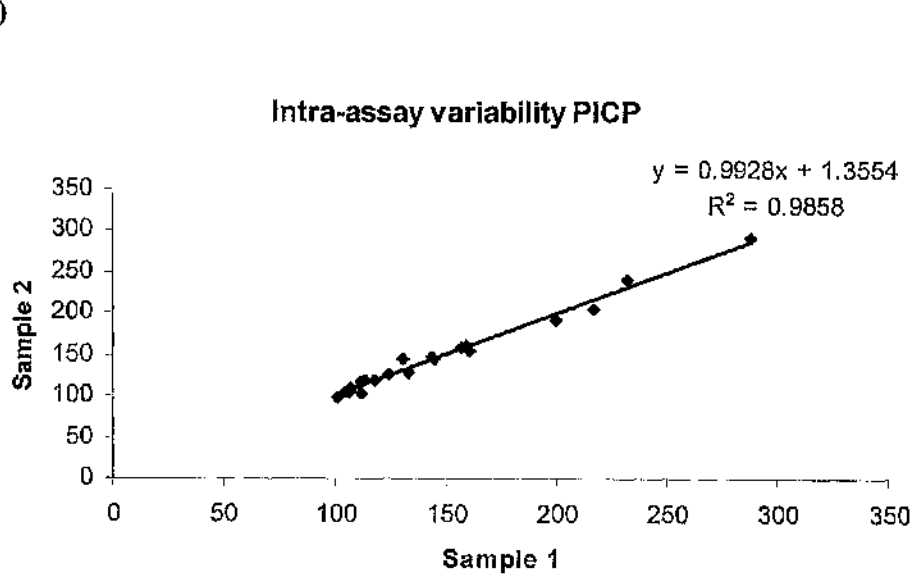
Table 3.3

sample 1	sample 2	Average	absolute difference	% difference
124	127	125.5	3.0	2.4
104	104	104.0	0.0	0.0
112	116	114.0	4.0	3.5
157	159	158.0	2.0	1.3
113	118	115.5	5.0	4.3
112	103	107.5	9.0	8.4
200	191	195.5	9.0	4.6
143	148	145.5	5.0	3.4
217	206	211.5	11.0	5.2
145	144	144.5	1.0	0.7
101	99	100.0	2.0	2.0
288	291	299.0	3.0	0.7
108	110	109.0	2.0	1.8
118	119	118.5	1.0	0.8
130	144	137.0	14.0	10.2
106	105	105.7	1	0.9
161	155	158.0	6.0	3.8
159	162	160.5	3.0	1.9
232	240	236.0	8.0	3.4
133	127	130.0	6.0	4.6
Mean			4.6ng/ml	3.1%

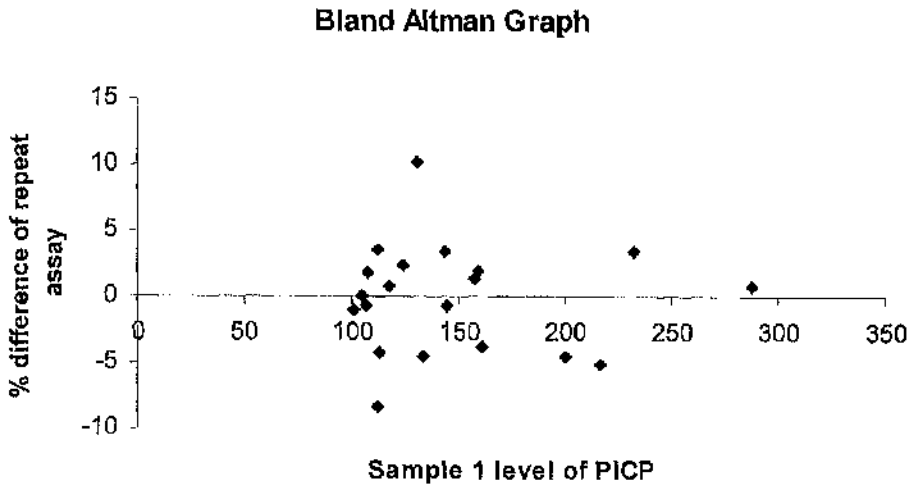
Legend - Intra-assay variability for plasma PICP comparing sample one and two. Absolute and percentage differences between values are also shown.

Figure 3.3

(a)



(b)



Legend - Intra-assay variability for plasma PICP in (a) scatterplot and (b) Bland-Altman form.

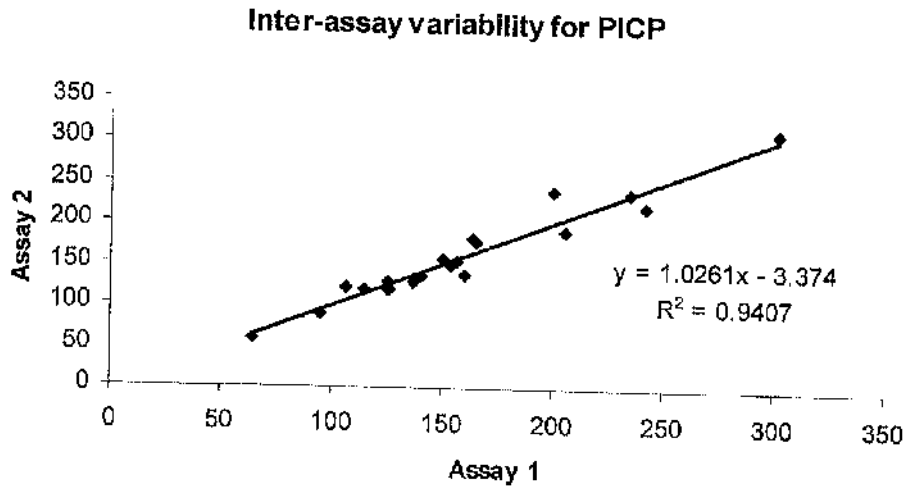
Table 3.4

PICP Assay 1	PICP Assay 2	Average	Absolute difference	% difference
160	138	149	22	14.8
242	222	232	20	8.6
163	183	173	20	11.6
165	178	171.5	13	7.6
205	192	198.5	13	6.5
64	59	61.5	5	8.1
156	154	155	2	1.3
235	237	236	1	0.5
125	128	126.5	3	2.4
137	128	132.5	9	6.8
95	88	91.5	7	7.7
106	122	114	16	14
140	135	137.5	5	3.8
200	240	220	40	18
150	157	153.5	7	4.6
302	312	307	10	3.3
114	120	117	6	5.1
154	149	151.5	5	3.3
125	118	121.5	7	5.8
126	119	122.5	7	5.7
Mean			**10.9ng/ml**	**6.9%**

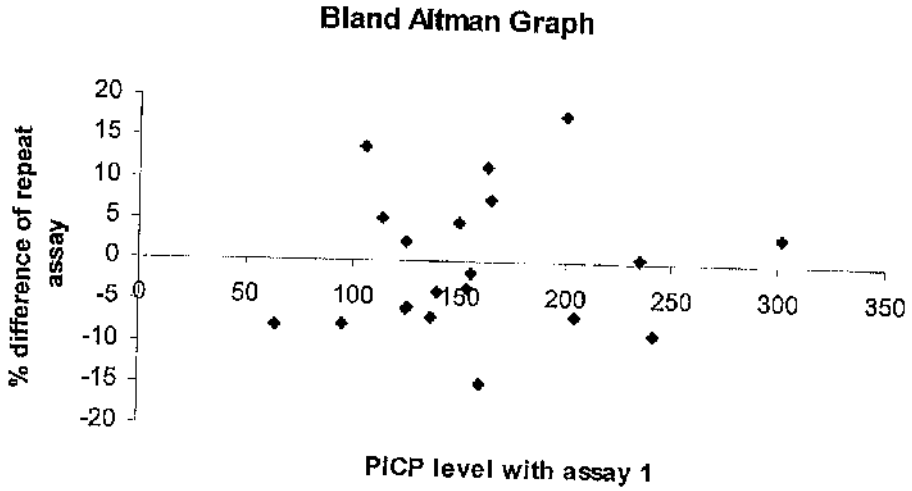
Legend - Inter-assay variability for plasma PICP comparing assay one and two. Absolute and percentage differences between values are also shown.

Figure 3.4

(a)



(b)



Legend - Inter-assay variability for plasma PICP in (a) scatterplot and (b) Bland-Altman form.

Intra and inter assay variabilities for plasma CITP were 3.7% (95% CI 2 – 5.4) and 6.4% (95% CI 4.5 – 8.3) respectively. Correlation co-efficients were 0.98 and 0.99. Intra-assay variability is demonstrated in figure 3.5 (a & b) with raw data in table 3.5. Figure 3.6 and table 3.6 show data for inter-assay variability. Again, no statistically significant differences were demonstrated using students t-test analysis of the means.

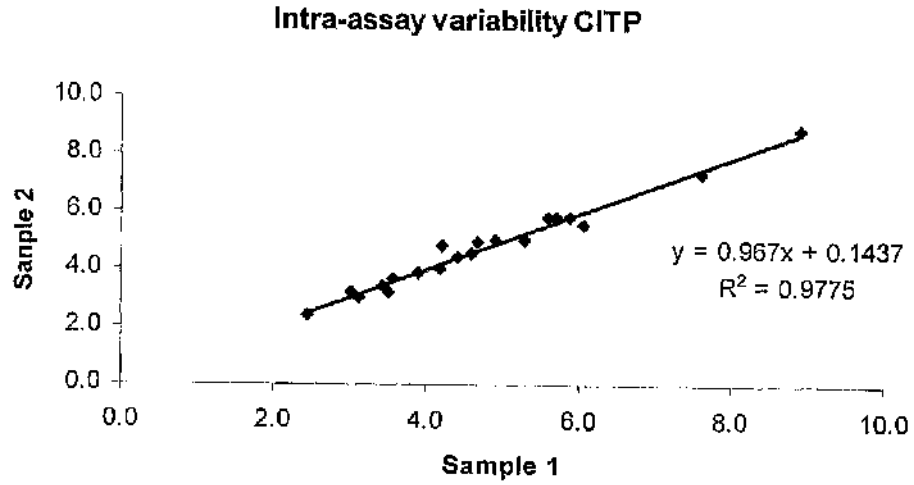
Table 3.5

CITP sample 1	CITP sample2	Average	absolute difference	% difference
5.7	5.8	5.75	0.1	1.7
5.6	5.8	5.70	0.1	1.8
3.4	3.4	3.40	0.0	0.0
6.1	5.6	5.85	0.5	8.5
8.9	8.9	8.90	0.0	0.0
3.9	3.8	3.85	0.1	2.6
4.7	4.9	4.80	0.2	4.2
3.6	3.7	3.65	0.1	3.5
4.2	4.8	4.50	0.6	13.2
4.2	4.0	4.10	0.2	4.2
2.4	2.4	2.40	0.0	0.0
4.9	5.0	4.95	0.1	2.0
3.5	3.2	3.35	0.3	9.5
7.6	7.3	6.45	0.3	4.3
3.1	3.0	3.05	0.1	3.2
4.6	4.6	4.60	0.0	0.0
4.4	4.4	4.40	0.0	0.0
5.3	5.0	5.15	0.3	5.2
5.9	5.8	5.85	0.1	1.4
Mean			0.17ng/ml	3.7%

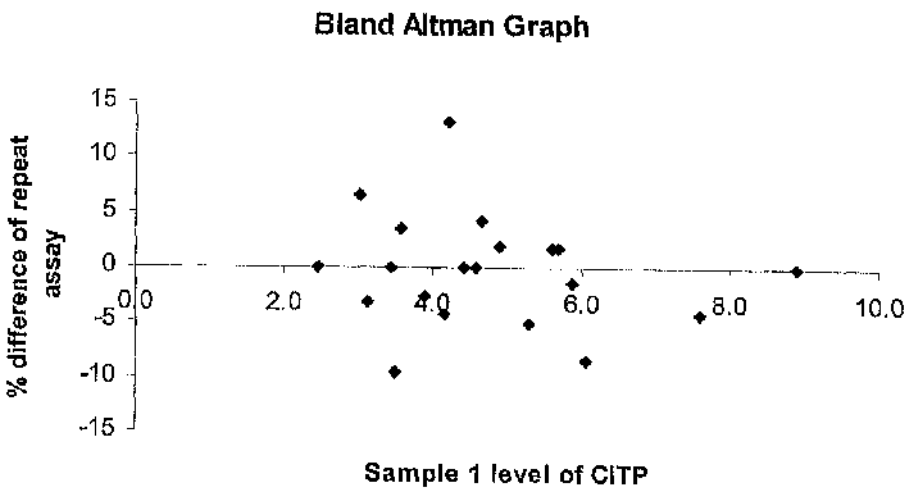
Legend - Intra-assay variability for plasma CITP comparing sample one and two. Absolute and percentage differences between values are also shown.

Figure 3.5

(a)



(b)



Legend - Intra-assay variability for plasma CITP in (a) scatterplot and (b) Bland-Altman form.

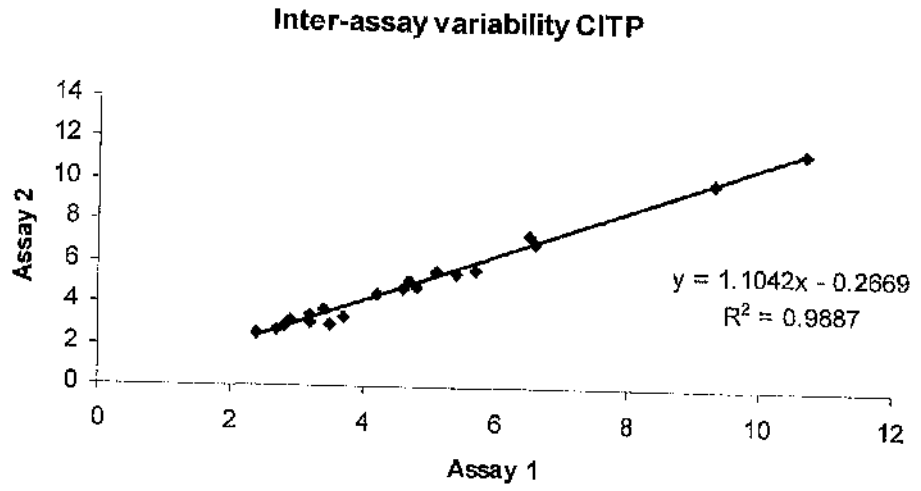
Table 3.6

CITP Assay1	CITP Assay2	Average	Absolute difference	% difference
2.8	2.9	2.85	0.1	3.5
3.4	3.7	3.55	0.3	8.5
3.5	3	3.25	0.5	15.4
3.7	3.4	3.55	0.3	8.5
2.7	2.7	2.7	0	0
5.1	5.6	5.35	0.5	9
4.8	4.9	4.85	0.1	2.1
6.5	7.4	6.95	0.9	12.9
2.9	3.2	3.05	0.3	9.8
3.2	3.5	3.35	0.3	9
9.3	10	9.65	0.7	7.25
10.7	11.5	11.1	0.8	7.2
4.2	4.5	4.35	0.3	6.9
6.6	7	6.8	0.4	5.9
5.4	5.5	5.45	0.1	1.8
2.4	2.5	2.45	0.1	4.1
3.2	3.1	3.15	0.1	3.2
4.7	5.1	4.9	0.4	8.2
4.6	4.8	4.7	0.2	4.3
Mean			0.32 ng/ml	6.4%

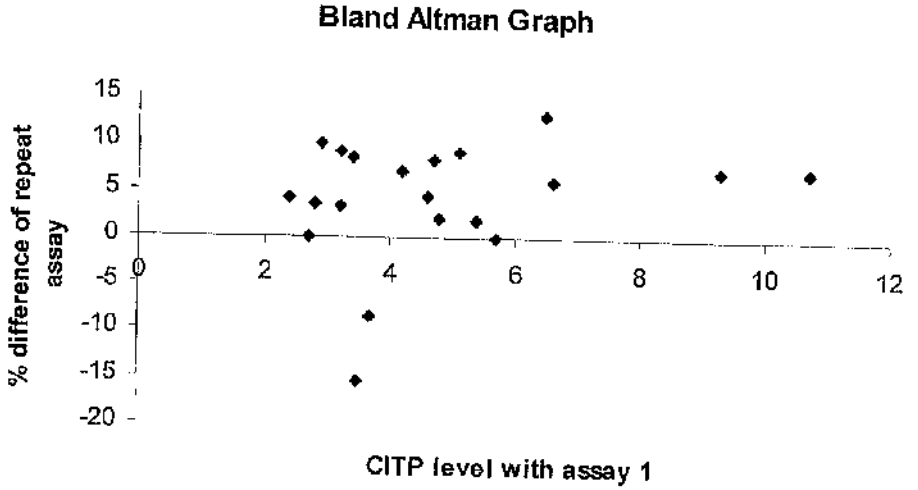
Legend - Inter-assay variability for plasma CITP comparing assay one and two. Absolute and percentage differences between values are also shown.

Figure 3.6

(a)



(b)



Legend - Inter-assay variability for plasma CITP in (a) scatterplot and (b) Bland-Altman form.

There was no significant correlation between the two Amersham assays for TIMP-1 (original and revised). We confirmed that absolute values were much higher with the revised assay with a large within assay variability. With regard to the Newcastle assay, there was a correlation between it and the original Amersham assay with a correlation coefficient of 0.77. Although some values were very similar using the two kits, the two assays varied by as much as 125% in some patients. Correlation and variability are demonstrated in figure 3.7 a & b respectively with raw data in table 3.7. The differences were significant using students t-test analysis, $p<0.0001$. Therefore, only the Amersham assay was used in any of the studies.

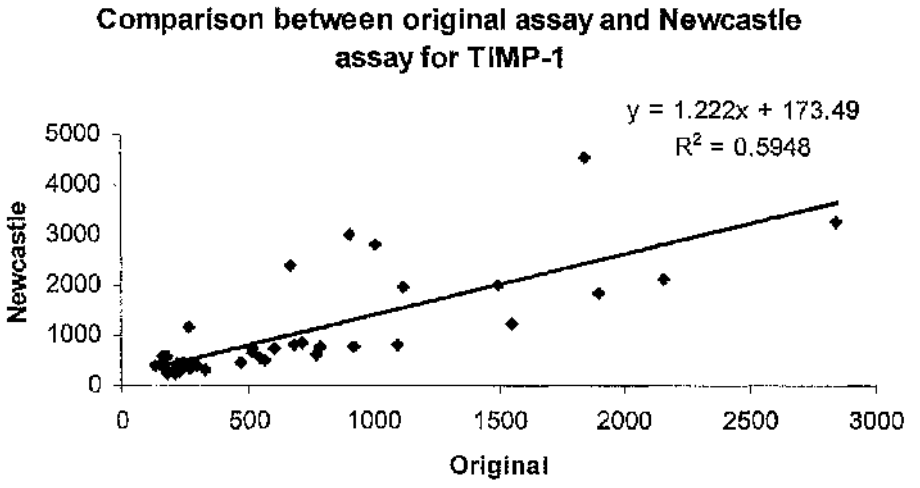
Table 3.7

Diagnostic group	Amersham	Newcastle	Absolute difference	% difference
Hypertension	266	410	144	42.6
Hypertension	160	410	250	87.7
Hypertension	176	310	134	55.1
Hypertension	165	580	415	111.4
Hypertension	265	1150	885	125.1
MI	2160	2100	-60	-2.8
MI	1894	1840	-54	-2.9
MI	475	460	-15	-3.2
MI	688	800	112	15.1
MI	714	860	146	18.5
MI	549	550	1	0.2
MI	787	780	-7	-0.9
MI	924	760	-164	-19.5
MI	1092	790	-302	-32.1
MI	1841	4548	2707	120.3
MI	522	740	218	34.5
MI	2841	3260	419	13.7
MI	1117	1980	863	55.7
MI	517	650	133	22.8
MI	1497	2010	513	29.2
MI	1007	2820	1813	94.7
MI	903	3010	2107	107.7
MI	564	500	-64	-12
MI	1548	1220	-328	-23.7
MI	774	610	-164	-23.7
MI	223	430	207	63.4
NORMAL	300	370	70	20.9
NORMAL	225	260	35	14.4
NORMAL	130	400	270	101.9
NORMAL	603	750	147	21.7
NORMAL	242	470	228	64
NORMAL	334	300	-34	-10.7
NORMAL	210	290	80	32
NORMAL	210	230	20	9.1
NORMAL	180	250	70	32.6
NORMAL	237	300	63	23.5
NORMAL	272	340	68	22.2
NORMAL	268	340	72	23.7
NORMAL	666	2370	1704	95
NORMAL	178	570	392	104.9
Mean			**386ng/ml**	**42.4%**

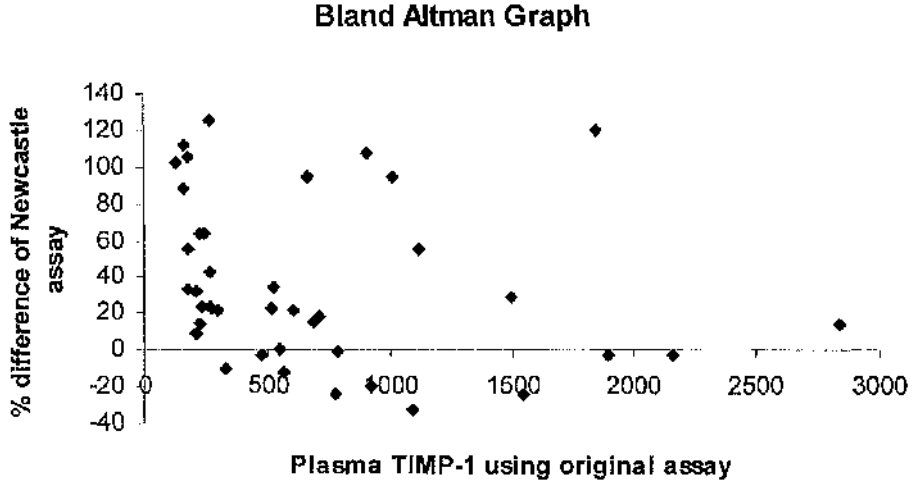
Legend - Comparison of Amersham and Newcastle assays for plasma TIMP-1 with absolute and percentage differences illustrated.

Figure 3.7

(a)



(b)



Legend – Variability between Amersham and Newcastle assay for plasma TIMP-1 in (a) scatterplot and (b) Bland Altman form.

3.3.2 Normal Ranges

20 volunteers donated blood for assay of plasma TIMP-1, PICP and CITP. Results are expressed as mean for the group +/- 1 standard deviation with 95% confidence intervals. The group consisted of 15 men and 5 women with a mean age of 59 years (range 36-86). All three assay results were normally distributed. Mean plasma TIMP-1 in this cohort was 202 ng/ml +/- 22 (95% CI 189 – 215) with a range from 164 to 267ng/ml. Normal range for plasma PICP was 80 to 207 ng/ml with a mean of 129 +/- 32 (95% CI 114 – 143). Mean plasma CITP was 2.6ng/ml +/- 0.3 (95% CI 2.4 – 2.8) with a range from 1.9 – 3.9. These values are expressed in tabular form in table 3.8.

Table 3.8

	Mean	SD	95% CI	Minimum	Maximum
TIMP-1	202	22	189 – 215	164	267
PICP	129	32	114 – 143	80	207
CITP	2.6	0.3	2.4 – 2.8	1.9	3.9

Legend - Normal mean with standard deviation and range of plasma TIMP-1, PICP and CITP in a group of 20 healthy volunteers.

3.3.3 Intra-individual Variability

10 people volunteered to have blood sampled on two occasions at different times of the day. Again results are expressed as percentage difference from mean with 95% confidence intervals. The within individual variability for plasma PICP was 6.1% (95% CI 3.9 – 8.3) with a correlation coefficient of 0.95. This is demonstrated in table 3.9 and figure 3.8 (a & b). The mean intra-individual variability for plasma CITP was 4.8% (95% CI 2.2 – 7.4) with a correlation coefficient of 0.98 (see table 3.10 and figure 3.9 (a & b)). The differences in mean values for the two samples were not statistically significant. Intra-individual assay was not performed for TIMP-1 as these studies were performed after the discontinuation of the Amersham assay.

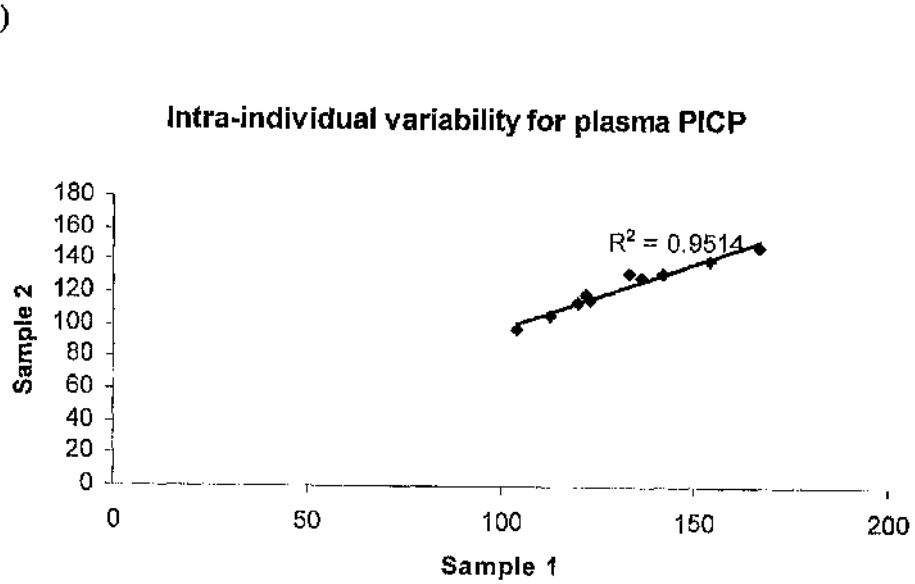
Table 3.9

Sample 1	Sample 2	Mean	Absolute difference	% difference
132	133	132.5	1	0.8
142	132	137	10	7.3
113	106	109.5	7	6.4
123	115	119	8	6.7
154	140	147	14	9.5
120	114	117	6	5.1
104	97	100.5	7	7.0
119	122	120.5	3	2.5
167	149	158	18	11.4
136	130	133	6	4.5
Mean			8ng/ml	6.1%

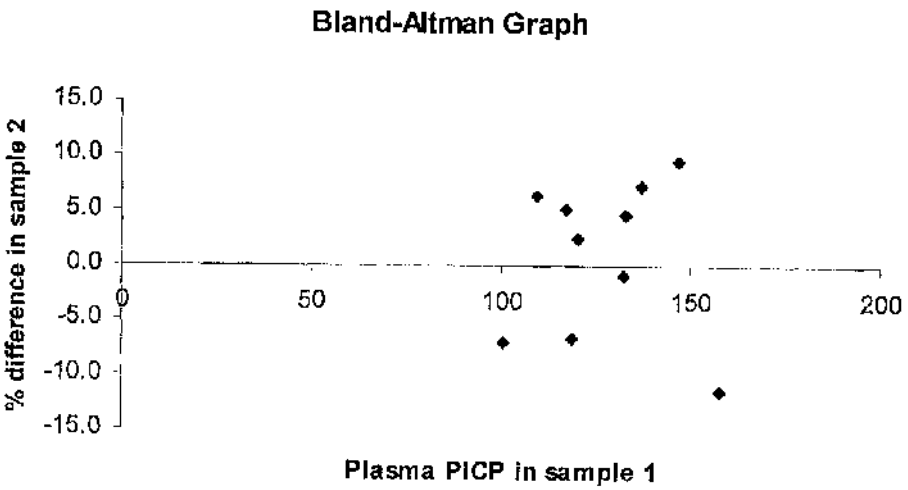
Legend - Within individual variability of plasma PICP from blood drawn on two separate occasions.

Figure 3.8

(a)



(b)



Legend - Intra-individual variability for plasma PICP in (a) scatterplot and (b) Bland-Altman form.

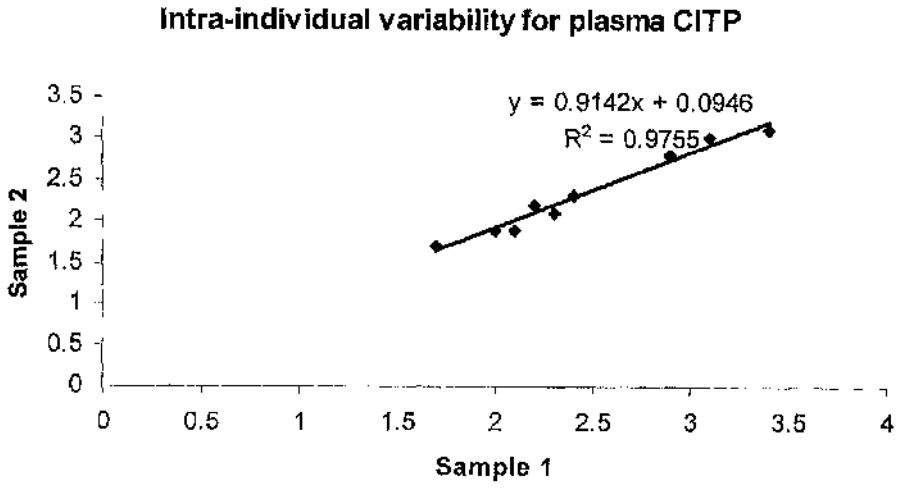
Table 3.10

Sample 1	Sample 2	Mean	Absolute difference	% difference
2.9	2.8	2.85	0.1	3.5
3.1	3	3.05	0.1	3.3
2.2	2.2	2.2	0	0.0
2.1	1.9	2	0.2	10.0
2	1.9	1.95	0.1	5.1
3.4	3.1	3.25	0.3	9.2
2.3	2.1	2.2	0.2	9.1
1.7	1.7	1.7	0	0.0
2.9	2.8	2.85	0.1	3.5
2.4	2.3	2.35	0.1	4.3
Mean			0.12ng/ml	4.8%

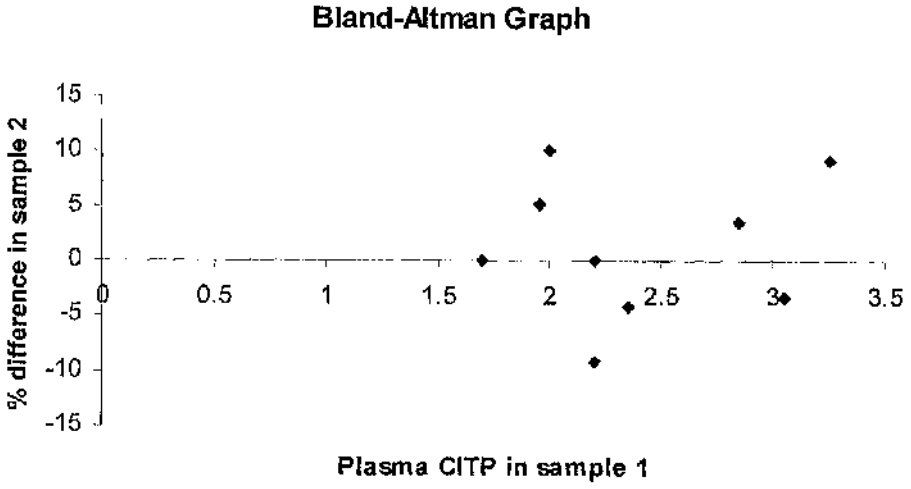
Legend - Within individual variability of plasma CITP from blood drawn on two separate occasions.

Figure 3.9

(a)



(b)



Legend - Intra-individual variability for plasma CITP in (a) scatterplot and (b) Bland-Altman form.

3.4 Discussion

We have demonstrated good quality control for assays of plasma PICP, CITP and TIMP-1 (Amersham assay) with intra-assay variabilities of less than 4% and inter-assay variabilities of <7%. Linearity across a large range of absolute values has also been demonstrated. The tight within assay variability allows comparison of absolute values and the good between assay variability allows accurate measurement of repeated values necessary for a longitudinal study.

However, regarding the assay for TIMP-1, there was great disparity between the original Amersham kit and the revised kit from Amersham. The differences noted may be due to different binding properties of the antibodies involved and their differing affinity for unbound or bound TIMP-1. Indeed the company provided data showing that the revised kit recognised TIMP-1 complexed with MMP-1, a feature not present in the original assay. In addition, the assays may have different cross reactivity with other TIMPs or TIMP-MMP complexes. The variation seen between the two assays was not limited to any one patient group and no reason for this became apparent. The differing results between the original Amersham kit and the assay based on the antibody from Newcastle are a little more difficult to explain. The antibodies were from the same cell line and the assay methodology the same except for the additional step of streptavadin bound peroxidase and biotin bound second antibody. The disparity between measured plasma TIMP-1 between the two assays may be due to alterations in binding to the second antibody due to the additional steps in the Newcastle based assay due to the interference of streptavadin or biotin. However, one would expect this result to be consistent and

predictable, yet there were wide variations absolute and percentage differences between the assays. This was not limited to one patient group and was demonstrated across a wide range of absolute values. Alternatively, it may have been due to an, as yet unknown, matrix effect. Whatever the explanation, only good quality control and reproducibility was demonstrated with the original Amersham kit and it was therefore decided that only those samples measured with this assay would be used in subsequent studies.

We have also established normal ranges for plasma levels of TIMP-1, PICP and CITP in a normal population to allow comparisons with experimental populations. These ranges are small with a small standard error of the mean, allowing use of the mean for the purposes of comparison to the mean of other populations.

Finally, we have shown no significant inter-individual variability for plasma levels of TIMP-1, PICP and CITP, excluding an effect of diurnal variation. This is important given the longitudinal nature of subsequent studies where samples are taken at different times.

3.5 Conclusions

I have demonstrated good quality control for the above three assays with tight intra and inter assay variabilities. I have also established normal ranges and excluded diurnal variation.

CHAPTER FOUR – TIME COURSE OF PLASMA MARKERS OF COLLAGEN TURNOVER FOLLOWING ACUTE MYOCARDIAL INFARCTION

4.1 Introduction

Ventricular remodelling following myocardial infarction is a powerful adverse prognostic indicator (White HD et al. 1987; Pfeffer MA and Braunwald E, 1990; Mahon et al. 1999), and changes to collagen content and structure within the extracellular matrix are important in its development (Whittaker P et al. 1991). Biochemical assessment of myocyte necrosis, by the measurement of serum Troponin or standard cardiac enzymes, is routinely performed following myocardial infarction, but to date there has been no similar focus on biochemical assessment of collagen turnover.

Type I collagen is the principal collagen type within the heart, with lesser amounts of types III and IV (Frank and Langer, 1974; Weber KT, 1989), and its metabolism can be assessed biochemically (Risteli L and Risteli J, 1990; Risteli J and Risteli L, 1995b). Procollagen type I carboxy-terminal propeptide (PICP) is cleaved in a 1:1 stoichiometric fashion from procollagen during the formation of Type I collagen and is therefore a marker of collagen synthesis (Melkko JT et al. 1990). Similarly, C-telopeptide for type I collagen (CITP) is released by endopeptidase cleavage by matrix metalloproteinases during collagen degradation and is a marker of breakdown (Risteli J et al. 1995a). Clearly, MMP activity must be tightly regulated and they are specifically inhibited by a family of naturally

occurring glycoproteins – the tissue inhibitors of metalloproteinases (TIMPs), which act by binding irreversibly to active MMPs, effectively halting protcolytic activity (Matrisian LM, 1990; Woessner JF, 1999; Woessner JF, 1999).

Previous studies have demonstrated increased levels of serum procollagen type III amino-terminal propeptide (PIIINP), a marker of collagen type III synthesis, following myocardial infarction peaking at day 7 and remaining elevated for many months, and that peak levels correlate positively with infarct size and inversely with survival (Jensen LT et al. 1990; Host NB et al. 1995). The prognostic significance of raised PIIINP following AMI has been confirmed in several studies, where both early and late elevation has been shown to correlate with indices of cardiac dysfunction, the development of heart failure and reduced survival (Uusimaa P et al. 1997; Modena et al. 1998; Poulsen SH et al. 2000). However, it is important to remember that net collagen content is a product of a dynamic equilibrium between collagen synthesis and degradation and that alterations to both processes may be important in the remodelling process.

It is also important to remember that it is type I collagen, rather than type III, which represents the most abundant collagen type within the heart (Medugorac and Jacob, 1983; Wei et al. 1999), and there are only limited data available on the time course of alterations in serum levels of markers of synthesis of type I collagen following myocardial infarction (Uusimaa P et al. 1997; Poulsen SH et al. 2001). Similarly, information regarding serological testing of collagen degradation is limited to one small study in 13 patients (Murakami T et al. 1998). We therefore sought to characterise the time course of changes in plasma markers

of turnover of type I collagen following myocardial infarction in a larger cohort of patients presenting with their first acute myocardial infarction. To facilitate full assessment of collagen flux, we measured sequential changes in plasma PICP as a marker of collagen synthesis, CITP as a marker of collagen degradation, and Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1) as a marker of inhibition of degradation. We also sought to determine whether plasma levels of these markers correlated with peak serum creatine kinase (CKmax) and whether they predicted adverse clinical outcomes of heart failure or death. Finally, we aimed to determine whether there was a relationship between plasma levels of these markers and plasma levels of renin and aldosterone given the importance of the renin-angiotensin-aldosterone system (RAAS) in ventricular remodelling (McAlpine HM et al. 1988; Lindpaintner et al. 1993), and the consequences of its effectors on fibroblast function and collagen metabolism (Kato H et al. 1991; Brilla CG et al. 1994; Brilla CG et al. 1995; Chua et al. 1996).

4.2 Methods

Detailed methodology can be found in Chapter 2, section 2.3.

4.2.1 Patient Population

The study population comprised of 71 patients presenting with a history of suspected acute myocardial infarction with electrocardiographic criteria for thrombolysis on the admission ECG - ST elevation or new left bundle branch block (Fibrinolytic Therapy Trialists, 1994). Patients with previous myocardial

infarction were not included in the study. Entry criteria consisted of chest pain for more than 20 minutes and presentation within 6 hours from the onset of pain. Seven patients were later excluded from the study when subsequent cardiac enzymes failed to confirm infarction, giving a study population of 64. Heart failure was defined as the development of crepitations in at least 1/3 of both lung fields or signs of pulmonary oedema on chest radiography during hospital admission. Exclusion criteria were concurrent fibrotic disease (pulmonary fibrosis, rheumatoid arthritis, treated hypertension, etc), conditions affecting metabolism of propeptides (renal disease defined as serum creatinine >130micromol/l, liver disease defines as ALT > 2x ULN, ACE inhibitor use, etc), active tumour, and the inability to provide informed consent. A full list of exclusion criteria can be found in Chapter 2 (table 2.1).

4.2.2 Blood Sampling and Biochemical Analyses

A cannula was inserted and sequential blood samples drawn at serial time points – on admission, at 12 hours, 24 hours, days 2, 3, 4 and 30. Venesection was performed with the patient in the recumbent position. 10mls of blood was collected in Lithium Heparin bottles and centrifuged at 3000rpm for 7 minutes. The separated plasma was divided and frozen at -70°C until use. A subgroup of 20 patients had additional samples taken on admission and day 3 for measurement of plasma renin and aldosterone.

Plasma PICP was measured by a radioimmunoassay technique utilising a polyclonal antibody directed against PICP (Orion Diagnostica, Finland) (Melkko

JT et al. 1990). In our laboratory, intra and inter assay variabilities are 3.1 and 6.9% respectively and the mean plasma level (+/- 1SD) in our local population is 129 +/- 32 ng/ml. Plasma CITP was measured using a similar radio-immunoassay technique (Orion Diagnostica, Finland) (Risteli J et al. 1993). Intra and inter assay variabilities are 3.7 and 6.4% respectively with a local population mean of 2.6 +/- 0.3 ng/ml. Plasma TIMP-1 was measured using a two-step sandwich ELISA technique (Amersham Pharmaceuticals, UK) (Plumpton TA et al. 1995). Due to the unexpected discontinuation of the original TIMP-1 ELISA kit by Amersham Pharmaceuticals (Chapter 3), only 30 patients had serial assessment of plasma TIMP-1. Intra and inter assay variabilities for plasma TIMP-1 are 4.0 and 4.4% respectively (Amersham assay) and the normal mean in the local population is 202 +/- 22 ng/ml. Plasma renin and aldosterone were measured by standard ELISA techniques. Measurements of creatine kinase were performed daily from admission and peak CK (CKmax) was determined for each patient. Mortality data was provided by the Common Services Agency, Edinburgh.

4.2.3 Statistical Analysis

All biochemical variables were tested for normality and log-transformed where necessary to allow parametric testing. All data are presented in the non-logarithmic format. Continuous variables are expressed as mean (SEM) and changes over time were tested for significance by repeated measurement of analysis of variance. Student's t test was employed for comparison of means and Pearson's correlation co-efficient tested correlation between variables. Statistical

analyses were performed using Minitab statistical software (Minitab Inc, Pennsylvania, USA) and a p value <0.05 was considered statistically significant.

4.3 Results

4.3.1 Baseline Characteristics

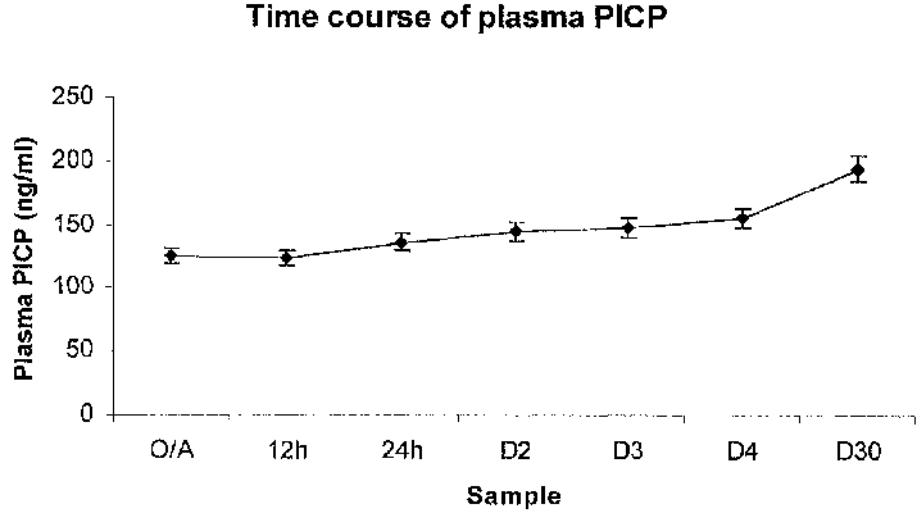
Forty-six patients (72%) were male. Mean age was 62 years, range 31 – 85. All presented within 6 hours of onset of pain, with a median time to presentation of 4.2 hours. The majority presented with inferior ST elevation – 40/64 (62%). Nineteen patients (30%) presented with anterior ST elevation, the remaining 5 (8%) having LBBB. Most patients (58/64) received intravenous thrombolytic therapy - 39 streptokinase, 19 tissue plasminogen activator. 71% of those thrombolysed had successful reperfusion based on ECG criteria (Hogg KJ et al. 1988). No patient had a second dose of thrombolytic therapy or underwent rescue angioplasty. Seventeen (27%) had clinical or radiographic evidence of congestive heart failure (CHF). Four patients (6%) died during the 30 day follow up period with a further five failing to survive to 6 months.

4.3.2 PICP

There was a sequential increase in plasma PICP over the study time period ($p<0.001$, figure 4.1). Mean admission level was within the normal range at 124 (4.4) ng/ml and increased slightly over the first few days, peaking on day 30 at 194 (9) ng/ml (O/A vs. D30 - $p<0.001$), more than two standard deviations above

the mean for a normal population. There was no correlation between admission or peak PICP and CKmax. PICP levels on admission were similar in those with CHF and those with no evidence of heart failure (121 vs. 126 ng/ml, p=0.67), as were peak levels at 30 days. Similarly, there was no significant difference in admission levels between non-survivors and survivors (127 vs. 124 ng/ml, p=0.83), nor between inferior, anterior LBBB infarcts (115 vs. 125 vs. 129 ng/ml, p=0.13). However, the pattern of change was different between infarct sites, with a mean increase in plasma PICP of 52 ng/ml for inferior infarcts vs. 82 ng/ml and 72 ng/ml for anterior and LBBB infarcts respectively (p=0.03, inferior vs. non-inferior). There was no difference in admission PICP levels or in pattern of change between those who reperfused and those who did not, nor between thrombolysis and no thrombolysis or between different thrombolytic agents.

Figure 4.1

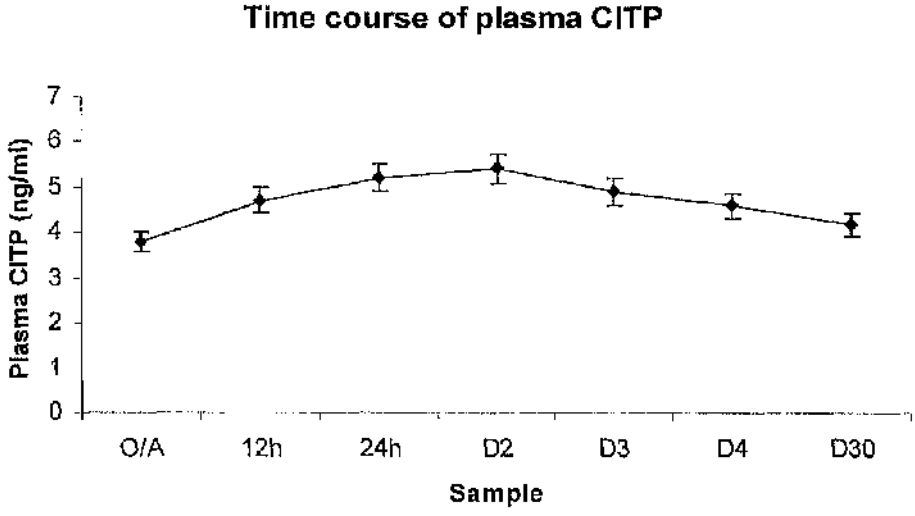


Legend - Time course of plasma PICP following myocardial infarction.

4.3.3 CITP

The group mean admission plasma CITP was elevated at 3.8 (0.25) ng/ml, more than two standard deviations higher than the normal population mean. There was a significant change over time (p=0.001, figure 4.2) with mean plasma CITP levels rising further from admission values within the first few days peaking at 5.2 (0.27) ng/ml on Day 2 (O/A vs. D2 - p<0.001). Plasma CITP returned towards admission levels by day 30, but remained elevated above the normal population mean. There was no correlation between plasma CITP at any time point and CKmax. Patients who developed CHF had significantly higher plasma levels of CITP on admission than those who remained free of heart failure (5.0 vs. 3.3 ng/ml, p=0.04) Non-surviving patients also had a higher admission CITP than surviving patients (5.7 vs. 3.4 ng/ml) but this did not reach statistical significance (p=0.06). Although there was no significant difference in admission CITP levels for the three infarct sites (inferior 3.3 vs. anterior 4.0 vs. LBBB 3.8 ng/ml, p=0.12), anterior and LBBB sites were associated with a higher peak level on day 2 (anterior 5.5 and LBBB 5.2 vs. 4.4 ng/ml, p=0.03). Again, there was no difference between those who reperfused and those who did not, nor between thrombolysis and no thrombolysis or between different thrombolytic agents.

Figure 4.2



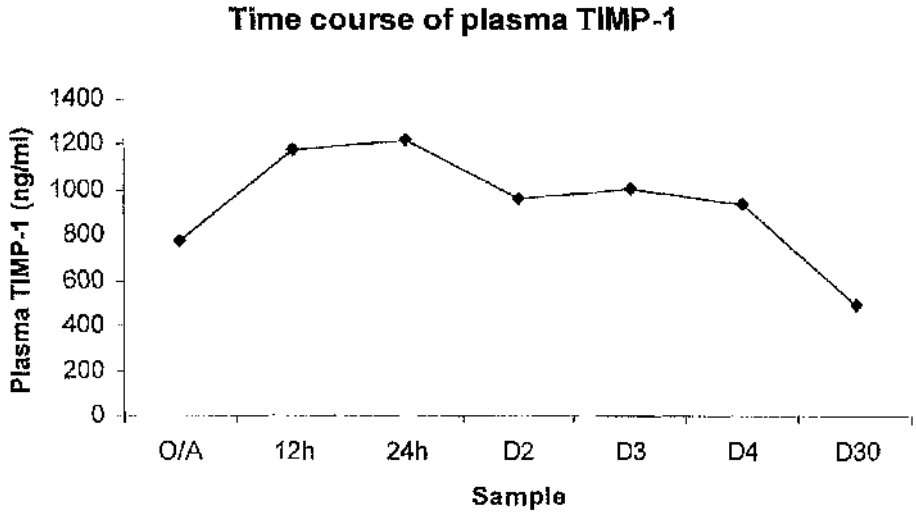
Legend - Time course of plasma CITP following myocardial infarction.

4.3.4 TIMP-1

In the 30 patients studied, mean plasma TIMP-1 was markedly elevated on admission compared to the mean of a normal population – 775 (101) vs. 202 (22) ng/ml. There was a sequential change over time ($p<0.001$, figure 4.3). Mean level increased further within 12 hours, peaking at 24 hours (O/A vs. 24h – $p<0.001$). Levels reached a plateau before falling substantially to a mean of 499 (45) ng/ml at day 30. However, this is still outwith the normal range and more than two standard deviations above normal population mean. Again no correlation with CKmax was observed. No significant difference in admission or peak TIMP-1 was found between those who developed heart failure and those who did not, survivors

and non-survivors, administration of thrombolysis or thrombolytic used. There was a trend to higher peak levels of TIMP-1 in anterior and LBBB sites compared to inferior site, but this did not reach statistical significance.

Figure 4.3



Legend - Time course of plasma TIMP-1 following myocardial infarction.

4.3.5 Renin/Aldosterone

On admission, plasma renin activity was within the normal range in 18/20 patients with a mean admission level of 49 (14) microunits/ml (range 3-270). This rose beyond the normal range by the third day to a mean of 107 (32) microunits/ml (range 4-480) but this rise was not statistically significant ($p=0.11$). However, of the 11 with heart failure, admission level was 92 (26) microunits/ml rising to 207 (55) on day 3, compared to 14 (3) on admission rising to 24 (4) in the nine patients with no clinical or radiographic evidence of failure, $p<0.05$. There was no

significant change in aldosterone in either group with admission and day 3 levels being within the normal range. There was no correlation between plasma levels of renin or aldosterone and plasma CITP, PICP or TIMP-1.

4.4 Discussion

4.4.1 Time Course

This is the largest study to date to utilise markers of both collagen synthesis and degradation in the non-invasive assessment of changes in collagen metabolism following myocardial infarction, and has demonstrated time-dependent changes in plasma concentrations of PICP, CITP and TIMP-1 following acute MI. Plasma levels of PICP display little change over the first few days, remaining within the normal range before peaking at day 30. In contrast, changes in plasma levels of CITP occur earlier and are more pronounced. Mean plasma CITP was elevated on admission, increasing further within the first few days and returning towards the normal range by day 30. These patterns of change are consistent with what is known regarding the pathophysiology of ventricular remodelling.

Early and late phases of the remodelling process are characterised by different changes within the extracellular matrix. Breakdown of collagen by matrix metalloproteinases is the key in early remodelling (Cannon et al. 1983; Whittaker P et al. 1991; Cleutjens JPM et al. 1995), allowing myocyte slippage, infarct expansion and LV dilatation (Olivetti G et al. 1990; Whittaker P et al. 1991; Kim et al. 2000). Indeed, there is as much as 25% collagen loss within 24 hours

following MI in the rat model (Cannon et al. 1983). Given that CITP is liberated from collagen during breakdown, the early elevation of plasma CITP demonstrated in this study is in keeping with these observations.

Conversely, late remodelling is characterised mainly by collagen synthesis and interstitial fibrosis, with further changes to LV geometry, structure and function (Fishbein MC et al. 1978; Pfeffer MA and Braunwald E, 1990). Again, the pattern of change of plasma levels of PICP, a marker of collagen synthesis, is consistent, with levels within the normal range in the early phase, peaking at day 30. This appears at odds with previous studies showing early elevation of PIIINP, another marker of collagen biosynthesis, following AMI (Jensen LT et al. 1990; Host NB et al. 1995; Uusimaa P et al. 1997; Poulsen SH et al. 2000). However, one should remember that unlike PICP, PIIINP is incompletely cleaved during the production of type III collagen and is also released when collagen is degraded. Levels are therefore influenced by the degree of collagen breakdown and this may explain the increased serum levels observed in these studies (Risteli J and Risteli L, 1995b).

Even though PICP is purely a marker of collagen synthesis, and type I collagen, not type III, is the principle myocardial collagen type, there are limited data in the published literature with regard to changes in serum levels of PICP in the setting of acute MI. Uusimaa *et al* examined serum levels of PICP and PIIINP following MI (Uusimaa P et al. 1997). They found no significant early change in PICP, mirroring the findings in our study. However, this is in contrast to their finding of early elevation of PIIINP, the levels of which, and area under the curve, correlated

with indices of cardiac dysfunction. This apparent disparity between these two markers of collagen biosynthesis could be explained in one of two ways. One mechanism would be that synthesis of type III collagen occurs early following AMI, with later synthesis of type I collagen. However, as previously discussed, type I collagen predominates in the heart, and indeed, there is a phenotypic shift from type III to type I collagen following AMI (Medugorac and Jacob, 1983; Wei et al. 1999), making this explanation unlikely. A more plausible mechanism for this disparity would be that the early elevation of PIIINP is a reflection of early collagen breakdown. This is supported by the finding of early elevation of CITP, a specific marker of collagen degradation, in our study. However, regardless of the mechanism, one should not focus on a solely on collagen synthesis. Remodelling should be regarded as a continuum and changes to both processes of synthesis and degradation will occur throughout the process, albeit with differing emphases at different time points.

I have also demonstrated that plasma levels of TIMP-1 display a time-dependent change following myocardial infarction. Levels are markedly elevated on admission, rising further before falling towards normal range by 30 days. If collagen breakdown is the principal finding during the early phase of remodelling, one may have expected that levels of TIMP-1 should be reduced, given that TIMP-1 is a marker of inhibition of degradation. However, the assay for TIMP-1 does not differentiate between free TIMP-1 and TIMP-1 complexed with MMP. MMP expression is up-regulated within a few hours of infarction and is associated with increased enzymatic activity (Carlyle et al. 1997; Herzog et al. 1998; Danielsen et al. 1998) and the importance of active MMPs in the pathophysiology of matrix

remodelling is well documented (Matrisian LM, 1990). This, coupled with the experimental evidence from this study of increased collagen breakdown, as manifested by raised CITP, suggests that the TIMP-MMP balance shifts towards increased MMP activity following MI. It is therefore likely that the rise in plasma levels of TIMP-1 is in response to increased collagen degradation and I hypothesise that these increased levels reflect TIMP-1 bound to MMP.

4.4.2 Relationship with Plasma Renin and Aldosterone

No relationship was observed between plasma levels of PICP, CITP or TIMP-1 and plasma renin or aldosterone on admission or at day 3. Although there is clear evidence of systemic activation of the RAAS following myocardial infarction (McAlpine HM et al. 1988; Rouleau et al. 1993; Lindpaintner et al. 1993), plasma levels do not peak for a few days (McAlpine HM et al. 1988). However, there is evidence of early de-novo generation of these effectors at a tissue level (Dzau, 1987) with associated increased tissue activity immediately following infarction (Sakharov et al. 1987). Given the importance of RAAS effectors on collagen metabolism (Brilla CG et al. 1995) (Lindpaintner et al. 1993) it is likely that both systemic and local RAAS activation have influence on remodelling following myocardial infarction. However, given the delay in mounting a systemic RAAS response, coupled with the rapidity of increased activity of ACE at a tissue level and increased gene expression at a cellular level, it would be reasonable to hypothesise that paracrine regulation of local tissue levels of RAAS effectors, independent of the systemic renin-angiotensin-aldosterone axis, plays an important role in the remodelling process, especially in the early stages. This may, in part,

explain the lack of relationship between plasma levels of the markers of collagen flux and plasma renin and aldosterone levels. It is also documented that increased extraction of circulating aldosterone is important in the remodelling process (Hayashi M et al. 2001b), and this study did not examine trans-cardiac gradients of any effectors of the RAAS, which may have unmasked a relationship with plasma levels of PICP, CITP or TIMP-1.

4.4.3 Site of Infarct, Thrombolyis and Reperfusion

In this study, admission levels of all three markers of collagen turnover were non-significantly higher in those presenting with anterior ST elevation or LBBB infarcts. Although there was a sequential rise in these markers in all three groups, the elevation was more marked in anterior and LBBB infarcts (significant for PICP and CITP, trend for TIMP-1). This study provides serological evidence of increased remodelling of the collagenous component of the heart in infarctions of these sites and is entirely in keeping with the fact that anterior ST elevation and new LBBB infarcts are associated with a worse prognosis compared to inferior infarcts (Hammermeister KE et al. 1979; Mahon et al. 1999).

Interestingly, there was no significant difference in plasma levels of these three markers, or in pattern of change, in those who had thrombolytic therapy vs. those who did not. It is well recognised that both streptokinase and tissue plasminogen activator have effects on collagen metabolism, probably mediated by plasmin (Kosow DP, 1979; Hoylaerts M et al. 1982; Nagase H et al. 1990), the effect of which is probably more significant with streptokinase (Rao AK et al. 1988).

Previous studies have demonstrated immediate elevation in serum levels of PIIINP during thrombolytic infusion, with later elevation in PICP (Peuhkurinen KJ et al. 1991; Peuhkurinen K et al. 1996). In our study, there was no observed difference in levels of any marker of collagen turnover between those who received thrombolysis and those who did not. However, only 6 patients did not receive thrombolytic therapy, allowing only limited comparison between the groups. Similarly, there was also no significant difference between streptokinase and tPA on levels of markers of collagen turnover in our study, unlike the study by Peuhkurinen *et al* (Peuhkurinen K et al. 1996). However, the early elevation in that study was seen during infusion of the thrombolytic agent and settled to baseline levels within hours of discontinuation of the drug, before rising again two to three days following infarction, the degree of which was similar between the two agents studied. In our study, the timing of blood sampling would have "missed" the acute elevation seen with thrombolytic infusion, as assays were performed on admission, prior to the onset of thrombolysis, with the second sample being taken at 12 hours, at which point, the acute effects of the agent would have not been apparent. Therefore, the direct effect of thrombolysis on plasma markers of collagen turnover can effectively be discounted in this study.

A somewhat surprising finding in this study was the apparent lack of effect of successful reperfusion on serological evidence of altered collagen turnover. Successful reperfusion is known to improve survival and limit remodelling (Jeremy et al. 1987; Touchstone et al. 1989; Marino et al. 1989; Fibrinolytic Therapy Trialists, 1994). However, the majority of patients in this study

reperfused (>70%), only allowing limited comparison between those who reperfused and those who did not.

4.4.4 Plasma Levels vs. Tissue Activity

One of my hypotheses was that plasma levels of PICP and CITP reflect the processes of collagen synthesis and degradation within the myocardium. Although this is speculative, the fact that the difference in the pattern of change of these markers over time following acute myocardial infarction is consistent with the pathophysiology of ventricular remodelling provides some indirect evidence to support this hypothesis. In addition, this study provides another strand of evidence to support plasma levels of these markers as reflections of changes in collagen flux at a tissue level. Collagen degradation is the principal process in early remodelling (Cleutjens JPM et al. 1995) which is associated with the development of heart failure and reduced survival (McKay RG et al. 1986; Pfeffer MA and Braunwald E, 1990; Korup et al. 1997). In this study, although admission and peak plasma CITP did not correlate with peak creatine kinase release, a surrogate of infarct size, levels were significantly higher in those who developed heart failure. There was also a trend towards higher levels in non-surviving patients compared to those who survived. No such association was observed between plasma PICP or TIMP-1. However, although these findings provide some supportive evidence of the use of plasma markers of collagen turnover as indicators of the remodelling process following myocardial infarction, further studies examining the relationship of these markers with functional changes within the heart will provide additional information in this regard.

4.4.5 Study Limitations

Although the assay methodologies are robust with good intra and inter-assay variabilities, the assay for TIMP-1 could not distinguish between free TIMP-1 and TIMP-1 bound with MMP, making interpretation of levels more difficult. Furthermore, due to the unexpected change in antibody in the ELISA for TIMP-1, only 30 patients had full biochemical assessment of collagen turnover, including TIMP-1, reducing statistical power to detect relationships between plasma TIMP-1 and effectors of the RAAS, development of heart failure and prognosis. In addition, levels of MMP-1 in plasma are below the reliable detection limit of the available assays and therefore could not studied. However, plasma CITP was studied as a marker of collagen breakdown and can be considered an indirect marker of MMP activity.

This study also employed peak CK as an indicator of degree of myocardial necrosis. A more robust method would have been calculation of CK release curves and measurement of area under the curve. However, due to logistical reasons, this was not performed. This may have influenced the relationship between CK and markers of collagen turnover.

4.5 Conclusions

This study demonstrates time-dependent changes in plasma levels of CITP, PICP and TIMP-1 following myocardial infarction. Raised CITP, a marker of collagen

breakdown, is associated with the development of heart failure and a trend towards an adverse outcome. Levels may reflect early remodelling and may be useful in monitoring the remodelling process.

CHAPTER FIVE – INCREASED COLLAGEN DEGRADATION PREDICTS EARLY VENTRICULAR REMODELLING FOLLOWING ACUTE MYOCARDIAL INFARCTION

5.1 Introduction

One of the most powerful predictors of adverse outcome following myocardial infarction is post-infarction ventricular remodelling which is characterised by alterations to ventricular size, shape and function (White HD et al. 1987; Pfeffer MA and Braunwald E, 1990; Mahon et al. 1999). Since the first description by Tennant and Wiggers in 1935 (Tennant R and Wiggers CJ, 1935), there has been an enormous body of evidence that alterations to LV function, structure and volume are important prognostic variables (Shanoff HM et al. 1969; Kostuk WJ et al. 1973; Pfeffer MA et al. 1979; McKay RG et al. 1986). These changes are evident within a few hours of the onset of symptoms and progress over time (Korup et al. 1997).

Early ventricular remodelling, defined as occurring within 72 hours (Sutton and Sharpe, 2000), is characterised by expansion and thinning of the infarct zone, development of wall motion abnormalities, LV dilatation and reduced ejection fraction (Pfeffer MA and Braunwald E, 1990; Sutton and Sharpe, 2000). Echocardiographic parameters such as end-systolic volume index, ejection fraction and extent of wall motion abnormalities all predict subsequent mortality (Kitamura et al. 1973; White HD et al. 1987; Kober L et al. 1994; Sutton et al. 1997; Migrino et al. 1997; Galasko et al. 2001). More recently, Doppler indices of LV

filling have been shown to provide additional prognostic information following infarction. The development of a restrictive filling pattern, a reflection of impaired diastolic function (Williamson et al. 1990; Chenzbraun et al. 1992), can be assessed by measurement of the mitral deceleration time (Little et al. 1995). Decreased mitral deceleration time is associated with larger infarcts and correlates with subsequent remodelling (Cerisano G et al. 1999).

Early identification of remodelling is important, as there are strategies available to reduce the remodelling process, such as maintaining the patency of the infarct related artery and the use of ACE inhibitors, which confer a survival benefit (Jeremy et al. 1987; Touchstone et al. 1989; Pfeffer MA et al. 1992; AIRE study investigators, 1993; ISIS-4 Collaborative Group, 1995). The early phase of remodelling following infarction is associated with changes to the extracellular matrix, both in the infarct zone and in the non-infarcted portion of the heart, manifested principally as increased collagen degradation (Cleutjens JPM et al. 1995). Indeed, there is as much as 25% net collagen loss within the first 24 hours following MI (Cannon et al. 1983) which facilitates myocyte slippage, infarct expansion and LV dilatation (Whittaker P et al. 1991; Kim et al. 2000).

Type I collagen is the principal collagen type within the heart (Weber KT, 1989), and its metabolism can be assessed biochemically (Risteli L and Risteli J, 1990; Risteli J and Risteli L, 1995b). During the formation of Type I collagen, the propeptide moiety, procollagen type I carboxy-terminal propeptide (PICP), is cleaved from procollagen and is therefore a marker of collagen synthesis (Melkko

JT et al. 1990). Similarly, during collagen degradation, C-telopeptide for type I collagen (CITP) is liberated and is a marker of breakdown (Risteli J et al. 1995a).

I have previously demonstrated time-dependent alterations in plasma levels of both PICP and CITP following acute myocardial infarction with early elevation of plasma CITP and late elevation of PICP. Admission and peak CITP were significantly higher in those who subsequently developed clinical or radiographic evidence of heart failure (Chapter 4). No such association was found with plasma levels of PICP. I hypothesise that raised plasma levels of CITP may reflect the increased degradation of collagen in the early stages following infarction and therefore may be a marker of early remodelling. To investigate this further, a cohort of patients presenting with their first acute myocardial infarction from the observational study previously described (Chapter 4) were studied in more detail with a comprehensive echocardiographic examination on the fourth day following infarction. Given the importance of abnormal wall motion index (WMI) on echocardiography performed in the sub-acute phase of AMI (Kober L et al. 1994), and its correlation with mortality, patients were dichotomised by the presence or absence of wall motion abnormality. In addition, I fully assessed collagen flux by the sequential measurement of plasma levels of PICP and CITP over time. I aimed to determine the relationship of plasma levels of PICP and CITP with echocardiographic indices of early remodelling including WMI and whether admission levels provide prognostic information with regard to its subsequent development.

5.2 Methods

Detailed methodology can be found in Chapter 2, section 2.3.

5.2.1 Patient Population

The study population comprised 51 patients presenting with their first myocardial infarction and eligible for reperfusion therapy based on the admission 12 lead electrocardiogram - ST elevation or new left bundle branch block (Fibrinolytic Therapy Trialists, 1994). Entry criteria consisted of chest pain for more than 20 minutes, presentation within 6 hours from the onset of pain. Exclusion criteria were concurrent fibrotic disease (pulmonary fibrosis, rheumatoid arthritis, treated hypertension, etc), conditions affecting metabolism of propeptides (renal disease defined as serum creatinine >130micromol/l, liver disease defines as ALT > 2x ULN, ACE inhibitor use, etc), active tumour, and the inability to provide informed consent. A full list of exclusion criteria can be found in Chapter 2 (table 2.1).

Patients were categorised on the presence or absence of early signs of remodelling on echocardiographic studies performed on the fourth day post event. Given that previous studies have demonstrated that the degree of wall motion abnormality in the sub-acute phase following AMI is the most powerful echocardiographic predictor of both short and long-term mortality (Kober L et al. 1994; Sutton et al. 1997; Migrino et al. 1997), this echo parameter was used to dichotomise patients into those with echocardiographic evidence of early remodelling and those with no such evidence. Biochemical variables were assessed in accordance with this

grouping, although correlation with other echocardiographic parameters was assessed with the group as a whole.

5.2.2 Echocardiographic Assessment of Remodelling

Detailed 2-dimensional and Doppler examinations were performed by a single observer (AMcG) on Day 4 following the index event, using a Vingmed System V Ultrasound Machine (General Electronics, Milwaukee, USA) connected to a PowerMacIntosh G3 computer with digital archiving facilities. Images were acquired in the four standard views with the patient in the left lateral position at end-expiration. Offline analysis was performed by a single observer (AMcG) blinded to the results of biochemical analyses.

(a) Wall Motion Index

A sixteen segmental scoring system was employed in accordance with the American Society of Echocardiography guidelines (Schiller et al. 1989). The sum of the scores from all segments were totalled and divided by sixteen giving a wall motion index (WMI). A score of 1 indicates normal movement of all segments, whereas a score of greater than one represents the presence of abnormal wall motion of one or more segments. The higher the score above one, the greater the degree of regional wall motion abnormalities and percentage of muscle affected. As mentioned previously, patients were dichotomised depending on the presence or absence of wall motion abnormality – i.e. WMI >1 or WMI=1.

(b) Mitral Deceleration Time

Pulsed wave Doppler signals were obtained through the tips of the mitral valve inflow in the apical four chamber view at end-expiration. Mitral deceleration time (Dt), defined as the time from the peak of the early mitral inflow (E-wave) to baseline, was measured in three cycles (avoiding extrasystolic or immediate post-extrasystolic beats) and a mean calculated for the purposes of analysis.

(c) Left Ventricular Volumes and Ejection Fraction

End diastole was identified from ECG gating, being defined as the onset of the R wave. End systole was identified from visual inspection, being defined as the smallest ventricular dimension in that cardiac cycle. Tracing of the endocardial contours in both diastole and systole was performed from stored 2 dimensional images acquired in the apical two and four chamber views and LV volumes calculated from the disc summation method or Simpson's rule (Schiller et al. 1979; Erbel et al. 1982). The mean of three cycles was calculated (avoiding extrasystolic or immediate post-extrasystolic beats) and normalised for body surface area, giving values for left ventricular end diastolic volume index (LVEDVI) and left ventricular end systolic volume index (LVESVI).

Ejection fraction was calculated by the following equation, which has been validated previously (Folland et al. 1979; Starling et al. 1981; Erbel et al. 1983):

$$\frac{\text{(LV end diastolic volume} - \text{LV end systolic volume)}}{\text{LV end diastolic volume}} \times 100$$

5.2.3 Blood Sampling and Biochemical Analyses

Blood was drawn from an indwelling cannula with the patient in a recumbent position at serial time points – on admission, at 12 hours, 24 hours, and days 2, 3, 4 and 30. 10mls of blood was collected in Lithium Heparin bottles, centrifuged at 3000rpm for 7 minutes and the separated plasma frozen at -70°C until use.

Plasma PICP was measured by standard radioimmunoassay (Orion Diagnostica, Finland) (Melkko JT et al. 1990). We have previously demonstrated intra and inter assay variabilities of 3.1 and 6.9% respectively with a local population mean (+/- 1SD) of 129 +/- 32 ng/ml (Chapter 3). Plasma CITP was also measured by radio-immunoassay (Orion Diagnostica, Finland) (Risteli J et al. 1993) with intra and inter assay variabilities of 3.7 and 6.4% respectively. Mean plasma level in our local population is 2.6 +/- 0.3 ng/ml.

5.2.4 Statistical Analysis

Statistical analysis was performed using Minitab statistical software (Minitab Inc, Pennsylvania, USA). Non-normally distributed variables were log-transformed to allow parametric testing. However, all data are presented in the non-logarithmic format. Continuous variables are expressed as mean (SEM) and changes over time were tested for significance by repeated measurement of analysis of variance. Student's t test was employed for comparison of means and Pearson's correlation co-efficient tested correlation between variables. Multiple regression models were

constructed using Minitab. A p value <0.05 was considered statistically significant.

5.3 Results

5.3.1 Baseline Characteristics

The majority of patients (74%) were male with a mean age of 59 years (range 31 – 85). The median time to presentation was 4.1 hours (range 2.4 - 5.8). Thirty-two (63%) presented with inferior ST elevation, 15 (29%) with anterior ST elevation, and the remainder (8%) having new LBBB. More than 90% (46/51) received intravenous thrombolytic therapy (28 streptokinase and 18 tissue plasminogen activator) with 72% having non-invasive evidence of successful reperfusion on 12 lead ECG performed at 90 minutes post thrombolysis (Hogg KJ et al. 1988). No patient had a second dose of thrombolytic or underwent rescue angioplasty.

Patients were dichotomised depending on the presence or absence of wall motion abnormalities on echocardiography. Twenty-three patients (45%) had no wall motion abnormalities and therefore had a wall motion index (WMI) of 1. The remaining 28 patients (55%) had evidence of wall motion abnormality and had a WMI greater than one (range 1.06 to 2.36). Baseline characteristics for both groups are shown in table 5.1. Mean age was similar in both groups with comparable percentage of diabetic patients, overall thrombolytic usage and reperfusion rates. However, there were significantly more anterior infarcts in the

abnormal wall motion group and therefore more received tPA rather than streptokinase as thrombolytic therapy in this group.

Table 5.1

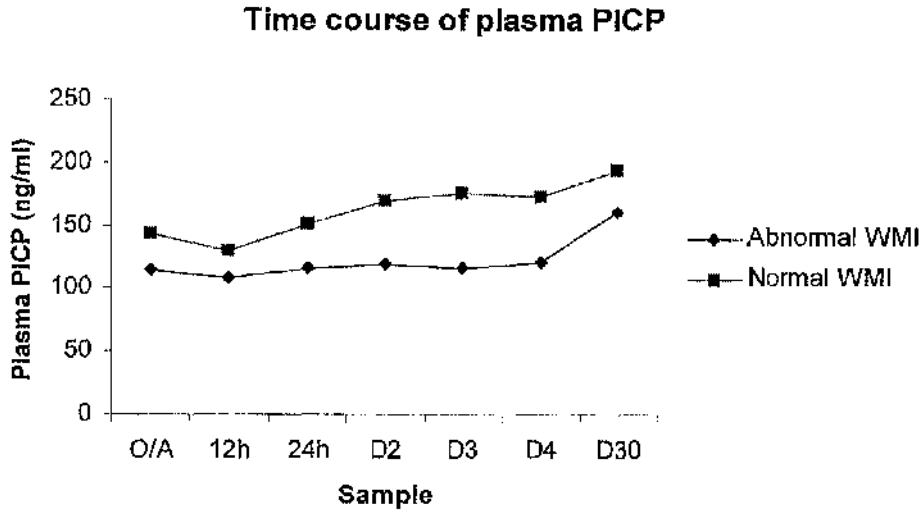
	Normal WMI N(%)	**Abnormal WMI N(%)**
Number	23	28
Mean Age	56	63
Male	16 (70)	22 (79)
Diabetes	5 (22)	8 (29)
Mean admission BP	148/90	146/90
Inferior MI	18 (78)	14 (50)
Anterior MI	4 (18)	11 (39)
LBBB	1 (4)	3 (11)
Thrombolysis	21 (91)	25 (89)
% tPA	19	50
% reperfused	76	68
% ACEI use	83	89

Legend - Baseline characteristics

5.3.2 PICP

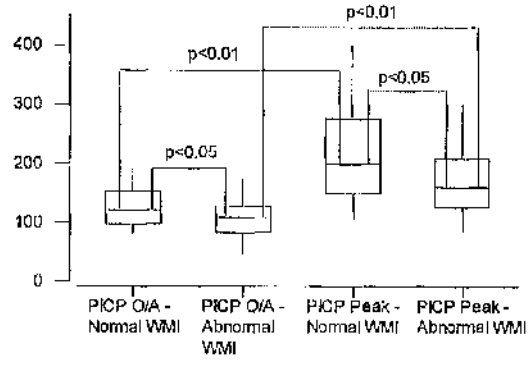
Both groups of patients demonstrated a sequential rise in plasma PICP over the study time period ($p<0.001$, figure 5.1). Admission means for both groups were within the 95th confidence interval for the mean of a normal population (114-143ng/ml), although admission PICP was lower in the abnormal wall motion group compared to the group with normal WMI (114 vs. 143ng/ml, $p<0.05$, figure 5.2). They displayed a similar pattern of change over the study period with a slight rise in the first few days, peaking by day 30, again with lower levels in the abnormal WMI group (160 vs. 195ng/ml, $p<0.05$, figure 5.2).

Figure 5.1



Legend - Time course of plasma PICP following myocardial infarction for patients with evidence of wall motion abnormalities (abnormal WMI) and with no wall motion abnormalities (normal WMI).

Figure 5.2

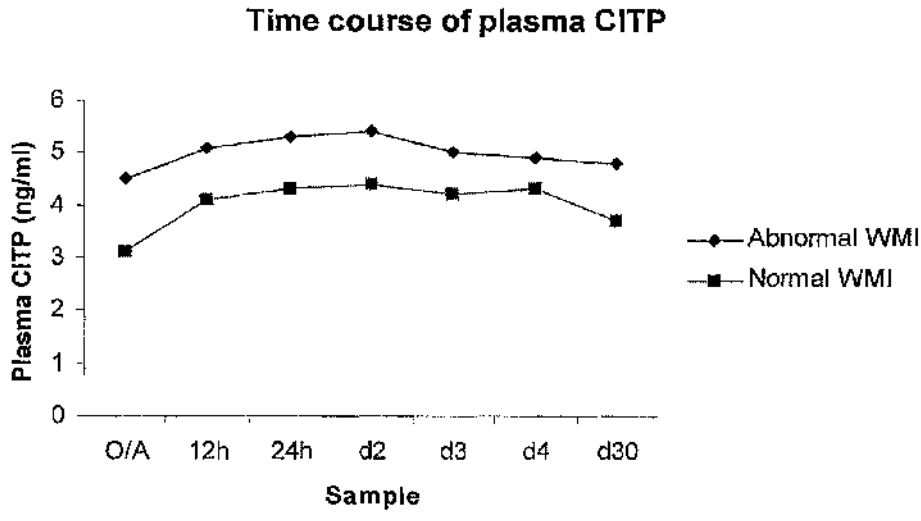


Legend - Comparison of group means (with 25th and 75th centiles and +/- 1SD shown) for plasma level of PICP on admission (O/A) and peak levels at day 30 (peak) for both groups.

5.3.3 CITP

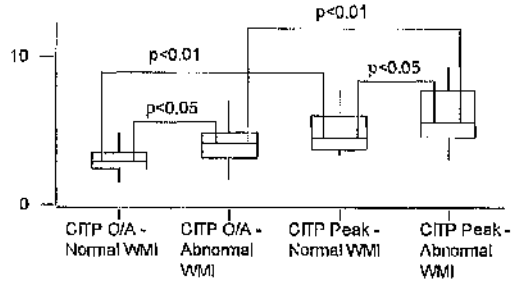
A time dependent rise in plasma levels of CITP was also seen in both groups with levels rising within 12 hours in both groups, reaching a platcau, before falling towards admission levels by day 30 ($p<0.001$, figure 5.3). The changes over time are parallel in the two groups with the main difference being plasma CITP on admission. The group with abnormal wall motion index had a mean admission level of 4.5 (0.6) ng/ml. This is outwith the normal range, being two standard deviations above the mean for a normal population (normal population mean 2.6 (SD 0.3) ng/ml, range 1.9 – 3.9). This was significantly higher than the admission mean for the group with no evidence of wall motion abnormalities which was within the normal range (4.5 vs. 3.1, $p<0.05$, figure 5.4). Peak levels of CITP were also higher in the abnormal WMI group (6.3 vs. 4.8, $p<0.05$, figure 5.4).

Figure 5.3



Legend - Time course of plasma CITP following myocardial infarction for patients with evidence of wall motion abnormalities (abnormal WMI) and with no wall motion abnormalities (normal WMI).

Figure 5.4

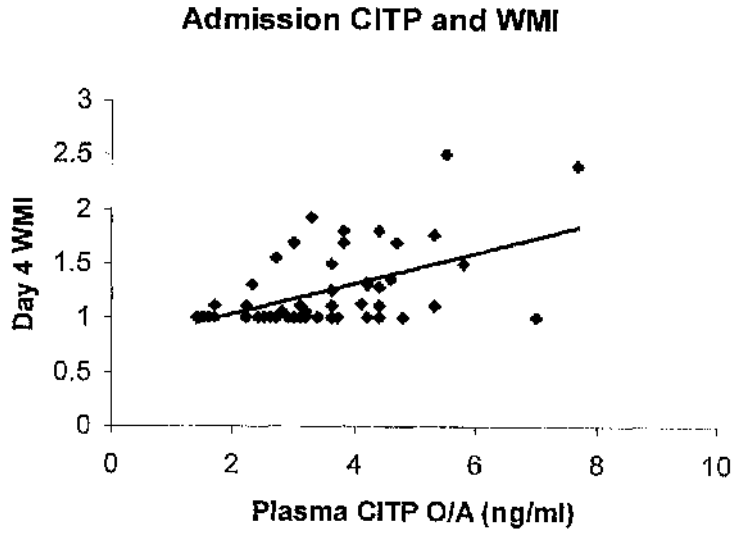


Legend - Comparison of group means (with 25th and 75th centiles and +/- 1SD shown) for plasma level of CITP on admission (O/A) and peak levels at day 30 (peak) for both groups.

5.3.4 Correlation with Wall Motion Index

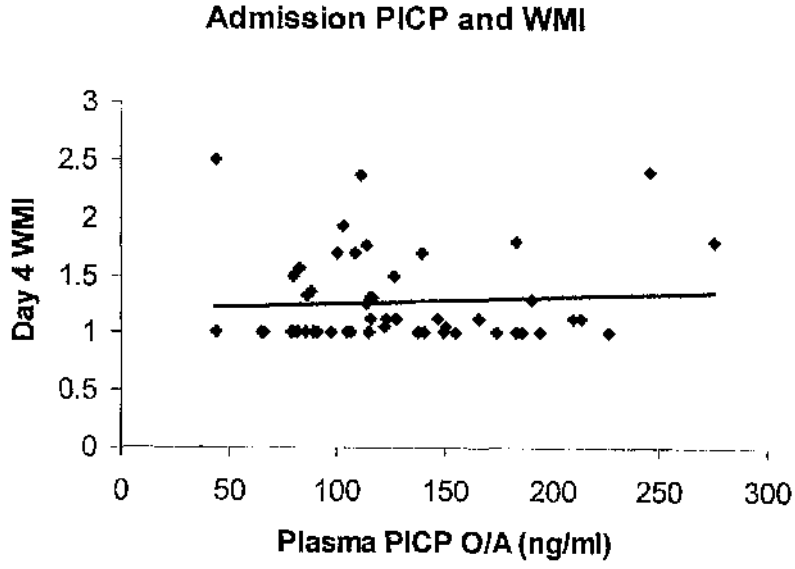
In addition to raised plasma levels of CITP and reduced levels of plasma PICP at all time points in the group with abnormal wall motion index, the absolute plasma level of CITP on admission positively correlated with wall motion index ($r = 0.53$, $p<0.01$, figure 5.5). Similar findings were found with peak CITP (data not shown). As both groups show similar pattern of change of plasma CITP over time, we assessed the use of admission CITP as a predictor of subsequent development of wall motion abnormalities. Using the mean plasma level of CITP of a normal population + 2 standard deviations as a partition value, an admission level of greater than 3.2ng/ml gives a positive predictive value of 74% for the development of wall motion abnormalities and a negative predictive value of 65%. In a multiple regression analysis, anterior infarct and admission CITP were independent multivariate predictors of wall motion score. No relationship was demonstrated with admission or peak plasma PICP (figure 5.6), peak CK, use of thrombolysis, thrombolytic agent or evidence of reperfusion.

Figure 5.5



Legend - Scatterplot of admission level of plasma CITP with wall motion index on day 4. Correlation coefficient 0.53, p<0.01.

Figure 5.6

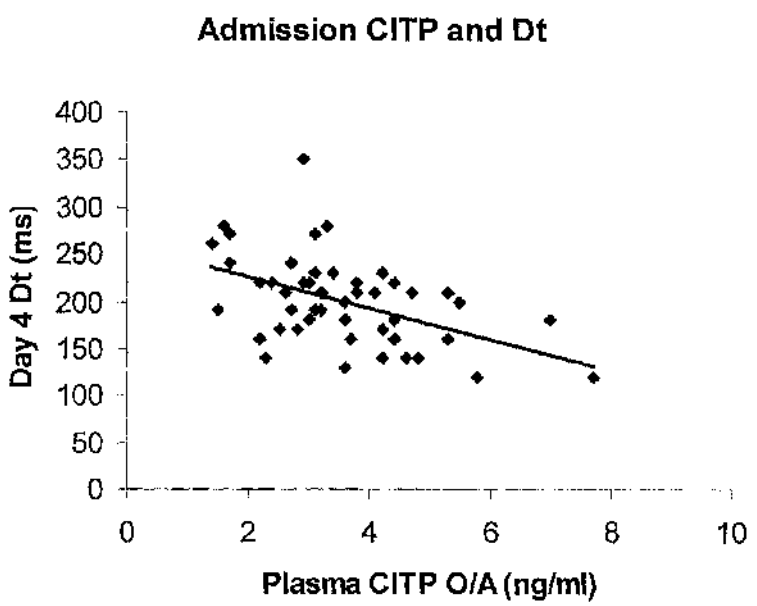


Legend - Scatterplot of admission level of plasma PICP with wall motion index on day 4. Correlation coefficient 0.07, p=NS.

5.3.5 Relationship with Mitral Deceleration Time

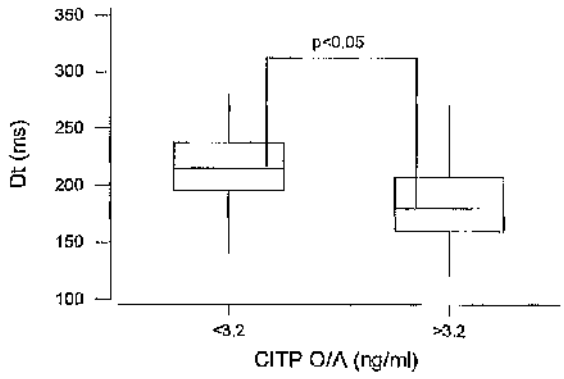
The group with wall motion abnormalities had a lower mean Dt than the group with normal wall motion (187 vs. 217ms, $p<0.05$). As with wall motion index, absolute level of admission plasma CITP correlated with mitral deceleration time (Dt), this time negatively ($r = -0.38$, $p<0.01$, figure 5.7). Using the same partition value as previously, Dt was lower in those with admission CITP >3.2 ng/ml compared to those with lower admission levels (183 vs. 221ms, $p<0.05$, figure 5.8). Again, no relationship with plasma PICP was demonstrated (figure 5.9). Dt was not related to infarct site, peak CK, use of thrombolysis or reperfusion status.

Figure 5.7



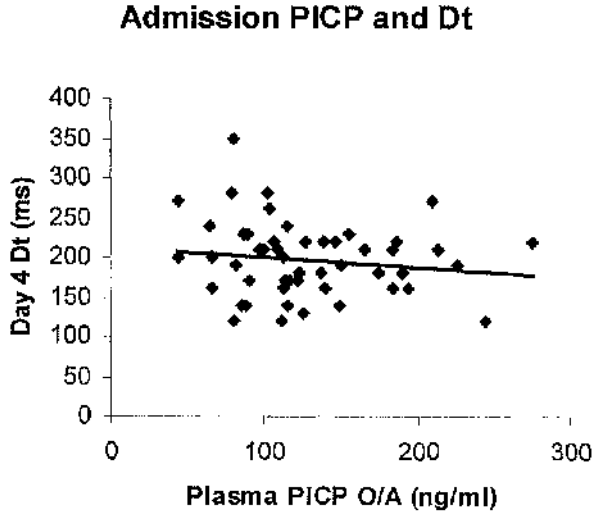
Legend - Scatterplot of admission level of plasma CITP with mitral deceleration time (Dt) on day 4. Correlation coefficient -0.38, $p<0.01$.

Figure 5.8



Legend - Comparison of Dt (mean +/- 1SD with 25th and 75th centiles shown) for Dt on day 4 using admission level of plasma CITP of 3.2ng/ml as a partition value

Figure 5.9

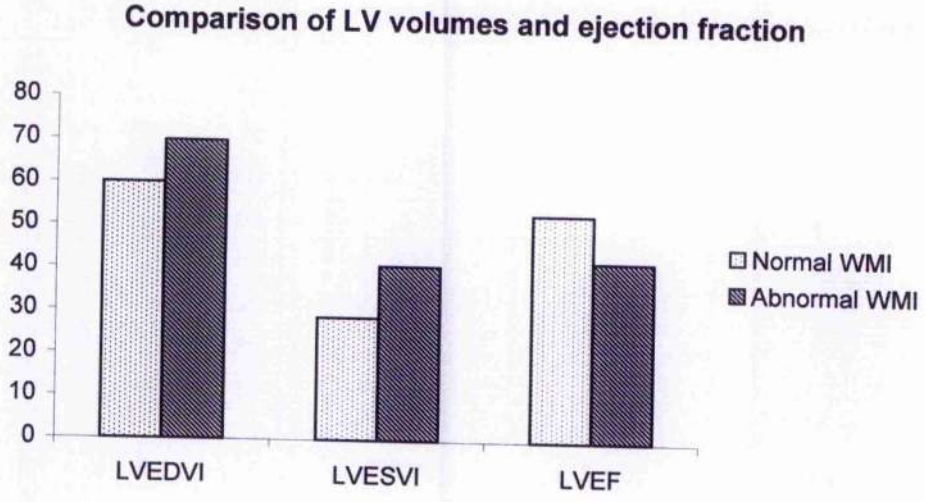


Legend - Scatterplot of admission level of plasma PICP with mitral deceleration time (Dt) on day 4. Correlation coefficient -0.13, p=NS.

5.3.6 Relationship with LV Volumes and Ejection Fraction

Mean end diastolic volume index was similar in the group with wall motion abnormalities compared to the group with normal WMI (69.8 vs. 60.0 mls/m2, p=NS). Mean end systolic index was higher in the abnormal WMI group (40.7 vs. 28.6 ml/m2, $p<0.05$) with a resultant lower ejection fraction (42% vs. 53%, $p<0.001$) (figure 5.10). However, there was no correlation between absolute admission levels of CITP or PICP with LV end diastolic or end systolic volumes or ejection fraction (figures 5.11 and 5.12) and no significant difference in these variables using 3.2ng/ml as a partition value for admission CITP.

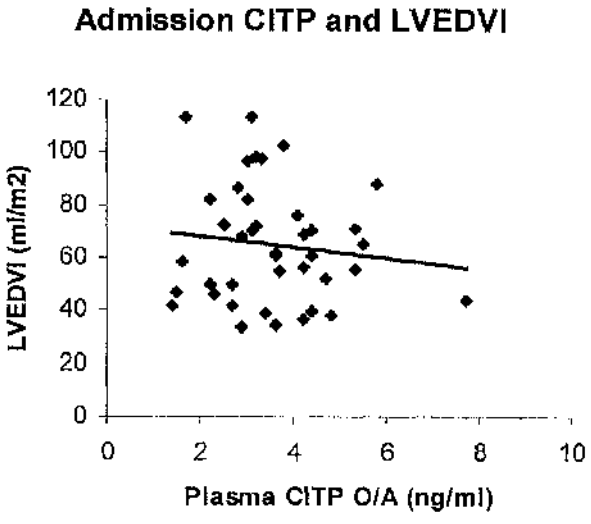
Figure 5.10



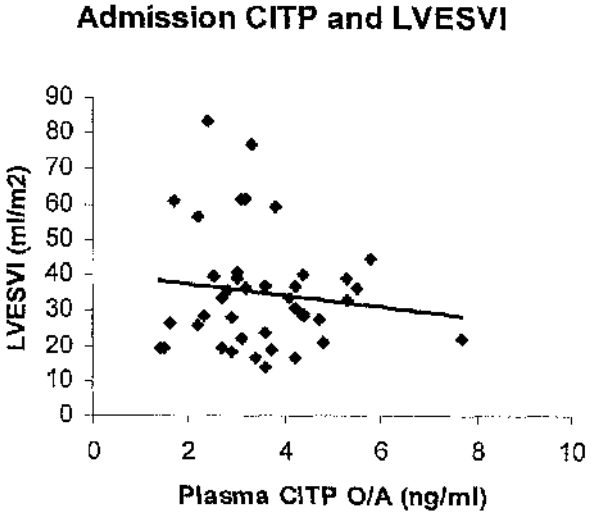
Legend – Comparison of mean LV end diastolic volume index (LVEDVI), mean end systolic volume index (LVESVI) and mean LV ejection fraction (LVEF) between the group with normal wall motion index vs. abnormal index.

Figure 5.11

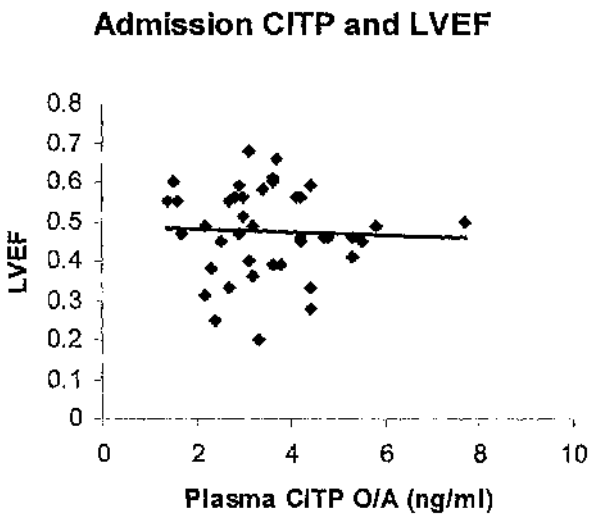
(a)



(b)



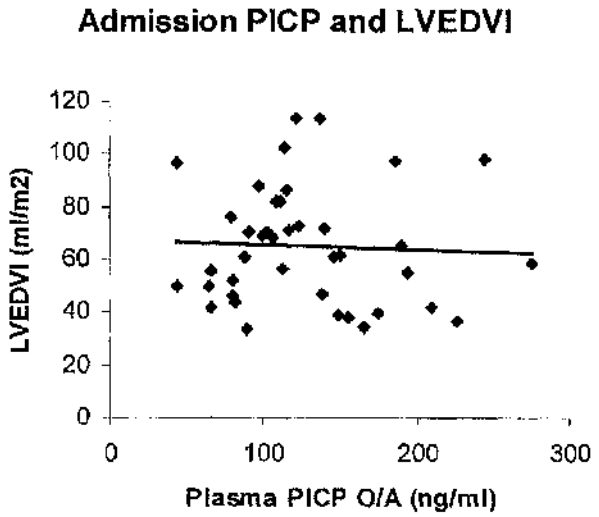
(c)



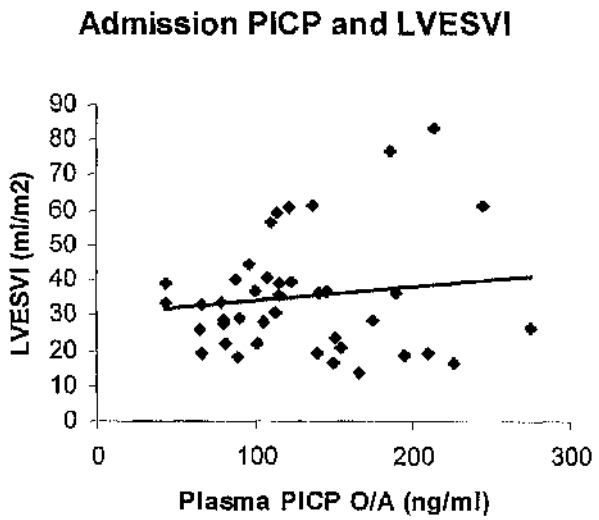
Legend - Scatterplot of admission level of plasma CITP with (a) LV end-diastolic volume index (LVEDVI) (Correlation coefficient -0.12, p=NS), (b) LV end-systolic volume index (LVESVI) (Correlation coefficient -0.12, p=NS) and (c) LV ejection fraction (LVEF) (Correlation coefficient -0.04, p=NS) on day 4.

Figure 5.12

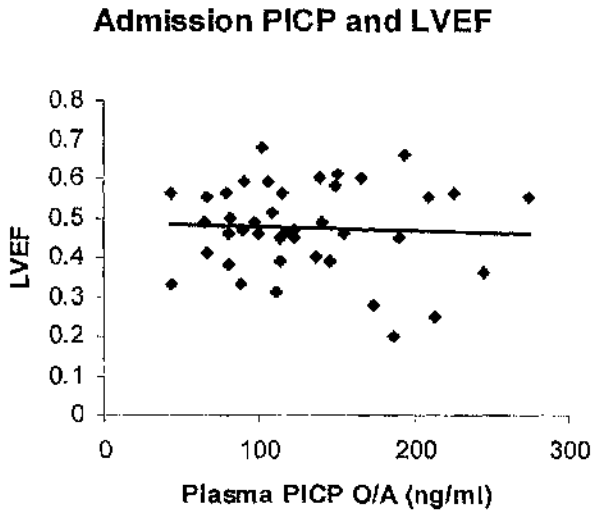
(a)



(b)



(c)



Legend - Scatterplot of admission level of plasma PICP with (a) LV end-diastolic volume index (LVEDVI) (Correlation coefficient -0.04, p=NS), (b) LV end-systolic volume index (LVESVI) (Correlation coefficient -0.13, p=NS) and (c) LV ejection fraction (LVEF) (Correlation coefficient -0.05, p=NS) on day 4.

5.4 Discussion

5.4.1 Normal vs. Abnormal Wall Motion Index

This study demonstrates time-dependent alterations in plasma markers of both collagen synthesis and degradation in patients presenting with their first acute myocardial infarction and delineates the relationship of these markers with established echocardiographic parameters of early ventricular remodelling. Patients with abnormal wall motion index on echocardiography on day 4 following infarction, the most powerful echocardiographic predictor of subsequent mortality (Kan et al. 1986; Sutton et al. 1994; Korup et al. 1997; Sutton et al. 1997; Migrino et al. 1997), displayed higher levels of mean plasma CITP on admission, and at all other time points, than the group with normal WMI. Higher peak level was also demonstrated in this group. With regard to mean plasma PICP, this was lower at all time points in the abnormal WMI group, although mean admission levels were within the normal range in both groups.

Although patients were categorised depending on the presence or absence of wall motion abnormalities, other echocardiographic parameters of remodelling were reflected within this dichotomy, supporting the use of wall motion as a dichotomous variable. Patients in the abnormal WMI group also displayed a significantly higher end-systolic volume index, lower Dt and lower ejection fraction than in those with normal WMI and a trend towards higher end-diastolic volume index, all of which is in keeping with changes seen in early remodelling.

5.4.2 Plasma Levels of CITP and PICP as Reflection of the Remodelling Process

We have previously demonstrated time-dependent alterations in plasma levels of PICP and CITP (Chapter 4). This current study provides evidence to support that plasma levels of these markers reflect functional changes within the heart. The differences found in plasma levels of PICP and CITP between the groups are consistent with the pathophysiology of remodelling following infarction. Early remodelling is characterised by a net loss of myocardial collagen content due to collagen degradation (Cannon et al. 1983; Cleutjens JPM et al. 1995), allowing the development of a restrictive ventricular filling pattern, progressive LV dilatation and reduced ejection fraction in addition to the development of wall motion abnormalities (Olivetti G et al. 1990; Whittaker P et al. 1991; Williamson et al. 1990; Kim et al. 1999). The early elevation in plasma levels of CITP, produced during collagen breakdown, demonstrated in this study in the group with echocardiographic evidence of early remodelling is likely to reflect increased collagen degradation. Collagen is primarily degraded by active matrix metalloproteinases (MMPs) (Birkedal-Hansen H et al. 1993). Previous studies have shown early up-regulation of MMP genetic expression within a few hours of infarction (Herzog et al. 1998) which is associated with increased gene product (Carlyle et al. 1997; Herzog et al. 1998; Danielsen et al. 1998). The finding in our study of raised plasma levels of CITP, a product of collagen breakdown, is in keeping with these previous studies.

Conversely, plasma levels of PICP, a marker of collagen synthesis is lower in the group with evidence of remodelling. This may be due to reduced synthesis in this

group or higher synthesis in the group who did not develop wall motion abnormalities. Although this study cannot answer that question directly, one must remember that net collagen content is a product of a dynamic equilibrium between synthesis and degradation. It is therefore clear from this study that the group with abnormal wall motion display evidence of increased degradation and reduced synthesis of collagen compared to those with normal wall motion, and therefore, the balance is shifted in favour of collagen breakdown. It is this shift in balance which supports that the observed changes in plasma levels of these propeptides are a reflection of remodelling, rather than a generalised increase in collagen turnover in a non-specific response to simple ischaemic injury.

It is clear from this study that this shift in favour of collagen degradation is apparent on admission. MMP expression is upregulated within hours of an ischaemic insult, resulting in immediate collagen breakdown(Cleutjens JPM et al. 1995; Herzog et al. 1998). The finding of elevated CITP on admission samples is entirely in keeping with this timescale. In contrast, although admission plasma levels of PICP were lower in the abnormal WMI group, absolute levels were within the normal range in both groups. Elevation above the normal range is not apparent until day 30, a finding consistent with the timescale of scar formation(Cleutjens JPM et al. 1995).

5.4.3 Relationship of Plasma CITP and PICP to Wall Motion Score and Mitral Deceleration Time

In addition to the differing levels of CITP and PICP between the groups with normal and abnormal WMI, absolute plasma levels of CITP on admission, and at peak, positively correlated with wall motion score and negatively correlated with mitral deceleration time (Dt). Indeed, using two standard deviations above a normal population mean as a partition value, an admission CITP level of >3.2ng/ml provided a 74% positive predictive value for the subsequent development of wall motion abnormality. Admission level less than this value provided a negative predictive power of 65%. Furthermore, those with admission CITP >3.2ng/ml had evidence of lower Dt than those with admission CITP <3.2ng/ml, reflecting the restrictive filling pattern seen in patients with remodelling (Popovic et al. 1996; Cerisano G et al. 1999). It is therefore clear that admission value of plasma CITP provides important information with respect to remodelling and is an independent predictor for the development of echocardiographic parameters of early remodelling. No such relationship was observed with admission levels of PICP, again reflecting that collagen degradation is the principal finding in the early phases following infarction.

Previous work by Poulsen *et al* has demonstrated early elevation of plasma PIIINP following AMI, the degree of which predicted LV remodelling, including the development of a restrictive filling pattern on echo (Poulsen SH et al. 2000). They concluded that this reflected increased collagen synthesis. However, as previously discussed, PIIINP is also liberated during collagen breakdown and levels may

reflect an increase in this process rather than increased synthesis (Risteli J and Risteli L, 1995b). Indeed, studies examining PICP, a pure marker of synthesis, show no such early elevation (Uusimaa P et al. 1997). PIIINP was not utilised in this study due to its lack of specificity to either process. This study related echocardiographic parameters of remodelling to specific markers of collagen synthesis or degradation and provides a clearer insight into the dynamics of collagen turnover following AMI and its relationship with the remodelling process.

5.4.4 Relationship of Plasma CITP and PICP to LV Volumes and Ejection Fraction

There was no association between absolute plasma levels of CITP or PICP on admission, or at any other time point, with LV end-diastolic or end-systolic volumes or ejection fraction. This may appear to be at odds with the finding that mean LVEDVI and LVESVI were higher and LVEF lower in the group with abnormal wall motion, who had higher mean CITP and lower PICP. It also appears to contradict previously published data suggesting that LV end-systolic volume and ejection fraction are powerful indicators of remodelling (White HD et al. 1987). However the lack of correlation with plasma markers of collagen turnover probably reflects the heterogeneity of the remodelling process and should not be regarded as an indication of failure of plasma markers to reflect the remodelling process. Indeed, recent results from the GISSI-3 echo sub-study showed that in-hospital dilatation and dysfunction were not associated with progressive dilatation and deterioration of function over time (Giannuzzi et al. 2001). This was thought to be due to the marked heterogeneity of dilatory and

compensatory haemodynamic responses to infarction, and therefore it is perhaps not surprising that absolute plasma levels of CITP or PICP were not associated with these parameters. However, elevated plasma CITP on admission is associated with the development of wall motion abnormalities which was found to predict outcome in the GISSI-3 study, including the development of progressive LV dilatation and reduction in global LV function. This is in keeping with the echo sub-studies of TRACE, SAVE and GUSTO-1 which also found wall motion score to be an independent predictor of subsequent mortality (Kober L et al. 1994; Sutton et al. 1997; Migrino et al. 1997).

5.4.5 Study Limitations

Given that the presence of wall motion abnormality was used to dichotomise patients, baseline characteristics of the two groups were not the same. Not surprisingly, anterior infarction and left bundle branch block were more common in the group who developed wall motion abnormalities, comprising half of this group compared to less than one quarter in those with normal wall motion. As a consequence, the rate of tPA usage as thrombolytic therapy was higher in this group. This is consistent with the fact that anterior and LBBB infarctions are associated with a poorer prognosis(Hammermeister KE et al. 1979; Mahon et al. 1999). However, even adjusting for these variables, admission CITP provides additional prognostic information, being an independent predictor for the development of wall motion abnormalities. No relationship was demonstrated with absolute values of PICP and echocardiographic parameters of remodelling. Given

the heterogeneity of plasma PICP within the patient population, a larger sample size may have increased power to detect such relationship.

As previously discussed, the differences in mean levels of both CITP and PICP between the groups is apparent from admission and changes over time parallel each other. It is therefore likely that the divergence of values for plasma CITP and PICP started before admission, even though all patients in this study presented within 6 hours of the onset of pain. Further studies examining changes in these levels in the very early phase following pain or in a model of acute coronary occlusion are required to investigate this further.

Finally, a single echocardiogram, rather than serial studies, was used to demonstrate a relationship between plasma markers of collagen turnover and remodelling. However, although remodelling is defined as a *change* in LV shape, structure and function, the process can be detected at an early stage and should be regarded as a continuum. The early phase is synonymous with infarct expansion and is characterised by the development of wall motion abnormalities, early dilatation and reduction in ejection fraction (Sutton and Sharpe, 2000). A single echocardiographic study performed within a few days of infarction allows identification of the early phase of remodelling and has been shown to correlate well with prognosis (Kitamura et al. 1973; Kan et al. 1986; White HD et al. 1987; Sutton et al. 1994; Kober L et al. 1994; Popovic et al. 1996; Migrino et al. 1997; Sutton et al. 1997; Cerisano G et al. 1999). This study would have been underpowered to detect change in LV dimensions over time.

5.5 Conclusions

This study demonstrates changes in plasma levels of CITP and PICP over time following myocardial infarction with raised levels of CITP and reduced PICP in patients with echocardiographic evidence of early remodelling. Admission CITP, a marker of collagen breakdown, predicts the development of subsequent wall motion abnormalities and is associated with reduced mitral deceleration time. It therefore provides early prognostic information following infarction and this study supports its use in the identification of patients at high risk of developing remodelling and raises the possibility of early initiation of cardioprotective strategies such as primary PCI, the early administration of ACE inhibitors, and potentially the use of synthetic MMP inhibitors.

CHAPTER SIX – SEROLOGICAL EVIDENCE OF EARLY REMODELLING IN "HIGH RISK" NON ST ELEVATION ACUTE CORONARY SYNDROMES

6.1 Introduction

Patients with non ST elevation acute coronary syndromes (ACS) represent a wide spectrum of risk of subsequent morbidity and mortality, with a 30 day mortality rate of up to 9% in high risk groups (Antman et al. 1996), and combined rate of death, MI or urgent revascularisation of more than 40% (Antman et al. 2000). Ischaemia sufficient to cause dynamic ST changes or sequential T wave changes on the 12 lead ECG is associated with an adverse outcome (Nyman et al. 1993; Cannon et al. 1997; Kaul et al. 2001), as is elevation of serum troponin (Hamm et al. 1992; Antman et al. 1996; Galvani et al. 1997).

In addition to causing these electrocardiographic changes and producing elevation in serum troponin, a high ischaemic burden also causes alterations to the collagenous component of the heart within the extra-cellular matrix, even in the absence of overt infarction (Zhao M et al. 1987). Collagen turnover can be assessed biochemically (Risteli L and Risteli J, 1990; Risteli J and Risteli L, 1995b), and I have previously shown changes in plasma markers of collagen turnover following myocardial infarction and demonstrated their value in the non-invasive assessment of ventricular remodelling (Chapters 4 and 5). I hypothesise that a degree of ventricular remodelling occurs in some patients presenting with acute coronary syndromes, even in the absence of myocyte necrosis. Due to the

marked heterogeneity of this patient population, remodelling is likely to occur at the more "high-risk" end of the spectrum and I hypothesisc that plasma markers of collagen turnover may reflect this.

I therefore examined changes in plasma levels of procollagen type I carboxy-terminal propeptide (PICP) and C-telopeptide for type I collagen (CITP), markers of synthesis and degradation respectively (Risteli J and Risteli L, 1995b), in a cohort of patients presenting with non-ST elevation acute coronary syndromes. Plasma levels of Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1), as a marker of inhibition of degradation, were also measured. Patients were dichotomised into high and low-risk groups based on the 12 lead ECG and troponin assay. High-risk was defined as the presence of dynamic ST depression/sequential T wave changes and/or troponin I >0.4ng/ml at 12 hours post admission. Low-risk was defined as the absence of ECG changes and a negative troponin.

6.2 Methods

Detailed methodology can be found in Chapter 2, section 2.4.

6.2.1 Patient Population

52 patients presenting to the Coronary Care Unit with a history suggestive of an acute coronary syndrome without ST elevation on a 12 lead ECG were considered for the study. Inclusion was based on history alone - chest pain typical of

myocardial ischaemia and occurring at rest starting within six hours of presentation and being present for at least 20 minutes. Patients with ECG fitting the criteria for reperfusion therapy or factors affecting assessment of ST/T segments, such as bundle branch block, concurrent digoxin therapy or the presence of a permanent pacemaker were excluded. Other exclusion criteria are listed in Chapter 2 (table 2.1).

6.2.2 Risk Stratification

Patients were dichotomised into high and low-risk groups based on ECG criteria and measurement of plasma troponin I as outlined above.

(a) Electrocardiography

12 lead ECGs were performed on admission and at 24 and 48 hours to assess ST segment deviation and T wave morphology. Additional ECGs were performed if the patient developed further pain. ST depression was defined as planar or down-sloping ST depression of 0.5mV, 80ms after the J point in two or more contiguous leads that was not there on prior ECGs (if available). Up-sloping ST depression was discounted. Sequential ECG changes were defined as new biphasic T waves or T inversion in two or more contiguous leads on ECGs performed subsequent to admission (Cannon et al. 1997).

(b) Troponin I

Cardiac troponin I was measured at 12 hours following admission using a microparticle enzyme immunoassay technique using a commercially available reagent pack (Troponin I No 3C29-66, Abbott Laboratories, Illinois, USA). The 95th percentile for a normal population in this assay is 0.4ng/ml and values above this were considered positive.

6.2.3 Blood Sampling and Biochemical Analyses

Sequential venous blood samples were taken for the measurement of plasma TIMP-1, PICP and CITP – on admission (O/A), at 12 hours, 24 and 48 hours. This time period was chosen as we have previously demonstrated that in ST elevation MI, changes in these plasma markers occur within the first 24 hours and reach a plateau by 48 hours (Chapter 4).

To avoid the discomfort of repeated venepuncture, blood was drawn from an indwelling cannula. Samples were spun at 3000rpm and the plasma frozen until use. Plasma PICP and CITP were measured by commercially available radio-immunoassays (Orion Diagnostica, Finland) using previously validated techniques (Melkko JT et al. 1990; Risteli J et al. 1993). Within assay variabilities are <4% with a <7% between assay variability. Due to the unexpected discontinuation of the original TIMP-1 ELISA kit by Amersham Pharmaceuticals (Chapter 3), only 33 patients had serial assessment of plasma TIMP-1. Intra and inter assay variabilities for plasma TIMP-1 are 4.0 and 4.4% respectively.

6.2.4 Statistical Analysis

Statistical analysis was performed using Minitab statistical software (Minitab Inc, Pennsylvania). All continuous variables are expressed as mean +/- one standard error of the mean. Changes over time were investigated by repeated measures of analysis of variance. Since biochemical variables were not normally distributed, log transformation was applied prior to statistical analysis. However, all variables are presented in the non-logarithmic format. Two-tailed Student's t test was used for comparison of means and a p value of <0.05 was considered significant.

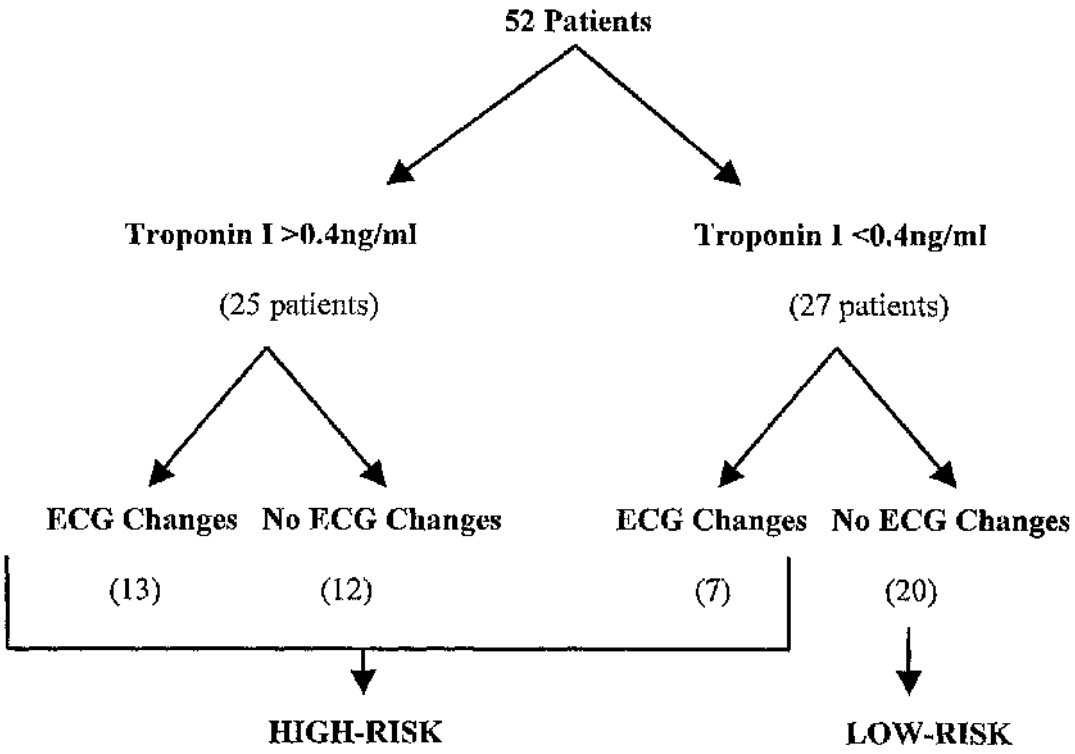
6.3 Results

6.3.1 Baseline Characteristics and Risk Stratification

Twenty of the 52 patients had no dynamic or sequential ECG changes and had levels of troponin I <0.4 ng/ml at 12 hours and were categorised as "low-risk". Of the 32 in the "high-risk" category, 13 had both ECG changes and positive troponin, 7 had only ECG changes and 12 were positive for troponin but had no ECG changes (figure 6.1). No patients had elevation in standard cardiac enzymes (defined as more than 2x upper limit of normal). Baseline demographics of the two groups are illustrated in table 6.1. Males comprised nearly two thirds of the patients in both groups. Mean age was similar as was admission blood pressure and number of diabetics. Slightly more patients in the high-risk group received low molecular weight heparin (94% vs. 85%) and IV GTN (25% vs. 10%), but similar rates of aspirin and beta-blocker usage were seen. This disparity is

probably due to the presence of dynamic ST/T changes in a cohort of the high-risk group.

Figure 6.1



Legend - Risk stratification based on troponin I and ECG findings.

Table 6.1

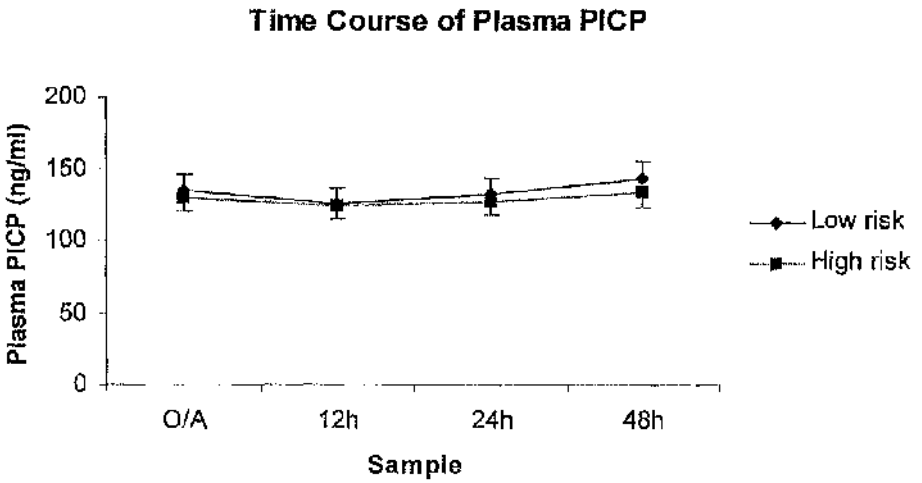
	HIGH-RISK N(%)	**LOW-RISK** N(%)
Number	32	20
Mean age	63	61
Male	21 (66)	13 (65)
Diabetes	6 (19)	3 (15)
Mean admission BP	144/88	146/80
Aspirin	32 (100)	19 (95)
LMWH	30 (94)	17 (85)
Beta-blockers	7 (22)	4 (16)
IV GTN	6 (25)	2 (10)

Legend - Baseline demographics of high and low risk groups.

6.3.2 PICP

Mean admission levels of PICP were within the normal range in both low and high-risks groups at 135 (7) ng/ml vs. 131 (1) ng/ml respectively (high vs. low, p=ns). There was no significant change in plasma level of PICP from admission to 48 hours in either group, with the values at each time point in one group being superimposed on the other (figure 6.2). Means at all four time points were within two standard deviations of the mean for a normal population. There was no correlation of plasma PICP with absolute level of plasma troponin.

Figure 6.2

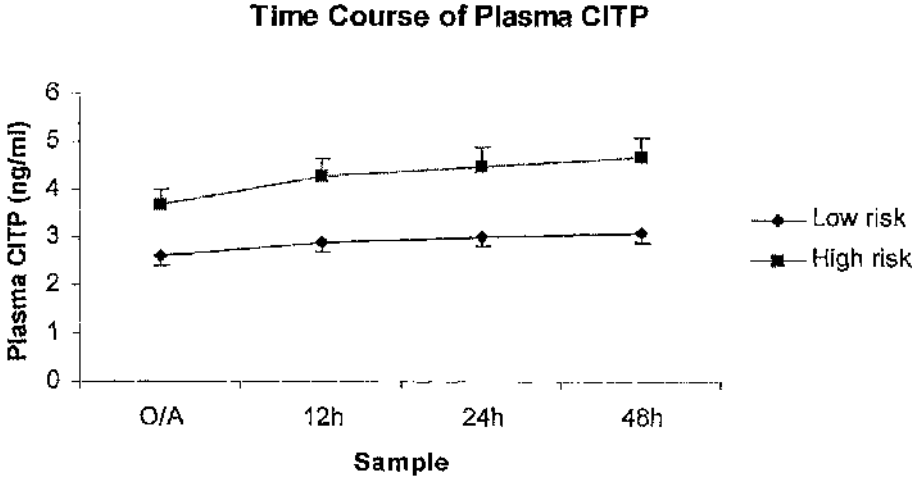


Legend - Changes (mean (SEM)) in plasma PICP over time.

6.3.3 CITP

The low-risk group showed no change over time and values at each time point were again within two standard deviations of the mean for a normal population at 2.6 (0.18) ng/ml. However, mean admission levels of CITP were higher in the high-risk group, 3.7 (0.16) ng/ml vs. 2.6 (0.18) ng/ml ($p<0.001$) and rose further in this group within 12 hours, reaching a plateau with a mean level of 4.7 (0.19) ng/ml at 48 hours (figure 6.3) (ANOVA, $p<0.01$). There was no significant change over time in plasma CITP from baseline in the low-risk group. Similar degrees of elevation were seen in those with positive troponin and those with negative troponin but with dynamic or sequential ECG changes. Of those with a positive troponin I, there was no significant correlation between absolute troponin level and admission CITP.

Figure 6.3

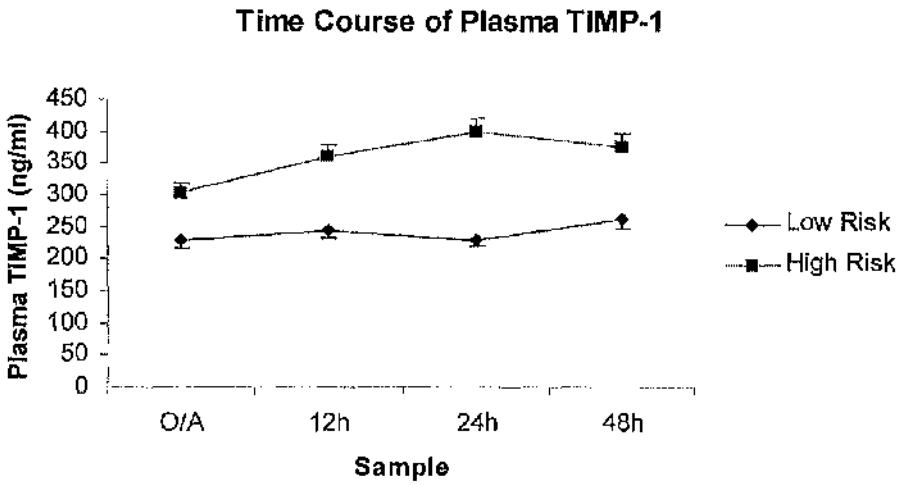


Legend - Changes (mean (SEM)) in plasma CITP over time.

6.3.4 TIMP-1

Mean plasma TIMP-1 in the low-risk group was also within two standard deviations of a normal population mean at 227 (19) ng/ml and showed no significant change over time. Mean TIMP-1 was within the normal range at all time points in this group. However, mean admission levels of plasma TIMP-1 were significantly elevated in the high-risk group at 302 (19) ng/ml (high vs. low, $p<0.01$) and rose further to a mean peak level of 400 (21) ng/ml (figure 6.4) (ANOVA, $p<0.01$).

Figure 6.4



Legend - Changes (mean (SEM)) in plasma TIMP-1 over time.

6.4 Discussion

6.4.1 Time Course

This is the first study to examine the pattern of change in plasma markers of collagen turnover in patients presenting with non-ST elevation acute coronary syndrome with no elevation in standard cardiac enzymes. We have demonstrated that patients with a diagnosis of non-ST elevation ACS based on history alone, in the absence of electrocardiographic changes and with a negative cardiac troponin I, display no time-dependent changes in any marker of collagen turnover. However, in those with high-risk features, there are sequential changes over time of plasma levels of both CITP and TIMP-1. No changes were seen in plasma levels of PICP. In this group, plasma levels of both CITP and TIMP-1 are

significantly higher at admission than levels in the low-risk group, being out with the normal range, and rise further over the next 48 hours.

6.4.2 Interpretation of Results

To allow interpretation of these findings, one must remember that CITP is produced during the degradation of Type I Collagen by endopeptidase cleavage by matrix metalloproteinases, and is therefore a marker of collagen breakdown (Risteli J et al. 1995a). Similarly, PICP is cleaved from procollagen during the production of Type I collagen and is consequently a marker of collagen synthesis (Melkko JT et al. 1990). This study therefore demonstrates serological evidence of increased collagen breakdown in the high-risk group with no associated alteration in synthesis, shifting the balance in favour of net collagen loss in the first 48 hours following ischaemia.

With respect to plasma TIMP-1, the elevation in the high-risk group may be explained by the fact that the assay does not differentiate between free, active TIMP-1 and inactive TIMP-1 bound with MMP. Therefore, the increase in TIMP-1 may be in response to increased MMP activity and be a reflection of TIMP complexed with MMP rather than increased free TIMP.

6.4.3 Comparison to ST Elevation MI Studies

I have previously demonstrated time dependent changes in plasma levels of PICP, CITP and TIMP-1 following ST elevation MI, and that early elevation in plasma

levels of CITP are associated with an adverse outcome and echocardiographic evidence of remodelling (Chapters 4 & 5). In this study of patients presenting with non-ST elevation acute coronary syndromes, low risk patients with no ECG changes and no evidence of myocardial necrosis had plasma levels of all three markers within the reference range. However, high risk ACS was associated with a time dependent rise in both CITP and TIMP-1. Admission levels of CITP in this group were similar to those with ST elevation MI with a normal wall motion index (3.7 vs. 3.1 ng/ml, p=0.3). Peak levels were also similar (4.7 vs. 4.8 ng/ml, p=0.9). This is in contrast to those presenting with ST elevation MI and early remodelling evident on echocardiography, where admission CITP was 4.5 ng/ml rising to 6.3 ng/ml at peak.

It is likely that the similar elevation in plasma CITP seen in the "high-risk" ACS and ST elevation MI with no echocardiographic evidence of remodelling groups are a reflection of changes occurring within the collagenous component of the heart. This may represent subtle remodelling of the extracellular framework, not sufficient to be evident on echocardiography. The similarity in CITP levels is also in keeping with the fact that mortality from high risk ACS approaches that of simple ST elevation MI (Antman et al. 1996; Levy, 1998; Antman et al. 2000).

6.4.4 The Role of Collagen Degradation

The pattern of change in the ST elevation MI studies (Chapters 4 & 5) is in keeping with the principal pathological finding of increased collagen degradation by matrix metalloprotcinases within the extracellular matrix in early phase of

remodelling following infarction (Cannon et al. 1983; Whittaker P et al. 1991; Cleutjens JPM et al. 1995). However, even in the absence of myocyte necrosis, severe ischaemia causes recruitment of inflammatory mediators from the blood and interstitial space, including interleukins, adhesion molecules and Tumour Necrosis Factors which upregulate the genetic expression of matrix metalloproteinases (Birkedal-Hansen H et al. 1993; Brilla CG et al. 1995; Schonbeck et al. 1997; Nicoletti A and Michel JB, 1999), thereby increasing proteolytic activity and causing net collagen loss. It is likely that this phenomenon is dependent on the degree of ischaemic burden and this is supported by our findings in this study of increased plasma levels of CITP, a marker of collagen breakdown, in patients at the "high-risk" end of the spectrum of non-ST elevation ACS. It is also interesting that we found similar evidence of collagen degradation in the group with positive troponin and those with negative troponin but with dynamic ECG changes. This suggests it is not cell death that is the stimulus for remodelling but may be the ischaemia itself.

6.4.5 Study Limitations

The ruptured plaque is the keystone of acute coronary syndromes (Davies, 2000), and increased MMP expression and activity have been implicated in the progression from stability to instability (Kaartinen M et al. 1994; Lee RT et al. 1996). Previous studies have shown increased levels of MMP-2 and TIMP-1 in the coronary sinus immediately following PTCA (Hojo et al. 2002), raising the possibility that this may be a reflection of plaque rupture and plaque remodelling. The degree of elevation in this study was mild (<10% change). In our study, the

degree of elevation in CITP in the high risk ACS group is much higher and is similar to that seen in ST elevation MI. It would appear more likely that the majority of change in CITP in our study is a reflection of collagenous remodelling of the cardiac extracellular matrix rather than of plaque remodelling in the coronary artery.

Another potential confounding variable is the use of low molecular weight heparin (LMWH). It is well documented that heparin administration is associated with suppression of aldosterone synthesis (Conn JW et al. 1966; Levesque H et al. 1990), and given the importance of the RAAS effectors on collagen metabolism (Brilla CG et al. 1995; Lindpaintner et al. 1993), one cannot discount the potential effect of LMWH on collagen homeostasis in this study. However, no change in plasma markers of collagen turnover was demonstrated in the low risk ACS group, the majority of whom (85%) also received LMWH. I would therefore propose that LMWH administration had little effect in this study.

6.5 Conclusions

In patients presenting with non-ST elevation acute coronary syndrome, time-dependent changes in plasma levels of CITP and TIMP-1 are associated with the presence of high-risk features of dynamic changes on the 12 lead electrocardiogram or a positive troponin. No changes are seen in patients with chest pain in the absence of these high-risk features. This may reflect a degree of remodelling in the high-risk group and may aid risk stratification of patients presenting non-ST elevation ACS.

CHAPTER SEVEN – TIME COURSE OF EARLY CHANGES IN PLASMA MARKERS OF COLLAGEN TURNOVER FOLLOWING PERCUTANEOUS TRANSLUMINAL CORONARY ANGIOPLASTY

7.1 Introduction

Changes to the collagen framework of the heart are important in the development of ventricular remodelling following myocardial infarction (MI) (Cannon et al. 1983; Whittaker P et al. 1991; Cleutjens JPM et al. 1995). Plasma markers of collagen turnover may be useful in the non-invasive assessment of left ventricular remodelling in patients presenting with acute coronary syndromes including MI (Chapters 4, 5 & 6), providing additional information with regard to prognosis (Chapter 5).

I have previously demonstrated time-dependent changes in plasma levels of procollagen type I carboxy-terminal propeptide (PICP) and C-telopeptide for type I collagen (CITP), markers of synthesis and degradation respectively (Risteli J and Risteli L, 1995b), following infarction (Chapter 4). The changes in plasma levels are rapid with CITP being elevated on admission with further change over the first few days. Admission values of CITP have a 74% predictive power for the subsequent development of wall motion abnormalities and are associated with other echocardiographic parameters of remodelling (Chapter 5). Plasma levels of

PICP also show time-dependent changes, but do not provide additional prognostic information.

These changes are in response to ischaemia and infarction due to abrupt coronary artery occlusion (Zhao M et al. 1987; Cleutjens JPM et al. 1995) which causes production and activation of matrix metalloproteinases (MMPs), the activity of which determines the extent of collagen breakdown (Carlyle et al. 1997; Herzog et al. 1998; Danielsen et al. 1998). Although previous studies have shown early up-regulation of matrix MMP genetic expression within a few hours of infarction (Herzog et al. 1998), which is associated with early collagen breakdown at a tissue level (Whittaker P et al. 1991; Cleutjens JPM et al. 1995), there are only limited data available on whether this is reflected in plasma levels of markers of collagen turnover (CITP and PICP). In my previous studies of myocardial infarction (Chapters 4 & 5), the differences in plasma levels of CITP and PICP between groups displaying evidence of remodelling and those with no such evidence were apparent from admission, suggesting the divergence in levels between the groups occurs early. Similar patterns of change were also demonstrated in high risk vs. low risk patients presenting with non-ST elevation acute coronary syndromes (Chapter 6). As all patients in these studies were recruited within 6 hours of the onset of symptoms, this divergence must start within a few hours of the ischaemic stimulus, for the difference in levels to be apparent at presentation.

I therefore sought to examine the early time course of plasma levels of CITP and PICP in a human model of controlled acute coronary artery occlusion. To this end,

I recruited a cohort of patients undergoing percutaneous coronary intervention and performed sequential blood sampling to assess temporal dynamics of plasma CITP and PICP.

7.2 Method

Detailed methodology can be found in Chapter 2, section 2.5.

7.2.1 Patient Population

Fourteen patients undergoing elective percutaneous coronary intervention (PCI) to a single lesion in a single coronary artery in our regional cardiac catheterisation laboratory were recruited for this study. Exclusion criteria included factors influencing collagen metabolism or elimination of propeptides, such as surgery within the last six months, hypertension, renal or hepatic dysfunction, in addition to factors making interpretation of the electrocardiogram difficult, such as the presence of a permanent pacemaker system, LBBB or concurrent digoxin therapy. All patients were naïve to ACE inhibitors, angiotensin II receptor blockers and spironolactone. To exclude changes in plasma markers of collagen turnover due to catheter manipulation in the aorta and coronary ostia or to the effects of intra-coronary injection of contrast media, a group of eight patients undergoing day-case elective coronary angiography were recruited as a control group (Control).

7.2.2 Blood Sampling and Biochemical Analyses

To avoid the discomfort of repeated venepuncture, blood was drawn from an indwelling venous cannula. In the angioplasty group, 6 sequential samples were taken for the measurement of plasma PICP and CITP at serial time points – pre-procedure (Pre), 1 minute following first balloon inflation (1 min), at the end of the procedure (End), 4 hours following procedure (4h) then at 8 and 16 hours. Given that elective diagnostic coronary angiography is performed as a day case, blood was drawn on only three occasions for the measurement of these propeptides – pre-procedure (Pre), at the end of the angiogram (End) and at 4 hours post procedure (4h). Blood was spun at 3000rpm and plasma frozen until use.

Plasma PICP and CITP were measured by commercially available radio-immunoassays (Orion Diagnostica, Finland), as described previously (Melkko JT et al. 1990; Risteli J et al. 1993). Within assay variability is <4% in our laboratory, with a <7% between assay variability. Troponin was measured by standard immunoassay (Bayer Corporation, New York, USA).

7.2.3 Assessment of Peri-procedural Ischaemia

The presence of peri-procedural ischaemia was identified in the cohort undergoing PCI by continuous electrocardiographic monitoring in the catheterisation laboratory and by the measurement of plasma troponin I at 16 hours. A positive troponin (>0.07ng/ml) or transient ST segment elevation or depression >1mm in

two leads during balloon inflation was taken as evidence of induced ischaemia. Cut off values for troponin I are different in this study compared to that used in Chapter 6 due to utilisation of different assays in the two studies (Chapter 2, section 2.5.4b).

7.2.4 Statistical Analysis

Statistical analysis was performed using Minitab statistical software (Minitab Inc, Pennsylvania, USA). All continuous variables are expressed as mean +/- one standard error of the mean. Given this study is designed to specifically look at the early temporal dynamics of serological markers of collagen turnover, results are also expressed as percentage change from baseline. Changes over time were investigated by repeated measures of analysis of variance. Biochemical variables were not normally distributed so underwent log transformation prior to using a two-tailed Student's t test for comparison of means, although all data are presented in the non-logarithmic format. A p value of <0.05 was considered significant. This study was designed with 90% power to detect a change in CITP from baseline of 25% and 90% power to detect a 50% change in PICP in the angioplasty group.

7.3 Results

7.3.1 Baseline Characteristics

Mean age in the PCI group was 60 years vs. 65 in the Control group undergoing diagnostic angiography with similar percentage of male patients in both groups

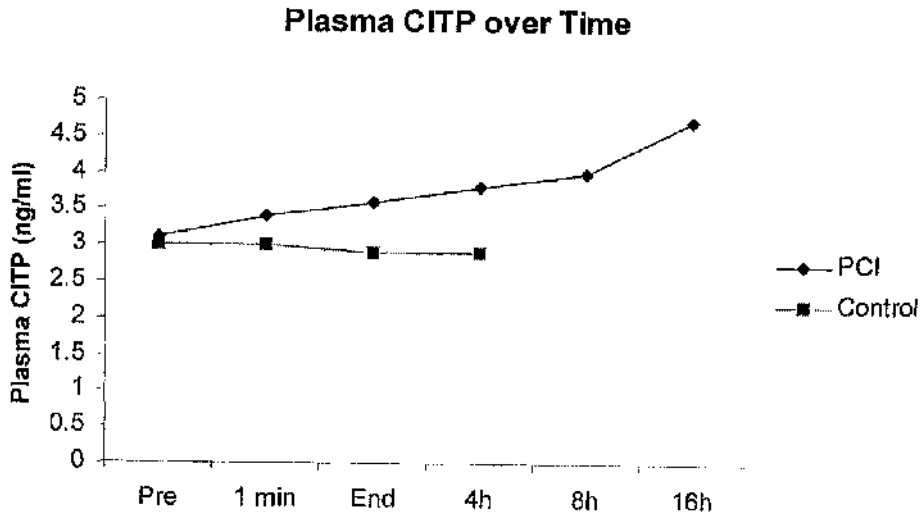
(64 vs. 62.5%). In the PCI group, the culprit artery was the left anterior descending in 6 patients, the right coronary artery in five and the circumflex artery in the remaining three. Intra-coronary stents were deployed in the majority of cases (12/14). The mean time of balloon inflation was 54 seconds with an average of 1.8 inflations per procedure. Ten had elevated troponin at 16 hours (mean 1.3, range 0.7 – 2.9 ng/ml) and nine had ST elevation or depression on the ECG during balloon inflation. Only one patient had no evidence of ST segment shift and a negative troponin, and was classed as not showing evidence of peri-procedural ischaemia.

7.3.2 CITP

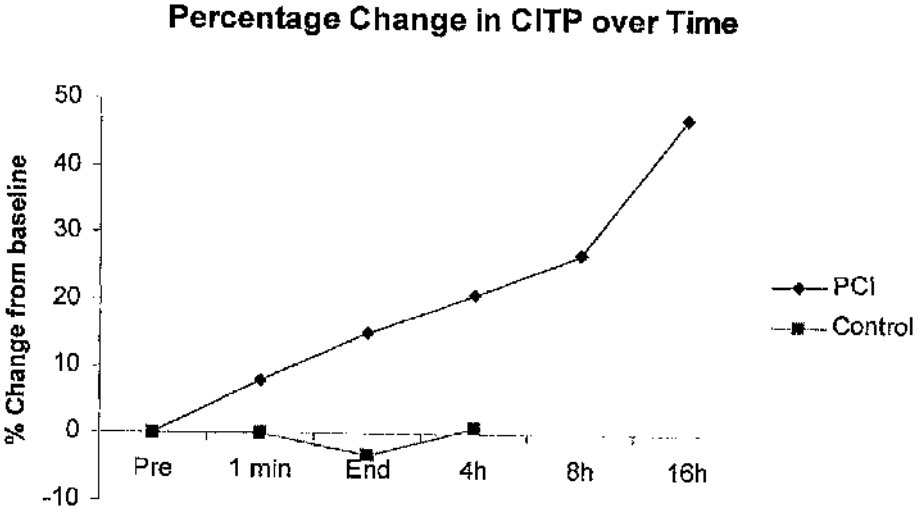
Mean CITP pre-procedure in the PCI group was 3.1 ng/ml with a sequential rise in group mean CITP over time, peaking above the normal range at 4.7ng/ml by 16 hours ($p<0.05$). Mean CITP in the Control group was within the normal range at all time points (figure 7.1a). With regard to percentage change from baseline, all patients bar one in the PCI cohort demonstrated a sequential rise in plasma CITP, starting immediately post balloon inflation and continuing thereafter. The largest proportional rise was between 8 and 16 hours with peak CITP at 16 hours being an average of 48% higher than pre-procedure values (figure 7.1b). There was no significant change over time in the Control group, although sampling was not extended beyond 4 hours. There was also no significant change in plasma CITP in the patient with negative troponin and no ECG changes.

Figure 7.1

(a)



(b)



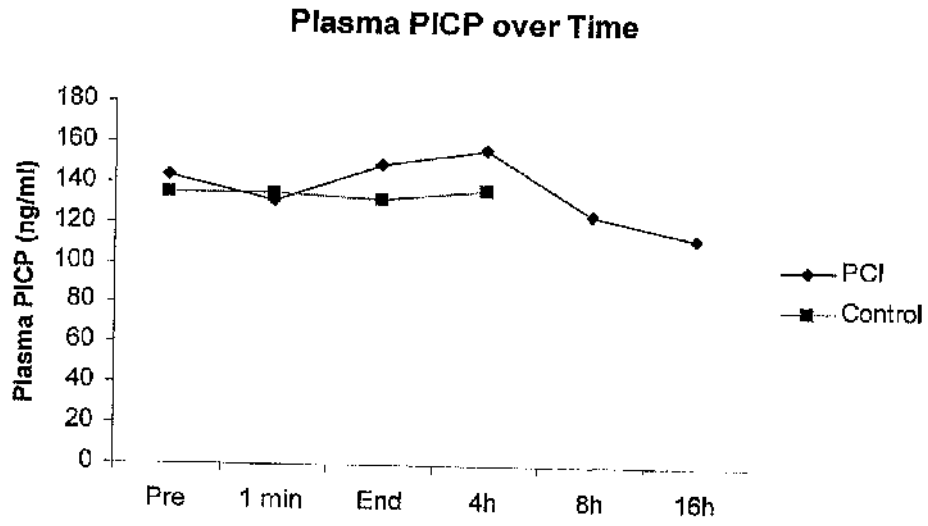
Legend - Pattern of change of plasma CITP in PCI and Control groups demonstrated as (a) Mean absolute values at each time point and (b) Mean percentage change from baseline.

7.3.3 PICP

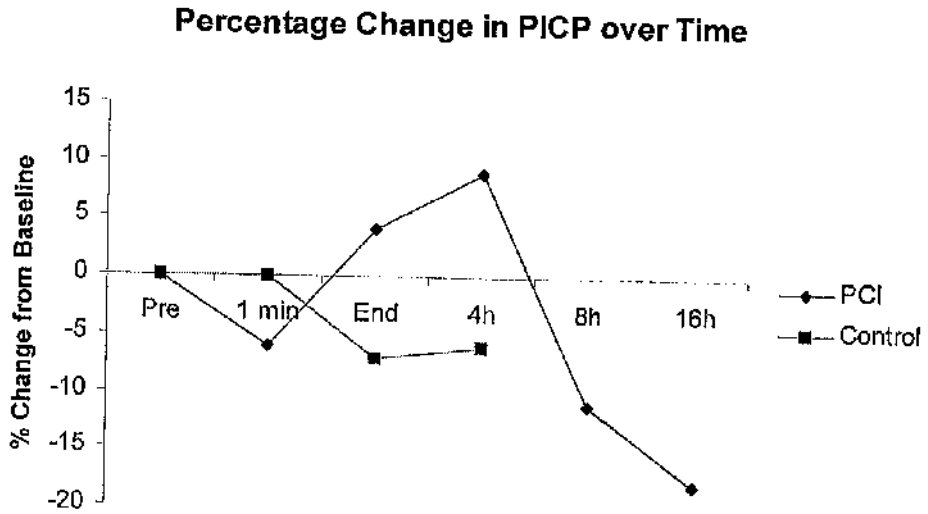
Changes over time of plasma PICP within the PCI group were initially heterogeneous with some patients displaying an early rise following angioplasty and others showing an early fall in plasma PICP. Group mean initially rose over during the procedure, then fell below pre-procedural values by eight hours, with a trough at 16 hours (figure 7.2 a & b), although this does not reach statistical significance (143 vs. 113ng/ml, p=0.09). There was no significant change over time in the Control group, although sampling was not extended to 16 hours.

Figure 7.2

(a)



(b)



Legend - Pattern of change of plasma PICP in PCI and Control groups demonstrated as (a) Mean absolute values at each time point and (b) Mean percentage change from baseline.

7.4 Discussion

I have demonstrated time-dependent changes in plasma levels of CITP following myocardial ischaemia caused by transient occlusion of a single epicardial coronary artery during percutaneous coronary interventional procedures. No such changes were demonstrated in the control group undergoing routine angiography. The ischaemic burden of the PCI group was high as manifested by the high proportion of dynamic ST segment shift and rate of positivity for troponin.

The changes to plasma levels of PICP were heterogeneous within the group undergoing PCI, with no significant change in the group mean within the first few hours. Although levels in the group decreased by 8 hours with a trough at 16 hours, this did not reach significance. The heterogeneity of plasma PICP response to ischaemia may be reflected by the wide normal range and standard deviations.

In contrast to PICP, the changes in plasma CITP were consistent within the group as a whole. Elevation in plasma CITP was evident as early as 1 minute following first balloon inflation, although did not reach statistical significance until 8 hours. There was a further increase at each time point, peaking at 16 hours at an average of nearly 50% higher than levels prior to procedure. This is in keeping with the studies of MMP activation discussed previously and illustrates the rapidity of collagen degradation following ischaemia and infarction (Zhao M et al. 1987; Whittaker P et al. 1991; Cleutjens JPM et al. 1995). Indeed, there is up to a 25% reduction in myocardial collagen content in the first 24 hours following AMI (Cannon et al. 1983). This is in contrast to increased collagen production which

occurs in the following days and weeks (Fishbein MC et al. 1978; Pfeffer MA and Braunwald E, 1990).

The early phase of histological evolution of myocardial infarction is therefore characterised by collagen degradation, and the finding in this study of early elevation of plasma levels of CITP is entirely in keeping with this. The rapidity of change of this propeptide may explain the divergence of plasma level of CITP at admission between patients who develop signs of remodelling and those who do not (Chapter 5) in the setting of ST elevation MI and between patients with high risk and low risk acute coronary syndromes (Chapter 6).

7.5 Study Limitations

There is some evidence of mild elevation in MMP and TIMP-1 levels following percutaneous intervention and the authors felt this was reflective of plaque remodelling rather than representing myocardial collagenous remodelling (Hojo et al. 2002). However, as discussed in section 6.5, the degree of elevation of plasma markers of collagen turnover seen in high risk ACS patients (Chapter 6) would appear to be far greater than one would expect from coronary arterial plaque remodelling. In the ACS study, "high risk" was defined as positive troponin or dynamic ST segment shift. It is therefore unsurprising that the pattern of elevation in plasma CITP at 16 hours in this PTCA study is similar to that seen in patients with high risk ACS, given that the majority had elevated Troponin (10/14), and only one patient had neither elevated Troponin or dynamic ECG changes. Therefore, the ischaemic burden is similar between the groups, and this is reflected

in a similar degree of collagen breakdown in the immediate post ischaemic period. The ischaemic burden of patients in Hojo *et al*'s study is not clear and may be significantly different to that experienced by this study population. Furthermore, they demonstrated only mild elevation in plasma levels of MMP and TIMP-1 (<10%) compared to the 50% increase in CITP seen in my study. I would therefore suggest that the majority of change in CITP is a reflection of myocardial collagen degradation rather than of plaque remodelling in an epicardial coronary artery.

7.6 Conclusions

The early dynamism of plasma levels of markers of collagen turnover further support their use in the non-invasive assessment of collagen turnover in ventricular remodelling following acute ischaemic events.

CHAPTER EIGHT – SUMMARY AND CONCLUSIONS

8.1 Summary of Results

The series of studies contained within this thesis demonstrates time-dependent alterations in plasma levels of markers of collagen turnover following a variety of ischaemic insults – ST elevation myocardial infarction, non-ST elevation acute coronary syndrome and coronary arterial occlusion during percutaneous coronary intervention. Our data supports our original hypotheses that levels of these markers would display an early change following ischaemic events and that they may be useful in the non-invasive assessment of the remodelling process (Chapter 1, section 1.6).

Chapter Three illustrated that the methodology for the assays is sound with good reproducibility, as reflected by the excellent intra- and inter-assay variabilities for all three markers (TIMP-, PICP and CITP), ranging from 3.1 – 6.9%. We also showed that there was no diurnal variation and defined a mean and range for all three markers in a local normal healthy population of a similar age and sex distribution to our study groups.

The remaining chapters were concerned with ischaemic coronary syndromes. As previously discussed, acute coronary syndrome is an umbrella term for all presentations with acute onset ischaemic chest pain, and represents a continuum from the traditional diagnostic categories of unstable angina through to acute myocardial infarction with a wide range of morbidity and mortality. Chapters Four

and Five studied those at highest risk, patients presenting with their first ST elevation acute myocardial infarction (MI). Chapter Four demonstrated time-dependent changes in plasma levels of CITP, PICP and TIMP-1 following MI. In this study, raised CITP on admission, a marker of collagen breakdown, was associated with the development of heart failure and a trend towards an adverse outcome. This study also showed that the changes in these markers of collagen turnover were not related to systemic activation of the renin-angiotensin-aldosterone system, although no examination of cardiac utilisation of RAAS effectors was made.

Chapter Five examined the relationship of plasma levels of CITP and PICP with the remodelling process following ST elevation MI. Patients were dichotomised using established echocardiographic parameters of remodelling. In this study, marked differences in plasma levels of both CITP and PICP were seen between the group with abnormal wall motion on echo at day 4 and those with no such echo evidence of remodelling, with raised levels of CITP and reduced PICP being apparent in the remodelling group. Furthermore, absolute levels of these markers provided information regarding the subsequent development of remodelling. Admission CITP predicted the development and extent of wall motion abnormalities and was associated with reduced mitral deceleration time, another echo parameter of remodelling. In summary, this study demonstrated that early measurement of a plasma marker of collagen breakdown correlated with, and predicted, the development of echocardiographic parameters of remodelling, thereby providing early prognostic information following infarction.

Chapter Six was concerned with patients presenting with non-ST elevation acute coronary syndromes (ACS), and demonstrated time-dependent changes in plasma levels of CITP and TIMP-1 in patients with high-risk features such as dynamic changes on the 12 lead electrocardiogram or a positive troponin. No changes were seen in patients with chest pain in the absence of these high-risk features and this would support our hypothesis that these markers of collagen turnover reflect a degree of remodelling in the "high-risk" end of the wide spectrum of risk encompassed by the term non-ST elevation ACS, and that it is likely that ischaemia is the stimulus rather than myocardial necrosis *per se*. Indeed, the degree of elevation in high risk ACS mirrors that of ST elevation MI without overt echo evidence of remodelling. It is well documented that collagenous remodelling within the extracellular matrix can occur without overt myocyte necrosis (Zhao M et al. 1987), and interestingly the prognosis in these two groups is similar (Antman et al. 1996; Levy, 1998; Antman et al. 2000).

In the non-ST elevation ACS study (Chapter 6), the divergence of plasma CITP between the high and low-risk groups was apparent from admission. The same is true of plasma levels of both CITP and PICP following ST elevation MI with divergence between those with echo evidence of remodelling and those without, also being evident at admission (Chapter 5). The immediate temporal dynamics of alterations in plasma levels of CITP and PICP following ischaemia were therefore examined in Chapter Seven, using percutaneous coronary intervention as a human model of acute coronary artery occlusion. This study demonstrated early elevation in plasma CITP following transient coronary occlusion. Although it took up to 8 hours for this rise to reach statistical significance, CITP rose steadily over time,

being apparent immediately following balloon inflation and persisting long after the cessation of the ischaemic stimulus. Therefore, the rapidity of change of plasma levels following ischaemia, coupled with the predictive value of plasma levels, make the measurement of this propeptide an attractive option in the early non-invasive assessment of the remodelling process.

8.2 Comparison with Previous Studies and the Pathophysiology of Remodelling

This is the largest series of studies examining the use of plasma markers of collagen turnover in the non-invasive assessment of remodelling following myocardial ischaemia. Most previous studies have focused on the markers of collagen synthesis, PICP and PIIINP, and have shown that increased plasma levels are associated with fibrosis in hypertensive heart disease and heart failure (Laviades C et al. 1994; Diez J et al. 1995; Klappacher et al. 1995). However, these are chronic conditions, with continual fibrogenic stimuli acting on the extracellular matrix (ECM). This is quite different from the acute changes to the ECM and myocardial collagen seen during remodelling following ischaemia and myocardial infarction.

With respect to plasma markers and myocardial infarction, PIIINP is the best studied and has been shown to be elevated immediately following infarction (Jensen LT et al. 1990; Host NB et al. 1995; Uusimaa P et al. 1997; Modena et al. 1998; Poulsen SH et al. 2000), and one might assume this to reflect collagen synthesis and subsequent fibrosis. However, although fibrosis is a key component

of the remodelled ventricle, this is a feature of late remodelling, occurring weeks to months to years after the initial event, probably reflecting end stage maladaptive response to injury (Fishbein MC et al. 1978; Pfeffer MA and Braunwald E, 1990; Wei et al. 1999). Therefore, the finding of increased levels of PIIINP early following infarction in these studies appears inconsistent with pathological data.

Conversely, our finding of early elevation in plasma levels of CITP is much more in keeping with the known pathophysiology of early remodelling following MI, a process characterised by net collagen loss (Cannon et al. 1983; Whittaker P et al. 1991; Cleutjens JPM et al. 1995). We also found late elevation of PICP at 1 month, again consistent with pathological data of scar formation which is complete 4-6 weeks after the event (Fishbein MC et al. 1978). One possible explanation for the apparent discrepancy between the results of PICP in our studies compared to the previous studies utilising PIIINP may be the fact that unlike PICP, PIIINP is incompletely cleaved during collagen production and is therefore also released when collagen is degraded (Risteli J and Risteli L, 1995b). As a result, levels are influenced by the degree of collagen breakdown, and the apparent early rise seen in these studies may be a consequence of increased degradation. Indeed, the time course of elevation of PIIINP in previous studies mirrors that of CITP in our studies.

One must also remember that net collagen content is the product of a dynamic equilibrium between synthesis and degradation, and these previous studies examined one marker in isolation. Our series of studies examined both sides of collagen balance, providing information with respect to both synthesis and

breakdown, increasing our understanding of the processes involved and assessing their use as indicators of the remodelling process, not only in myocardial infarction, but in other ischaemic chest pain syndromes.

8.3 Potential Applications and Areas for Further Study

Acute coronary events are associated with significant morbidity and mortality and there are many strategies available to reduce this risk in both the settings of ST elevation myocardial infarction and non-ST elevation acute coronary syndromes. However, the key lies in identifying those most likely to benefit from these interventions, so as to avoid unnecessary risks and best utilise limited resources.

Ventricular remodelling following ST elevation myocardial infarction is a powerful adverse prognostic indicator. Strategies to limit the remodelling process are available, including achieving patency of the infarct related artery and inhibition of the renin-angiotensin-aldosterone and sympathetic nervous systems (ISIS-1 (First International Study of Infarct Survival), 1988; Pfeffer MA et al. 1992; AIRE study investigators, 1993; ISIS-4 Collaborative Group, 1995; Fibrinolytic Therapy Trialists, 1994; Grines et al. 1999b). The extent of benefit of these strategies is dependent on the absolute risk of the patient, with high-risk groups having potentially the most to gain. Selection of patients suitable for each intervention is therefore paramount and should be based on the risk:benefit ratio of the individual rather than on a population basis. At present this is difficult to achieve although historical, electrocardiographical and clinical features help in the early assessment of risk (Hammermeister KE et al. 1979; Fibrinolytic Therapy

Trialists, 1994; Mahon et al. 1999). Echocardiography is a powerful predictor of both morbidity and mortality following infarction (Sutton et al. 1997; Migrino et al. 1997). However, echo identifies early remodelling when it has already begun, and therefore its use as an aid to risk stratification in the acute phase is limited.

In contrast, our studies support the potential use of biochemical markers of collagen turnover as an early identifier of those at risk of remodelling in the immediate period following infarction (Chapter 5). The measurement of plasma levels of CITP at admission provides additional prognostic information to clinical and other variables, identifying those most at risk of remodelling, and therefore at risk of a poor outcome. The early measurement of CITP may allow better targeting of therapy, reducing the risk of potentially serious side effects in those at low risk of an adverse outcome, and ensuring those liable to benefit from these interventions are identified early. For example, the level of plasma CITP at admission may influence the choice between thrombolytic or mechanical based reperfusion strategies and perhaps help in the decision regarding acute administration of ACE inhibitors, aldosterone antagonists, beta-blockers or statins. Although the use of plasma CITP in the early identification of high-risk patients is an attractive prospect, further work is required to determine whether interventions based on levels confer a clinical benefit.

Serological markers of collagen turnover may also help in the risk stratification of patients presenting with non-ST elevation acute coronary syndromes. As previously discussed, ischaemia can cause alterations to the ECM in the absence of overt myocyte necrosis (Zhao M et al. 1987; Charney et al. 1992). We have

demonstrated early elevation in plasma CITP in high-risk patients (Chapter 7), a feature not present in those subsequently deemed to be low risk. This divergence of plasma CITP between high and low risk groups apparent on admission sampling may allow very early identification of those at highest risk. Further work is required to determine whether measurement of CITP provides any additional prognostic information to routine investigations such as the 12 lead ECG and troponin measurement, and whether early measurement of plasma CITP could be used to guide interventions.

Finally, as early remodelling is characterised by collagen degradation, which is dependent on the activity of matrix metalloproteinases, there has been much interest in the potential use of synthetic inhibitors of matrix metalloproteinases as a method to attenuate the remodelling process. Manipulation of the MMP-TIMP system following MI is an attractive concept, and two animal studies have examined the use of synthetic MMP inhibitors in this setting (Rohde et al. 1999; Creemers et al. 1999). As previously discussed, although these studies demonstrated decreased LV dilatation and improved fractional shortening, one study also demonstrated delayed infarct healing (Creemers et al. 1999). It is important to remember that MMPs have diverse biological actions, being involved in the regulation of biologically active growth factors such as TNF-alpha, TGF-beta and IL-1 (Gearing et al. 1994; Schonbeck et al. 1998). Therefore, inhibition of MMPs may have potent adverse effects on other cellular and matrix components. Furthermore, MMP inhibition may also reduce fibroblast activation and paradoxically reduce collagen synthesis. Like all therapeutic interventions, the risk:benefit ratio of MMP inhibition following MI must be established. It is likely

that biochemical assessment of collagen turnover will play a key role in the future when considering the use of MMP inhibitors. Further work in this field is required.

8.4 Conclusions

The series of studies in this thesis provide further insight into the pathophysiology of the remodelling process and support the use of plasma markers of collagen turnover in the non-invasive assessment of remodelling following acute ischaemic events.

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