Platelet Reactivity, Polymorphisms and Premature Myocardial Infarction

Thesis submitted for the degree of
Doctor of Medicine
at the University of Leicester

by

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Author's declaration

I declare that the work has been done and the thesis composed by myself, and that the books and papers cited were all consulted by me personally.

I was responsible for writing the grant proposals to secure the British Cardiac Society David de Bono Award, The British Medical Association Edith Walsh Award and The British Medical Association Geoffrey Holt and Ivy Powell Cardiovascular Research Award. I directly contacted all potential subjects and planned the co-ordination of recruitment, phenotyping and laboratory investigations. I carried out the majority of sample preperations for the analyses and all the DNA extractions as well as the bulk of the platelet function flow cytometry.

The statistical calculations were initially analysed by myself and the methods employed verified by Dr. Martin Tobin and Professor John Thompson.

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Chapter 1

Introduction

1.1 Importance and Impact of Myocardial Infarction

"Every two minutes someone in the UK suffers a heart attack.
Every four minutes someone in the UK dies from a heart attack" [1]

Coronary heart disease (CHD) is the single largest cause of mortality in the Western World and in the United Kingdom (UK) (Figure 1). Although, death rates are decreasing in most Western countries globally the incidence continues to increase and by the year 2020 CHD will become the number one public health problem on the planet (Figure 2) [2]. Acute myocardial infarction (MI) is the most important complication of CHD. There are 300,000 MIs annually in the U.K. leading to 125,000 deaths of which 45,000 are premature (under the age of 65).

Figure 1 ***Deaths by cause, men and women, 2000, United Kingdom***

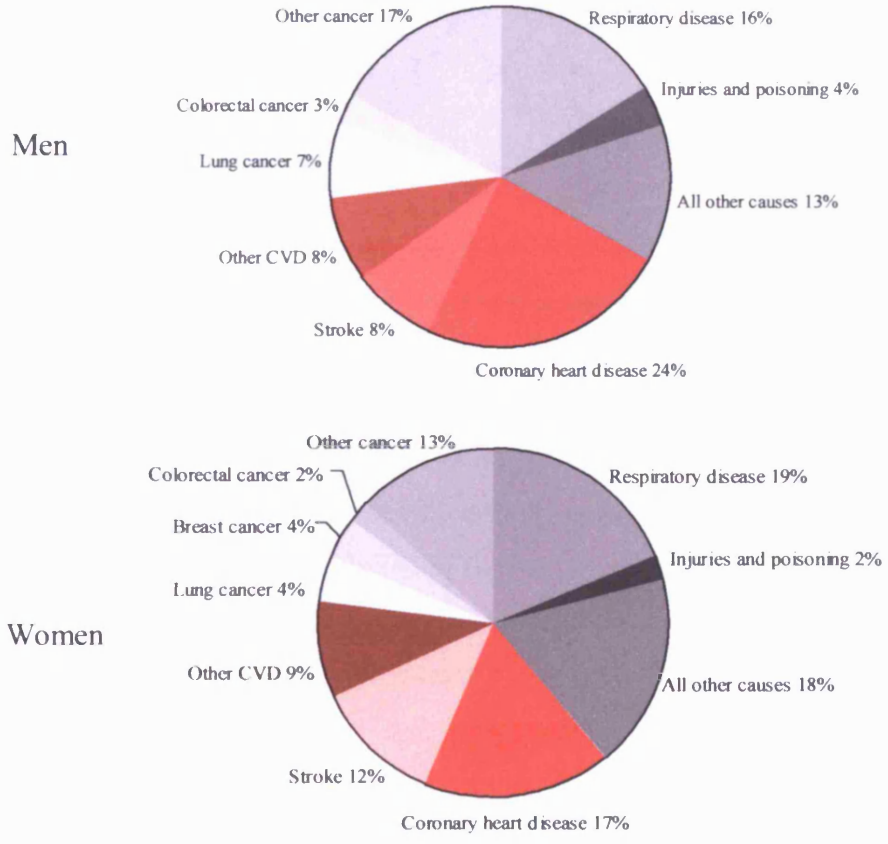

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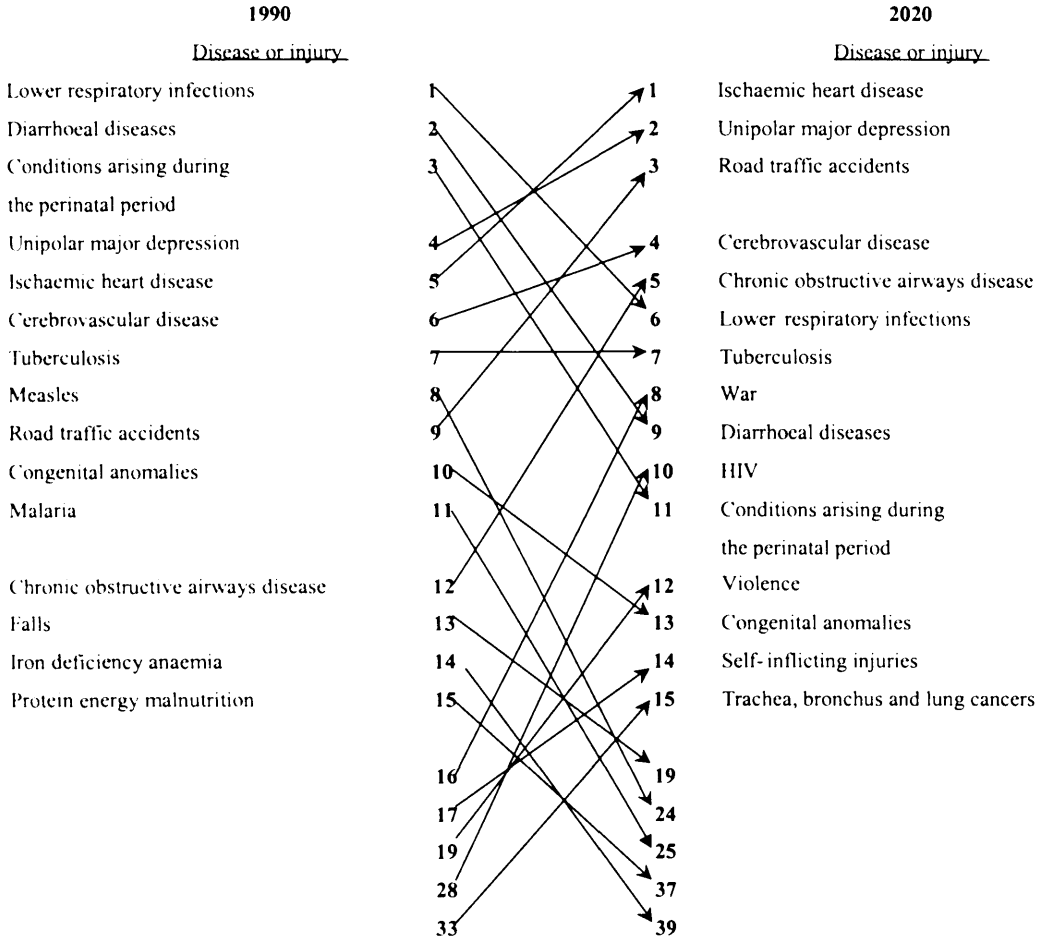
Figure 2 ***Change in the rank order of disease burden for 15 leading causes worldwide, 1990-2020***



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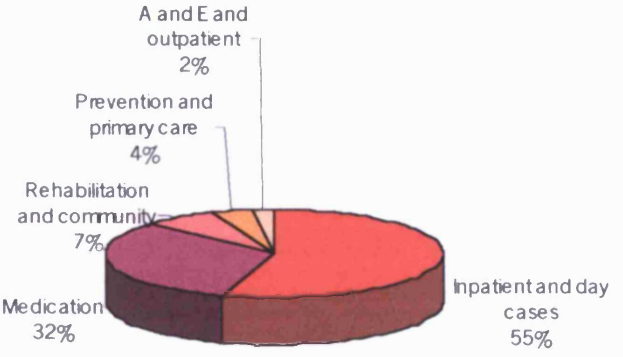
The burden and impact of CHD on the Health Service is tremendous with a total of 1.4 million angina patients as well as the 300,000 yearly MIs. The annual cost to the health service is estimated at £1.6 billion of which the vast majority is spent on inpatient care (55%) and medication (32%). Only 1% of the budget goes towards primary prevention (Figure 3). The costs in terms of production losses, as a result of mortality (£1.8 billion), morbidity (£4.8 billion) and informal care (£2.2 billion) totals a staggering £8.5 billion[1].

The British government has targeted coronary heart disease as a top-level priority for improved prevention, diagnosis and delivery of treatments in its 10-year blueprint for the Health Service, The National Service Framework[3].

Figure 3 ***Costs of CHD to the National Health Service and social care system, 1996, United Kingdom***

	£ million	% of total
Prevention	11.6	1
Primary care	57.9	4
Accident and emergency care	5.2	0.3
Outpatients	19.5	1
Inpatients	878.6	54
Day cases	11.4	1
Medication	527.0	32
Rehabilitation	22.5	1
Community health services	26.5	2
Social services	69.8	4
Total	1,630.0	100



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1.2 Pathophysiology of Myocardial Infarction

Definition A myocardial infarction is a result of an acute occlusion of one of the coronary arteries leading to irreversible myocardial damage through ischaemia and necrosis. The usual substrate leading to an MI is atherosclerosis.

1.2.1 Atherosclerosis

Atherosclerosis, a disease of large and medium-sized arteries, is the primary underlying cause of angina, MIs, strokes and peripheral vascular disease. Epidemiological studies have revealed several important environmental and genetic risk factors associated with atherosclerosis (Section 1.2.2). The disease has a complex aetiological basis, but modern molecular investigative tools, including genetically modified mouse models, has resulted in a clearer understanding of the molecular mechanisms that connect altered cholesterol metabolism and other risk factors to the development of the atherosclerotic plaque. Atherosclerosis is no longer considered an inevitable degenerative consequence of aging, but rather a chronic inflammatory condition that can be converted into an acute clinical event by plaque rupture and/or thrombosis.

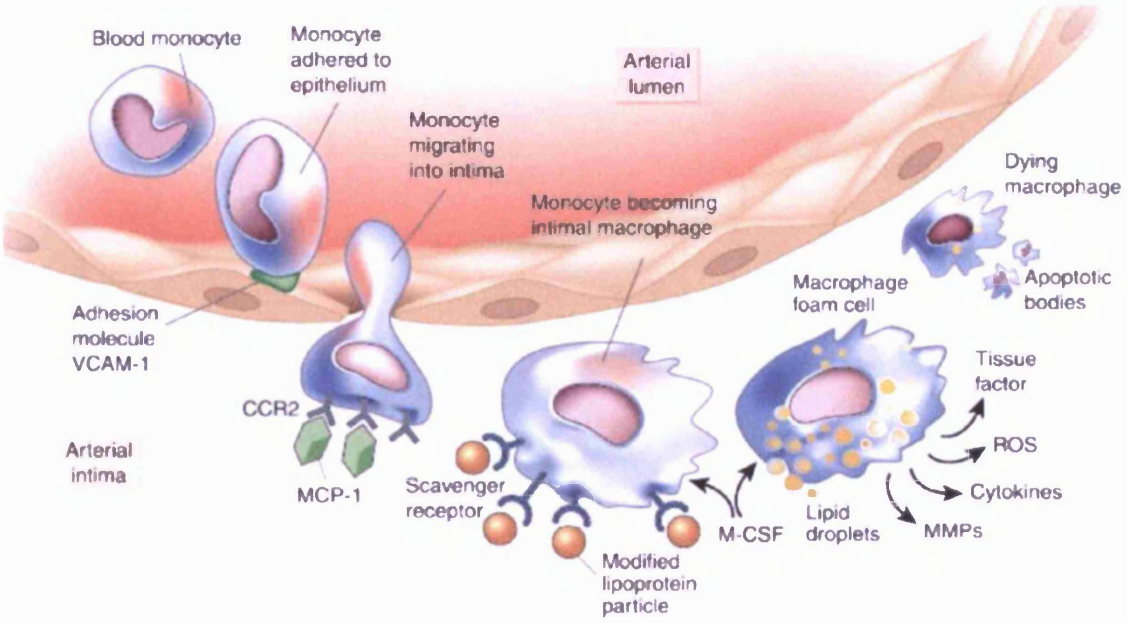
1.2.1.1 The biology of atherosclerosis

The hallmark of atherosclerosis is the accumulation of lipids and fibrous elements in the large arteries. The early lesions of atherosclerosis consist of subendothelial accumulations of cholesterol-engorged macrophages, called 'foam cells'. In humans, such 'fatty streak' lesions can usually be found in the aorta in the first decade of life[4] and are the precursors of more advanced lesions characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells (SMCs). Such 'fibrous lesions' typically have a 'fibrous cap' consisting of SMCs and extracellular matrix that encloses a lipid-rich 'necrotic core'. Plaques can become increasingly complex, with calcification, ulceration at the luminal surface, and haemorrhage from small vessels that grow into the lesion from the media of the blood vessel wall. Although advanced lesions can grow sufficiently large to block blood flow, the most important clinical complication is an acute occlusion due to the formation of a thrombus or blood clot, resulting in myocardial infarction or stroke. Usually, the thrombosis is associated with rupture or erosion of the lesion.

1.2.1.2 Initiation of atherosclerosis

In animal experiments soon after initiating a diet rich in cholesterol, light microscopy reveals attachment of blood leukocytes to the endothelial cells that line the intima, the innermost layers of the arteries[5]. Under ordinary circumstances, the endothelial monolayer in contact with flowing blood resists firm adhesion of leukocytes. However, the early adhesion of mononuclear leukocytes to arterial endothelium, at sites of atheroma initiation, is mediated through endothelial-leukocyte adhesion molecules and in particular vascular cell adhesion molecule-1 (VCAM-1) (Figure 4). VCAM-1 is the primary implicated adhesion molecule as it binds particularly to those classes of leukocytes found in nascent atheroma: the monocyte and the T lymphocyte; and endothelial cells express VCAM-1 in response to cholesterol feeding selectively in areas prone to lesion formation[6]. In addition, VCAM-1 rises before leukocyte recruitment begins in animal models of cholesterol-induced lesion formation[7]. Hypomorphic variants of VCAM-1 introduced into mice rendered susceptible to atherogenesis (by inactivation of the apolipoprotein E gene) show reduced lesion formation[8].

Figure 4 ***Initiation of atherosclerosis***



This figure schematizes steps (from left to right) in the recruitment of mononuclear phagocytes to the nascent atherosclerotic plaque and some of the functions of these cells in the mature atheroma.

From reference 4

The mechanism of VCAM-1 induction is thought to depend on inflammation instigated by modified lipoprotein particles accumulating in the arterial intima in response to the hyperlipidaemia. Constituents of modified lipoprotein particles can induce transcriptional activation of the VCAM-1 gene mediated in part by nuclear factor-κB (NF-κB)[9]. Pro-inflammatory cytokines such as interleukin (IL)-1β or tumour necrosis factor α (TNF-α), found in human atherosclerotic lesions, induce VCAM-1 expression in endothelial cells by this pathway. Finally, Massberg et al.[10] provide evidence to support the crucial role of the platelet in the initiation of atherosclerotic lesion formation. Using Apo E gene deficient mice, they showed platelet-endothelial cell interaction through GPIbα and GPIIbIIIa coincided with inflammatory gene expression and preceded atherosclerotic plaque invasion by leukocytes.

Mechanisms of leukocyte migration

Once adherent to the endothelial cells, leukocytes enter the intima by diapedesis between endothelial cells at their junctions. This phenomenon of directed migration of leukocytes through the endothelium are mediated by families of chemoattractant cytokines (chemokines) capable of recruiting leukocytes into the arterial intima. For example, monocyte chemoattractant protein-1 (MCP-1), overexpressed in human and experimental atheroma, can recruit the mononuclear phagocytes that characteristically accumulate in the nascent atheroma (Figure 4). Mice, susceptible to atherosclerosis, lacking MCP-1 or its receptor CCR2 show striking decreases in mononuclear phagocyte accumulation and local lipid levels[11,12]. IL-8 has a similar role as a leukocyte chemoattractant during atherogenesis[13]. Other chemokines overexpressed in atheromas that may contribute to lymphocyte recruitment, include a trio of CXC chemokines induced by interferon-γ (IFN-γ)[14], and eotaxin a CC chemokine that attracts mast cells[15].

Mechanism of leukocyte activation in the intima

Once resident in the arterial intima, monocytes increase expression of scavenger receptors for modified lipoproteins such as the scavenger receptor A (SRA) and CD36, and then internalize modified lipoproteins, such that cholesteryl esters accumulate in cytoplasmic droplets (Figure 4). These lipid-laden macrophages, known as foam cells, characterize the early atherosclerotic lesion. Macrophages within atheroma also secrete a number of growth factors and cytokines involved in lesion progression and complication. Examples include macrophage colony-stimulating factor (M-CSF)[16] and granulocyte-macrophage colony-stimulating factor (GM-CSF)[17] both of which promote inflammation by increasing production of cytokines and growth factors by these cells and also serve as survival and co-mitogenic stimuli.

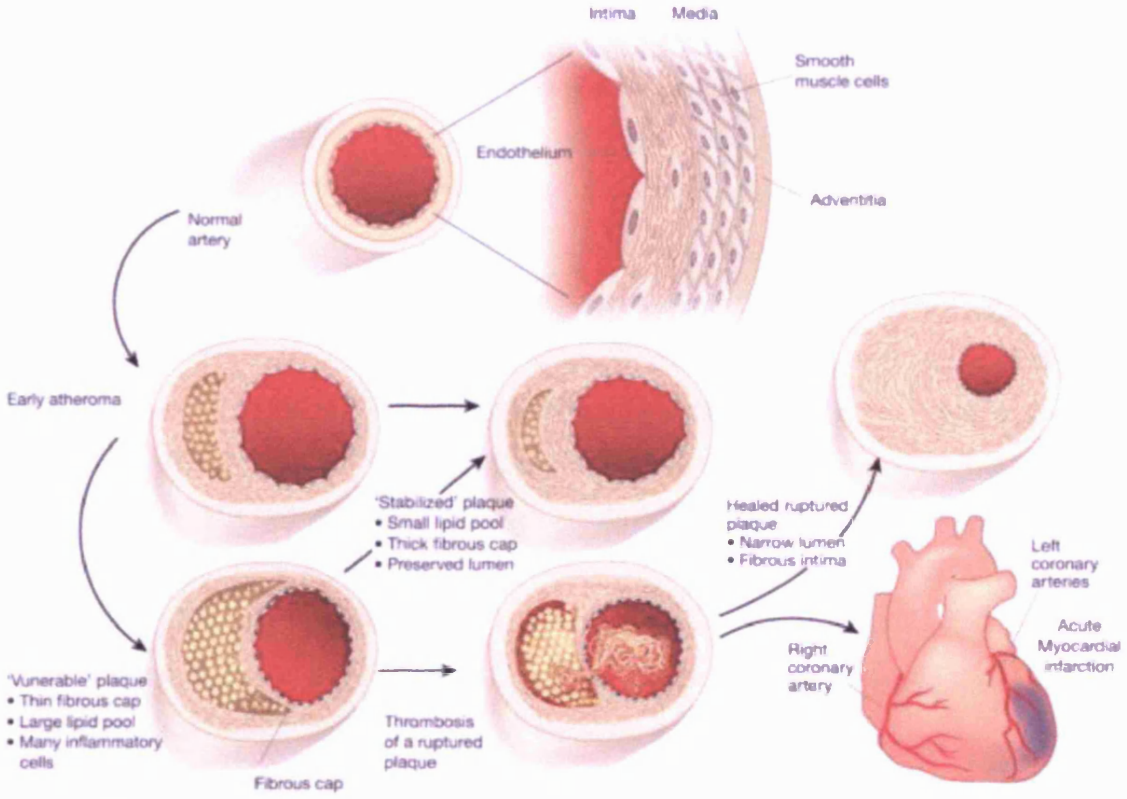
1.2.1.3 Atheroma progression and development of the acute coronary syndromes[4]

After formation of the fatty streak, the nascent atheroma typically evolves into a more complex lesion, which may eventually lead to clinical manifestations. As the lesion becomes more bulky, the arterial lumen narrows until it hampers flow and leads to clinical manifestations: in the coronary circulation, angina pectoris, or acute myocardial infarction.

The normal human coronary artery has a typical trilaminar structure - the endothelium (endothelial cells and basement membrane), the tunica intima (few smooth muscle cells scattered within the intimal extracellular matrix) and the tunica media (multiple layers of smooth muscle cells embedded in a matrix rich in elastin and collagen) (Figure 5). In early atherogenesis, recruitment of inflammatory cells and the accumulation of lipids leads to formation of a lipid-rich core, as the artery enlarges in an outward, ablumenal direction to accommodate the expansion of the intima. If inflammatory conditions prevail and risk factors such as dyslipidaemia persist, the lipid core can grow, and proteinases secreted by the activated leukocytes can degrade the extracellular matrix, while pro-inflammatory cytokines such as interferon-γ (IFN-γ) can limit the synthesis of new collagen. These changes can thin the fibrous cap and render it friable and susceptible to rupture. If the plaque ruptures, blood coming in contact with the tissue factor in the plaque coagulates. Platelets activated by thrombin generated from the coagulation cascade and by contact with the intimal compartment instigate thrombus formation. If the thrombus occludes the vessel persistently, an acute myocardial infarction can result. The thrombus may eventually resorb as a result of endogenous or therapeutic thrombolysis. However, a wound healing response triggered by thrombin generated during blood coagulation can stimulate smooth muscle proliferation. Platelet-derived growth factor (PDGF) and transforming growth factor-β (TFG-β), released from activated platelets, stimulates smooth muscle cell migration and interstitial collagen production respectively. This increased migration, proliferation and extracellular matrix synthesis by smooth muscle cells thickens the fibrous cap and causes further expansion of the intima, often now in an inward direction. Stenotic lesions produced by the lumenal encroachment of the fibrosed plaque may restrict flow, leading to ischaemia, commonly provoking symptoms such as angina pectoris.

Advanced stenotic plaques, being more fibrous, may prove less susceptible to rupture and thrombosis. Lipid lowering can reduce lipid content and calm the intimal inflammatory response, yielding a more 'stable' plaque with a thick fibrous cap and a preserved lumen.

Figure 5 ***Progression of atherosclerosis and its clinical consequences***



This figure schematises the life history of an atheroma. In general stable progressive plaques lead to angina, whereas unstable or vulnerable plaques lead to thrombus formation and the acute coronary syndromes.

From reference 4

Traditionally, it was regarded that this process, a result of smooth muscle accumulation and multiplication and the laying down of extracellular matrix in the plaque, occurred in an inevitable and progressive fashion gradually over time. However, today atherosclerosis is regarded as a 'response to injury', with lipoprotein or other risk factors as the injurious agents[18] (Table 1), and angiographic studies show periodic 'bursts of growth' leading to a discontinuous progression of lesions[19]. The trigger for

these rapid growth phases is thought to be due to physical disruption of plaques[20] and may be divided into three models[21]:

Superficial erosions, or microscopic areas of desquamation of endothelial cells that form the monolayer covering the intima, occurs frequently in both humans and animals with experimentally induced atherosclerosis. This results from both the cytolytic attack of the basement membrane cells by activated killer T cells as well as the expression and activation of matrix metalloproteinases leading to degradation of the sub-endothelial basement membrane[22]. Such areas of limited endothelial desquamation often form the nidus of a platelet thrombus as they uncover sub-endothelial collagen and von Willebrand factor that promote platelet adhesion and activation[23]. Although common and most often asymptomatic[24], such superficial erosion may account for approximately one-quarter of fatal coronary thromboses. In individuals with fewer conventional risk factors (of atherosclerosis) and in particular the young, due to a lower atherosclerotic burden, this maybe the dominant mechanism leading to an occlusive thrombus and therefore MI. In these particular groups platelets activation and degree of response to injury maybe paramount in determining the clinical outcome.

Disruption of the microvessels that form in atherosclerotic plaques provides another scenario for sudden plaque progression[25]. Atheromata develop microvascular channels as a result of neo-angiogenesis. This is promoted by the release of angiogenic mediators, from macrophages, such as acidic and basic fibroblast growth factor and vascular endothelial growth factor (VEGF)[26,27]. The new blood vessels in the plaque are particularly fragile and prone to micro-haemorrhage. Multiple lines of evidence support thrombosis in situ within plaques during human atherogenesis. Intra-plaque deposition of fibrin and fibrin-split products and haemosiderin provide evidence of intra-plaque haemorrhage. The thrombosis in situ leads to thrombin generation, which, in addition to cleaving fibrinogen, can potently stimulate smooth muscle migration and proliferation. Thrombin triggers platelet release of growth factors such as platelet-derived growth factor (PDGF) from their alpha granules, further stimulating smooth muscle migration and proliferation. Activated platelets also elaborate transforming growth factor β (TGF-β), the most potent stimulus known for interstitial collagen synthesis by smooth muscle

cells. In this manner, a silent microvascular haemorrhage within the atherosclerotic intima can give rise to a growth spurt in the evolution of the plaque.

Plaque rupture is the third and most common mechanism of plaque disruption and this process also involves inflammation (Figure 5). The plaque's fibrous cap usually serves to sequester the thrombogenic lipid-rich core of the atheroma from the bloodstream, which contains circulating coagulation proteins. Fracture of the fibrous cap results from decreased tensile strength due to reduced collagen levels within the cap. Inflammatory cytokines, such as IFN-γ, inhibit collagen production by smooth muscle cells, the principle source of this extracellular matrix macromolecule in the arterial wall[28]. Interstitial collagen fibrils also undergo proteolytic degradation by interstitial collagenases (MMP-1, -8 and -13) all of which are overexpressed in atheromatous plaques[29,30]. Fissure of the fibrous cap allows the coagulation factors contact with tissue factor, the main pro-thrombotic stimulus found in the lesion's lipid core. Although the ruptured fibrous cap causes some three-quarters of acute myocardial infarctions, like the other forms of plaque disruption, most episodes probably cause no clinical symptoms. When the prevailing fibrinolytic mechanisms outweigh the pro-coagulant pathways, a limited mural thrombus, rather than an occlusive and sustained blood clot, forms. With healing, however, resorbtion of the mural thrombus and the release of PDGF and the anti-inflammatory mediator TGF-β combine to engender a healing response that leads to fibrous tissue formation. The consequent smooth muscle accumulation and collagen accretion allow rapid evolution of a fatty lesion to one of more fibrous character (Figure 5).

These examples illustrate the inextricable links between thrombosis, and therefore the platelet, and lesion progression. The platelet plays a pivotal role not only in atherosclerosis disease progression but also its potential clinical thrombotic consequences, and indeed may be one of the key determinants to clinical outcome

1.2.2 Risk factors

1.2.2.1 Risk factors for coronary atherosclerosis and myocardial infarction

The concept of risk factor identification and modification is based on the premise that exposure to certain host and environmental factors increases the statistical risk of developing the disease and alteration of these conditions decreases the risk.

Epidemiological studies over the past 50 years have revealed numerous risk factors for coronary atherosclerosis and MI (Table 1). As atherosclerosis is usually a pre-requisite to MI, the two processes are inextricably linked and share a number of risk factors. These can be grouped into factors with an important genetic component, and those that are largely environmental. However, this concept is an oversimplification as similar amounts of exposure to 'environmental' risks such as smoking and high-fat diets show different inter-individual susceptibilities, which is probably genetic mediated. The relative abundance of the different plasma lipoproteins appears to be of primary importance, as raised levels of atherogenic lipoproteins are a prerequisite for most forms of the disease. With the exception of gender, and the level of lipoprotein(a), each of the genetic risk factors involves multiple genes. The genetic dissection of complex multifactorial disease such as CHD continues to remain a major challenge, however significant progress is being made with the use of novel and rapidly advancing technology[45,46]. Another level of complexity involves the interactions between risk factors. Frequently, these are not simply additive; for example, the effects of hypertension on CHD are considerably amplified if cholesterol levels are high[41].

Table 1 ***Genetic and environmental factors associated with coronary atherosclerosis and myocardial infarction***

Factors with a strong genetic component	
Elevated levels of LDL/VLDL	Associations demonstrated in epidemiological studies and supported by studies of genetic disorders and animal models. Clinical trials have shown benefit of cholesterol reduction[31].
Reduced levels of HDL	Associations demonstrated by numerous epidemiological studies of genetic disease and animal models[32].
Elevated levels of lipoprotein (a)	Associations observed in many, but not all, epidemiological studies. Animal studies have been contradictory[33].
Elevated blood pressure	Associations observed in epidemiological studies. Clinical trials have demonstrated benefits of blood pressure reduction[31,34].
Elevated levels of homocysteine	Associations have been observed in epidemiological studies, and homocystinuria results in severe occlusive vascular disease[35].
Family history	When all well-known risk factors are controlled for, family history remains a very significant independent factor[36,37].
Diabetes and obesity	Associations observed in epidemiological studies and in studies with animal models[31].
Elevated levels of haemostatic factors	Significant independent associations have been observed with elevated levels of fibrinogen, plasminogen activator inhibitor type 1 and platelet reactivity[31].
Depression and other behaviour traits	Associations observed in several population studies[38].
Gender (male)	Below age 60 men develop CHD at more than twice the rate of women[39].
Systemic inflammation	Elevated levels of inflammatory molecules such as C-reactive protein are associated with CHD. As are inflammatory disease such as rheumatoid arthritis[40].
Metabolic syndrome	This cluster of metabolic disturbances, with insulin resistance as a central feature, is strongly associated with CHD[41].
Environmental factors	
High-fat diet	Population migration and epidemiological studies indicate strong associations with lifestyle, and diet appears to be the most significant factor. High-fat, high-cholesterol diets are usually required for development of atherosclerosis in experimental animals[31].
Smoking	Strong associations observed in numerous epidemiological studies. Clinical trials have demonstrated the benefit of stopping smoking[31]
Low antioxidant levels	Results of clinical trials with antioxidants have not been conclusive. Fat-soluble antioxidants protect against atherosclerosis in experimental animals, however[42].
Lack of exercise	Significant independent association with CHD[31].
Infectious agents	Epidemiological studies provide suggestive evidence for association with various infectious agents such as Chlamydia pneumoniae and Helicobacter pylori. Preliminary animal studies support this relationship[43,44].

1.2.2.2 The Familial aggregation of myocardial infarction

The familial aggregation of myocardial infarction has been appreciated for almost a century[47]. Most studies estimate the risk of having a positive family history (a surrogate

for genetic risk) in a first degree relative to a 2-fold increase in the risk of MI. However, this risk increases further with younger ages of onset of disease in affected family members[37]. Marenberg *et al.*[48] followed up 10,502 pairs of Swedish twins for 26 years with respect to susceptibility to death from coronary heart disease. In men, the relative hazard of death from coronary heart disease when one's twin died before the age of 55 years, compared with the hazard when one's twin did not die before 55, was 8 for monozygotic twins and 4 for dizygotic twins. Among the women, when one's twin died before the age of 65 years the relative hazard was 15 for monozygotic and 3 for dizygotic. Sorensen *et al.*[49] followed up 960 Danish adoptees placed at an early age (>90% by 2 years of age) with their adopted parents to assess genetic and environmental influences on premature death. The death of a biological parent before the age of 50 was associated with a relative risk of death in the adoptees of 5 for cardiovascular and cerebrovascular deaths. The same classification for adopted parents was associated with a relative risk of 3. This study underlines the genetic and environmental components of cardiovascular disease and the inevitable overlap between the two in complex multifactorial disease such as MI.

Within a population, the heritability of atherosclerosis (the fraction of disease explained by genetics) has been high in most studies, frequently exceeding 50%. Population migration studies, on the other hand, clearly show that the environment explains much of the variation in disease incidence between populations. Thus, the common forms of CHD result from the combination of an unhealthy environment, genetic susceptibility (gene-environment interaction) [49] and our increased lifespan.

1.2.2.3 Candidate genes for myocardial infarction

Although, a familial aggregation of coronary artery disease has long been appreciated[47], until recently tools were not available to identify the specific genetic components responsible. Therefore, historically, a 'positive' family history has been viewed by physician and patient alike, as a 'non-modifiable' coronary risk factor, like age and sex. However, progress in molecular biology has dramatically altered this perspective in the last decade. The ability to elucidate the role of variation in potentially relevant genes

has fuelled a plethora of research into the genetics of CAD. Several exciting observations have already been made that provide valuable insight regarding how genetic factors may act to increase the risk of MI. However, many, remain to be confirmed, and conflicting data have raised important issues regarding study design and interpretation. The ultimate hope that genetic characterization may help to improve prevention and treatment, particularly for specific individual subjects, remains to be realised.

The search for genetic factors predisposing to acute myocardial infarction has, to date, largely been guided by current understanding of the underlying pathophysiology. Thus, the main targets have been genes for molecules involved in lipid homeostasis, blood coagulation, platelet function, and more recently, vessel wall biology (as processes related to plaque stability and rupture are better defined). The most common approach used has been to see if a variant in a particular gene (a polymorphism) is present more (or less) commonly in cases (usually of MI) compared with non-affected controls (case-control study), the implication being that if this is the case, either the polymorphism itself, or another polymorphism with which it is in linkage disequilibrium, has a functional effect that influences the risk of MI. In this manner, polymorphisms in genes in each of the above categories have now been associated, in one or more studies, with risk of MI, and the list continues to expand rapidly. Examples include the angiotensin converting enzyme (ace) insertion/deletion polymorphism thought to modulate blood pressure through ace levels and the fibrinogen promoter G-455A polymorphism influencing thrombosis by increasing plasma fibrinogen. Although such studies are relatively straightforward to set up they are prone to errors and principle advantages and disadvantages of such studies are discussed in detail in Chapter 5 and 7. A literature review of the field of genetic polymorphisms in myocardial infarction at the outset of this study is given in 'What is known about the genetics of acute coronary syndromes?' by Samani and Singh in Challenges in acute coronary syndromes[37].

1.3. The Platelet

1.3.1 History

The identification of platelets as a class of blood corpuscles was initially described by Bizzozzero in 1882 and the importance of platelets for the formation of a haemostatic plug was first reported by Eberth and Schimmelbusch in 1888. However, it was the work of Aschoff in 1925, whose opinion still provides two keys to the understanding of thrombogenesis:

1.) '...aggregations of platelets as they are present in a thrombus can only be sedimented as long as the blood is flowing.'

2.) '...formation of fibrin is not a primary event in thrombosis, but is preceded by important changes of the corpuscular elements of the blood. To understand the mechanism of thrombosis it is the latter changes that have to be understood.'

1.3.2 Platelet production and circulation

The origin of platelets is the bone marrow, where megakaryocytes - as the result of mitotic proliferation of a committed progenitor cell - liberate platelets as the end product of protrusions of their membrane and cytoplasm, a process known as thrombopoiesis. The physiological number in the circulation varies from 150,000 to 400,000/mm^3 blood.

Platelet production is mainly under the control of thrombopoietin, a 353 amino acid polypeptide produced by the liver and kidney, the gene for which was discovered in 1994[50]. Plasma concentrations of thrombopoietin vary inversely with the platelet count. An increased level of thrombopoietin cause megakaryocytes to increase in size and number and stimulates their cytoplasm to mature more rapidly[51]. Not only does this produce more platelets but also results in platelets with a raised mean platelet volume (MPV) and number of glycoprotein surface receptors[52], giving rise to increased

metabolic and haemostatic activity[53]. It has been suggested that a raised MPV may be an independent risk factor for MI[52-54].

The normal lifespan of the platelet is 8-11 days. The spleen continually but transiently sequesters about a third of circulating platelets. Splenomegaly greatly increases the fraction of platelets retained in splenic sinusoids, without decreasing overall platelet survival time. Most platelets are removed from the circulation after senescence, but a constant small fraction is continually removed by involvement in the maintenance of vascular integrity[55].

1.3.3 Platelet structure

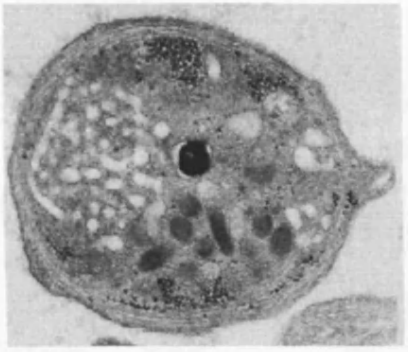
Platelets circulate as quiescent biconvex, anucleate discs about 2-4μm in diameter. The main components of the platelet are the external membrane bearing various glycoprotein molecules, intracellular granules, the cytoskeleton, and interrelated enzyme systems. As they are anucleate, platelets have very limited capacity for protein production, and cannot regenerate intracellular enzymes. The plasma membrane is covered by an outermost layer of glycacalyx, and is extensively invaginated to form the open canalicular system (OCS), which greatly increases the platelet surface area. Various glycoproteins are embedded in the plasma membrane, and some extend into the cytoplasm. Beneath the plasma membrane lies the cytoskeleton, composed of microtubules, to which the membrane is anchored at specific points.

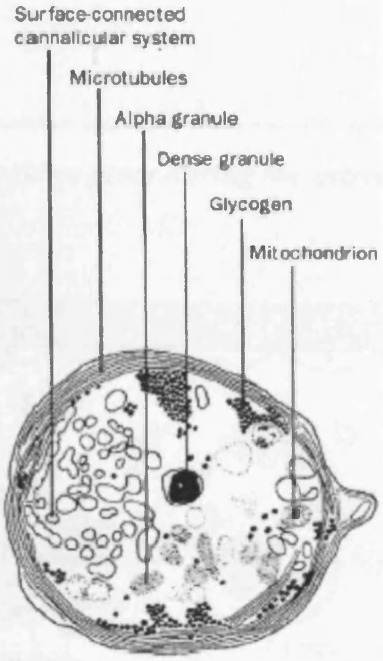
Platelet organelles include mitochondria, lysosomes, dense bodies and α-granules (Figure 6). The latter two are platelet specific. Each platelet contains approximately 50 α-granules ranging 300-500nm in diameter, surrounded by a membrane containing glycoprotein (GP) IIbIIIa and P-selectin. Alpha granules contain platelet specific proteins such as β-thromboglobulin (β-TG) and platelet factor 4 as well as ligands such as von Willibrand factor (vWF) and fibrinogen, which also occur in the plasma. During secretion, α-granules contents are exocytosed, and they appear to be secreted more readily than dense bodies or lysosomes, making β-TG a sensitive marker for activation. In contrast each platelet contains 5-6 dense bodies, 200-300nm in diameter containing

serotonin, ATP, ADP, pyrophosphate and Ca^{++}, the last accounting for their electron dense appearance. Agonists other than thrombin stimulate dense granule secretion.

Figure 6 ***The resting platelet and its components***

Electron micrograph (x20,000) and schematic representation of a resting platelet and its components in cross-section.





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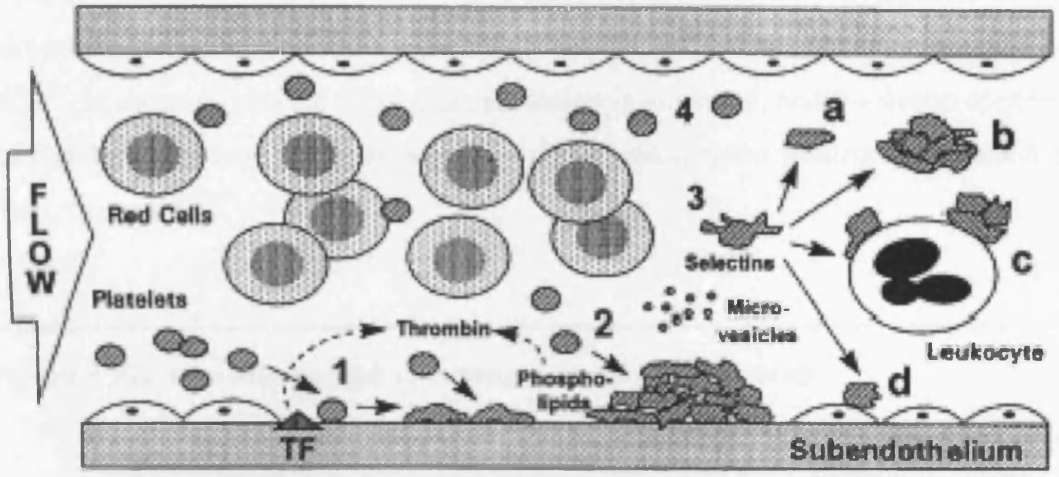
1.3.4 Platelet activation

Platelets play a key role in early phases of hemostasis. Fundamental mechanisms in this process include: adhesion, aggregation, internal contraction and secretion (Figure 7). Furthermore, phospholipids present in the platelet membranes are important for blood coagulation. Interest in platelet function goes beyond their physiologic role in the arrest of bleeding. It is widely accepted that thrombotic events occur because of a dysregulation in mechanisms involved in primary hemostasis. Finally, platelets also participate in other physiological and pathophysiological processes such as inflammation and wound healing[56].

Individual platelets circulate in a resting (inactivated) state. When platelets are exposed to vessel wall injury, alterations of blood flow, or stimulation by platelet specific

agonists (e.g. thrombin, ADP, collagen, platelet activating factor, serotonin, adrenaline, gram negative bacteria endotoxin), they manifest a triad of linked functional responses (Figure 7): adhesion, secretion and aggregation, which represent platelet activation. The functional responses occur via a series of elaborately coordinated signaling pathways that convert extracellular stimuli, via specific surface receptors, into biochemical-metabolic and morphological changes[55,57].

Figure 7 ***Diagram representing the series of events taking place during the activation of platelets.***



During the formation of a mural thrombus under flow conditions, some platelets interact with the subendothelium (adhesion and spreading) (1) and become recruited into the aggregate (2). A relatively small proportion of the platelets will become moderately activated (3) and will leave the forming aggregate. They will either remain in the circulation when the activation is minimal (a, b), or will interact with leucocytes (c) or endothelium (d) if they express selectins on their surface. The vast majority of platelets remain unaffected (4).

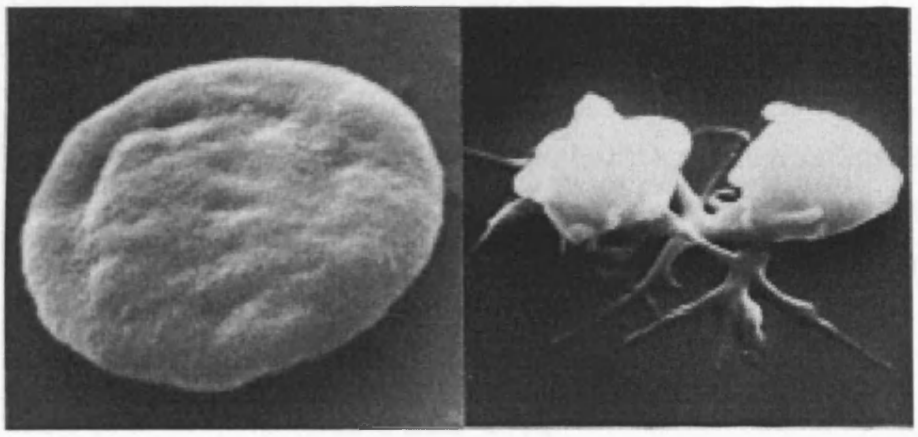
Adapted from reference 58

The functional responses during platelet activation are closely linked processes[55]. Shape change, as a result of microtubule contraction, converts the platelet from a disc into an irregular tiny sphere with long pseudopods (Figure 8). Changes in the surface contour are accompanied by movement of randomly dispersed organelles towards the platelet center. Elements of the OCS become more prominent in activated platelets as

membranes of the alpha-granules fuse with those lining OCS channels, thus facilitating release[55,58].

Platelet adhesion and spreading represent the processes by which platelets attach to, and then cover, non-platelet surfaces, such as a damaged endothelium or subendothelial matrices. Platelet secretion results in the release of granule contents via granule fusion with the plasma membrane or directly, through the OCS; the released substances may recruit and activate more platelets and other blood cells, and thus amplify the responses. Platelet aggregation, by which platelets adhere to one another to form a haemostatic plug, is a metabolically active process. During several of these events, the distribution of the platelet membrane glycoproteins is also altered. GPIb-IX-V complexes become drawn into the cell center and are removed from the surface into the remnants of the OCS[59]. In contrast, surface GPIIbIIIa expression is increased, and P-selectin appears on the platelet membrane, as the result of granule fusion with the plasma membrane during platelet secretion[60].

Figure 8 ***Electron micrograph of resting and activated platelets***



The photographs demonstrate the disc shape of the normal circulating platelet and the more spherical form of the activated platelets with the many long pseudopodia

Adapted from reference 55

Platelet activation can, under certain conditions, also result in alterations of the phospholipids distribution in the platelet membranes. In resting platelets, the

phospholipids are asymmetrically organized, with negatively charged phospholipids almost exclusively present in the inner leaflet. During platelet activation by collagen, thrombin or complement C5b the negatively charged phospholipids, especially phosphatidylserine, may be exposed on the platelet surface, which creates a prothrombotic surface and membrane vesiculation may occur and generate platelet-derived microparticles by membrane shedding[61].

1.3.5 Platelet glycoprotein receptors

There are many important platelet membrane glycoprotein receptors, including, GPIb-IX-V, GPIaIIa and GPIIbIIIa. These receptors mediate adhesion to subendothelial tissue and subsequent aggregation to form the initial haemostatic plug[55].

1.3.5.1 Nomenclature

The largest glycoprotein was designated I, the smallest IX. Letters a and b were added when better techniques allowed resolution of single protein bands on electrophoresis into two separate bands (e.g., glycoprotein I became glycoprotein Ia and Ib).

1.3.5.2 The glycoprotein Ib-IX-V receptor complex

GPIb is the receptor for VWF and forms a complex with GPIX and GPV (GPIb-IX-V) on platelet membranes. GPIb-IX-V is a constitutively active receptor responsible for immediate and initial platelet attachment to exposed perivascular von Willebrand factor at high shear flow rates. There are about 25,000 copies of GPIb per platelet[62]. The receptor for VWF is located in the alpha subunit of GPIb. GPIX is necessary for expression of the GPIb molecule on the platelet surface and may act by stabilizing covalent bonds between alpha and beta subunits of GPIb. GPV has been proposed as a receptor for thrombin and also functions as a stabilizing molecule for the GPIb-IX structure[62]. GPIb is connected across the membrane via actin binding protein to the submembrane actin cytoskeleton. Absence of this connection results in the giant platelets seen in patients with the Bernard-Soulier syndrome (BSS). These platelets are not only softer and more easily deformable but have a deficiency of GPIb receptors,

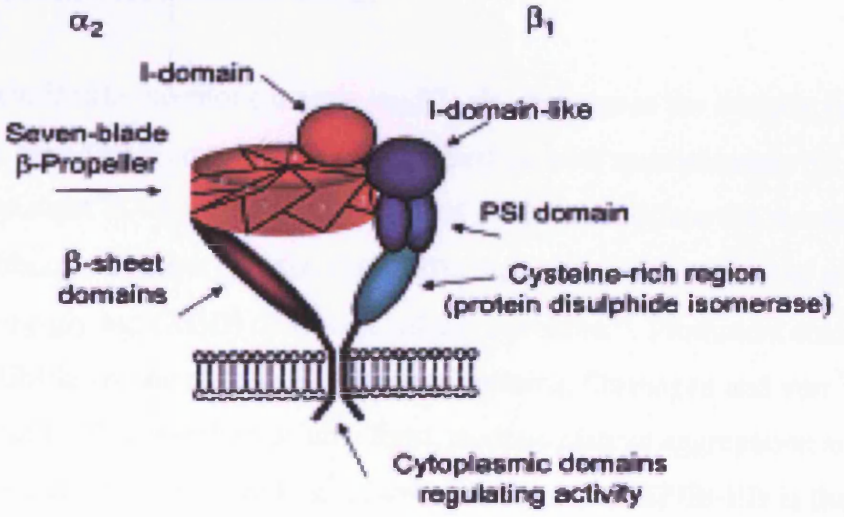
which results in markedly impaired platelet adhesive capacities and therefore the bleeding disorder[63].

The GPIb-IX-V complex is a product of four genes and to date no clinically important polymorphisms (variations in the gene coding sequence) have been discovered.

1.3.5.3 The glycoprotein IaIIa receptor

The glycoprotein IaIIa complex (also known as $\alpha_2\beta_1$), a member of the integrin family of receptors, is constitutively active and involved in initial platelet adhesion and anchoring to the subendothelial matrix by directly binding to collagen (both fibrillar (types I-III and V) and nonfibrillar (types IV, VI, VII and VIII))[64]. There are approximately 900-2300 molecules per platelet and deficiency of the receptor leads to impaired collagen induced aggregation leading to clinically mild bleeding disorders[64].

Figure 9 ***Model of GPIaIIa integrin structure based on sequence similarities between the domains and other molecules***



Adapted from reference 64

The structure of the GPIa IIa receptor comprises of the α_2 and β_1 subunits each with a number of separate domains (Figure 9), the exact molecular structure of which is still under investigation[64]. In brief, the collagen binding outer I-domain of the α_2 subunit is inserted between repeats 2 and 3 of the seven β-propeller structures, which in turn contacts a putative MIDAS (metal ion dependent adhesion site) or I-domain-like structure in the β_1 -subunit. The N-terminus of the subunit folds into a plexinsemaphorin-integrin-like (PSI) domain under the putative MIDAS domain near a domain with no known sequence similarities. The domain of β_1 above the membrane is very rich in disulphide bridges and has endogenous protein disulphide isomerase activity responsible for regulating conformational changes of the integrin in response to signalling via the cytoplasmic domains. These changes alter the conformation of the α_2 I-domain and its avidity for collagen[64,65].

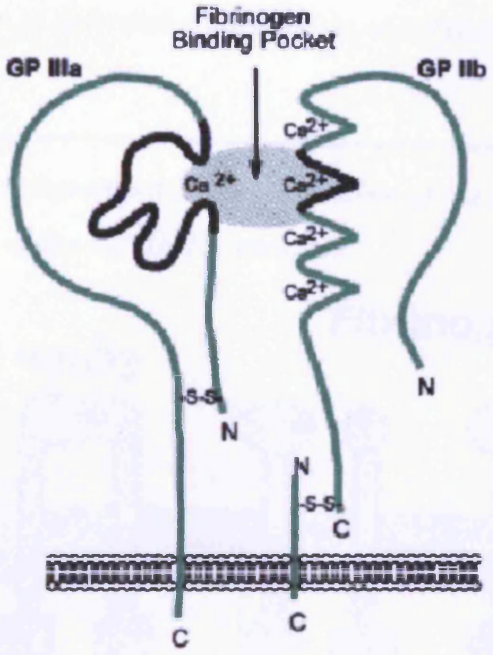
Unlike the GPIb-IX-V (and GPIIbIIIa) there are large variations in inter-individual platelet receptor expression of GPIaIIa (up to tenfold)[66]. Moreover, a large proportion of this variation has been linked to two linked silent polymorphisms (C807T and G873A, see section 1.6.1), which may have important clinical consequences to platelet function and therefore risk of thrombosis.

1.3.5.4 The glycoprotein IIbIIIa receptor

The glycoprotein IIbIIIa receptor (integrin $\alpha_{IIb}\beta3$) also belongs to the integrin family and is the most abundant receptor on the platelet surface with approximately 50,000 molecules per platelet of which 70% are expressed on the cell surface the remainder are within the membranes of the α granule. GPIIb-IIIa receptors interact with the amino acid sequence arg-gly-asp (RGD) of several adhesive proteins[60]. Prominent among the ligands for GPIIbIIIa are the multivalent adhesive proteins, fibrinogen and von Willebrand factor (vWF), which in soluble form, mediate platelet aggregation and, in solid phase, mediate adhesive spreading[67]. Severe deficiency in GPIIb-IIIa is the cause of a lifelong bleeding disorder, Glanzmann's thrombasthenia, related to the severely impaired spreading and aggregating capacities of these platelets in response to all physiological agonists and absent clot retraction.

GPIIbIIIa is a Ca^{++} dependent heterodimer, which consists of a single polypeptide chain β subunit GPIIIa and a unique α subunit GPIIb. GPIIb consists of two chains linked by a disulphide bridge, of which only the smaller (light chain) $GPIIb_{\beta}$ is inserted into the platelet membrane[68]. The heavy chain of GPIIb is entirely extracellular and contains four putative cation-binding motifs of 12 amino acids. The fibrinogen-binding site consists of the metal ion dependent adhesion site (MIDAS) domain of the β_3 and the second calcium ion-binding repeat of αIIb (Figure 10).

Figure 10 ***Model of GPIIbIIIa integrin structure***



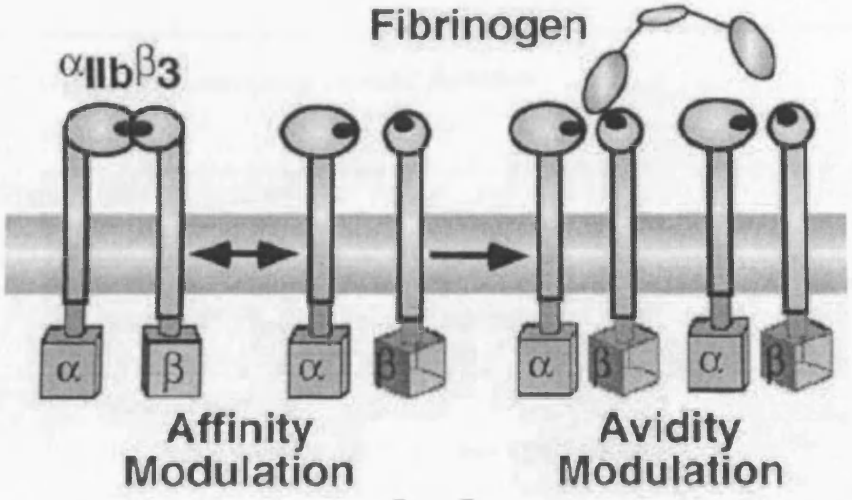
Adapted from reference 68

GPIIbIIIa is normally in a low activation state on the surface membrane of resting platelets. As a result, there is little or no binding of soluble fibrinogen or vWF, in resting platelets. The affinity/avidity of the receptor for these ligands increases rapidly following platelet exposure to soluble agonists (e.g., thrombin, ADP, epinephrine, thromboxane A2) or to adhesive proteins immobilized within the subendothelial matrix (e.g., collagen, vWF) (Figure 11). These structurally diverse excitatory agonists interact with specific membrane receptors in human platelets, including the protease-activated

receptors for thrombin (PAR1 and PAR4), P2Y1, and $P2Y_{12}$ purinergic receptors for ADP, α_2-adrenergic receptors for epinephrine, GP VI and integrin GPIaIIa for collagen and GP Ib-IX-V for immobilized vWF. Counteracting these proadhesive stimuli are inhibitory agonists, among them certain prostaglandins, nitric oxide and possibly platelet-derived growth factor (PDGF). These inhibit or reverse the process of GPIIbIIIa affinity/avidity modulation[67].

The use of flow cytometry with specifically developed monoclonal antibodies has facilitated the recognition of resting and activated conformations in GPIIbIIIa. PAC1 is an antibody that binds only to activated platelets, and appears to be specific for the recognition of activated conformation of GPIIbIIIa[69].

Figure 11 ***Diagrammatic representation of the conformational change upon activation of the GPIIbIIIa receptor***



This diagram represents the conformational change within the GPIIbIIIa receptor upon activation. In the resting state the receptor cannot bind to appropriate ligands as the fibrinogen/ligand-binding site are only exposed once the platelet has been activated.

Adapted from reference 66

A number of polymorphisms within the coding sequences of the GPIIbIIIa receptor have been described[70]. Of these, the C196T (leu33pro) polymorphism, within the GPIIIa gene, is responsible for the formation of the PL^{A1} and PL^{A2} alloantigenic

determinants and the most studied for clinical and functional consequences (Section 1.6.2).

1.4 Assessment of platelet activation

While there is an increasing realisation that platelet activation plays a prime role in the increasing heart disease burden of society (sections 1.5.3 and 7.3.1), there is still no generally accepted ideal measure of platelet activation that would indicate a state of 'high risk'.

There are numerous techniques (Table 2) of assessing platelet function and they are discussed briefly here. Platelet activation may be quantified in a number of ways. These include change in shape, tendency to aggregate, surface receptor expression and/or occupation and by the blood and urine levels of relevant platelet metabolic products.

Table 2 ***Methods of assessing platelet function***

Platelet flow cytometry
Platelet shape change
Resting expression of glycoproteins such as P selectin, receptors, etc.
Expression of glycoproteins in response to ADP, collagen, epinephrine.
Fibrinogen binding in response to ADP, collagen, epinephrine, thrombin.
Platelet aggregation
Platelet shape change, spontaneous aggregation
Aggregation in response to ADP, collagen, epinephrine, thrombin.
Flow chambers
Adhesion to a substratum such as collagen or endothelial cells
Soluble markers (plasma and/or urine)
Beta thromboglobulin
Platelet factor 4
Soluble P selectin
Soluble glycoprotein V
Thromboxane(s)

1.4.1 Markers of platelet activation

1.4.1.1 Platelet shape change

Platelet shape change (Figure 8) can be measured by flow cytometry or electron microscopy[71]. Activation leads to a change from a discoid to a more spherical shape with many pseudopodia vastly increasing the surface area of the platelet. This can also be detected by the increase of light transmission and the disappearance of oscillations of stirred platelet suspension in the platelet aggregometer[72].

1.4.1.2 Platelet metabolic products

Detection of platelet specific alpha granule contents in the plasma such as β thromboglobulin and platelet factor 4 can easily be performed using an ELISA or radioimmunoassay. These proteins are specific to platelet release[73] and have been suggested as a means of detecting increased platelet activation in vivo[74].

These proteins may be influenced by the platelet count and a correlation between platelet count and beta thromboglobulin and platelet factor 4 levels have been reported[75]. Beta thromboglobulin levels are raised in renal failure as it is normally metabolised by the kidney[76], and in this situation platelet factor 4 may serve as a more accurate marker for platelet activation. Abnormally elevated levels of platelet factor 4 have been reported with concomitant heparin therapy[77] as this leads to platelet factor 4 to be released from its binding sites on endothelial cells.

1.4.1.3 Platelet surface receptors expression

The platelet-endothelial interaction is mediated through adhesion molecules on the surface of platelets, which belong in the families of selectins (P-selectin), integrins (GPIa, GPIb, GPIIbIIIa) and immunoglobulins. P-selectin levels are raised in a number of thrombotic disorders[78] and are not influenced by various anticoagulants, renal dysfunction and methods of preparation of plasma[79]. Integrin expression can be measured using fluorescence-activated flow cytometric techniques in combination with

monoclonal antibodies[80]. This technique allows a detailed assessment of platelet activation at the molecular level.

1.4.2 Techniques used to assess platelet function

1.4.2.1 Platelet aggregation

The original method described by Born[81] in 1962 soon became the 'gold standard' for platelet function testing. Blood is centrifuged gently to obtain platelet rich plasma (PRP), which is stirred in a cuvette at 37°C between a light source and a photocell within the aggregometry instrument. Upon addition of an agonist, platelets aggregate and the transmission of light increases and is detected and recorded on a chart with time. The addition of a panel of platelet agonists at a range of concentrations, triggers classical platelet responses including shape change, primary and secondary aggregation.

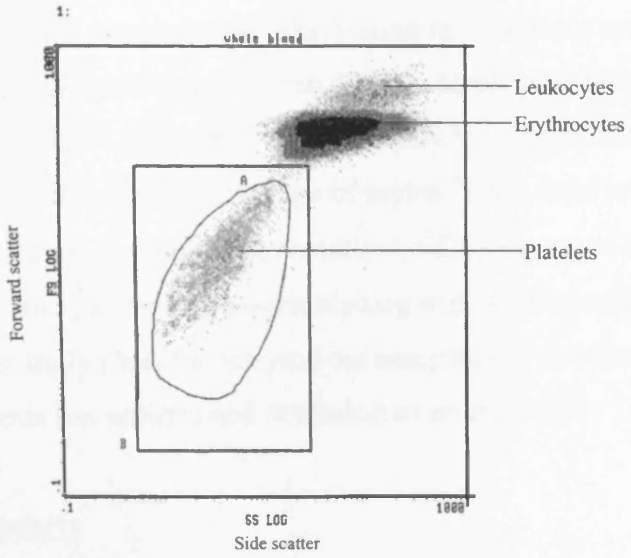
Although an enormous amount of information can be obtained with aggregometry, and despite the development of computer controlled multichannel capability, the test remains labour intensive, requires sample preparation, quality control and a fair deal of technical expertise both to perform and interpret the results. There is also the uncertainty of whether the ex vivo conditions in the platelet aggregometer truly reflect in vivo aggregation[82].

1.4.2.2 Flow cytometry

The key principle of flow cytometry is the detection of individual particles, usually cells, passing in a fluid stream across a 488nm laser beam one at a time (achieved by means of ''hydrodynamic focusing''). The size and granularity of the particle cause the light to be scattered forward and sideways respectively. Dyes may also be bound within or on the surface of a particle. These dyes absorb the laser light energy and emit fluorescence of different colours, which can then be detected. Photo-multiplier tubes detect this scattered and coloured light and convert it into voltage pulses. A plot of pulse heights of one measurement (e.g. size) versus the pulse heights of another measurement (e.g. granularity), called a dot density plot, is generated by the flow

cytometer (Figure 12). A population within this plot can then be isolated and data collected based on that population alone, a process known as gating. The chosen gate is assigned to histograms and the final process is the analysis of histogram statistics. The cytometer calculates the statistics for the region such as mean, median, % within the region and count (see section 4.1 for further explanation of flow cytometry results).

Figure 12 ***Dot density plot allowing differentiation of platelets using flow cytometry***



This figure taken from a flow cytometer shows how by using detection of forward scatter (y axis) and side scatter (x axis) of the laser beam, on a sample of whole blood; the method is able to differentiate between leukocytes, erythrocytes and platelets. The ellipsoidal region around the platelet population is the 'gated' region chosen for further analysis.

For platelet analysis the procedure is performed on unseperated whole blood. Phosphate buffered saline is used for incubation and washing. Formaldehyde is used to fix whole blood samples[83].

Flow cytometry can detect activated platelets by a number of methods including (i) the change in shape of activated platelets[84], (ii) the detection of specific antigens on the membrane of activated platelets (p-selectin, GPIIbIIIa) and (iii) the detection of platelet surface bound proteins (such as fibrinogen)[85]. For example, on activation the GPIIbIIIa receptor undergoes a conformational change, which exposes epitopes to allow

fibrinogen binding (Figure 11). This mechanism is used by the monoclonal antibody PAC 1[69] which specifically binds to activated GPIIbIIIa receptors only. The platelet-bound antibodies are then detected with streptavidin conjugated with fluorecein isothiocynate (FITC). Multiple fluorescent stained monoclonal antibodies can be used to measure activated platelets, leukocyte platelet aggregates and procoagulant platelet-derived microparticles simultaneously[86,87].

If carried out by well-trained and experienced staff, flow cytometry provides an unrivalled detail of platelet function, allowing direct platelet analysis under physiological conditions (whole blood assay) using small sample amounts. Flow cytometry has very high sensitivity and can directly detect activation of as little as 0.8% of the platelet population[69]. Further, it has previously been demonstrated that the technique is unaffected by concomitant use of aspirin, when used to quantify P-selectin expression (evidence of α-granule degranulation), CD63 expression (evidence of lysosomal release) and platelet fibrinogen binding with ADP stimulation[88,89]. Therefore, the method is potentially ideal for carrying out comparative platelet function tests in a cohort of MI patients (on aspirin) and controls (not on aspirin).

1.4.2.3 Flow chambers

The use of parallel plate flow chambers with anticoagulated whole blood allows the assessment of platelet aggregation and adhesion under varying conditions of shear stress. The dimensions of the chamber and the use of mechanical pumps recreate physiological shear flow rates over premounted sections of endothelium containing antigens such as collagen or von Willebrand factor. Labelled platelets are quantified for rates of aggregation, adhesion or thrombus formation using either direct microscopy or more recently a computerized epifluorescence video microscopy system.

1.4.3 Comparison of platelet function tests

There can be discrepancy between different methods of quantification of platelet activation and it should be borne in mind that the different types of platelet function tests described above actually reflect different aspects of platelet physiology. For

example, methods such as platelet aggregation quantify in vitro platelet activation in response to agonists, whereas the measurement of platelet release products in the plasma quantifies in vivo platelet activation.

Venepuncture can potentially influence all types of platelet activation assessment[90] apart from measurement of urinary metabolites, which is the only method that does not require blood letting. Platelet shape change is also influenced by a variety of factors including temperature and contact with a number of substances[91].

More than one test could be applied to assess the state of platelet activation in disease states, although interpretation must be confined to the relationship between the method used and disease state studied. Use of plasma markers of platelet activation is easier to undertake in large-scale epidemiological investigations compared to more time consuming methods that require expensive equipment such as flow cytometry. However, the latter offers a far more sophisticated and direct assessment of platelet function including the ability to quantify specific platelet surface receptor expression and activity (fibrinogen binding to the GPIIbIIIa receptor). For this reason, flow cytometry was the platelet function study of choice for the purposes of this project.

1.5 The role of platelets in the pathogenesis of myocardial infarction

1.5.1 Historical background

There is considerable evidence that platelets play a direct role in the pathogenesis of myocardial infarction. In 1912, Dr. J.B. Herrick[92] was the first to associate the clinical presentation of an acute infarction with a thrombotic coronary occlusion. The finding of platelet thrombi, emboli and aggregates in the circulation of the myocardium after sudden MI deaths was reinforced by a number of autopsy studies from the late 1940's onwards [93-98]. The very specific description of plaque disruption leading to an acute MI was mainly established by Constantinides in 1966[99]. The series of autopsies reported by Davies et. al. in 1984[96], showed that in a 100 victims of sudden coronary death (within 6 hours from onset of symptoms), coronary thrombi were found in 74 cases. Among the 26 cases without an intraluminal thrombus, plaque fissuring was found in 21, leaving

only 5 cases with no acute arterial lesion. These findings confirmed that the pathological process in the majority of sudden ischaemic deaths involves a rapidly evolving coronary-artery lesion in which plaque fissuring and resultant platelet rich thrombus formation are present.

With the development of selective angiography in 1959 by Sones et. al.[100] a new tool became available to directly visualise the coronary circulation in the setting of an acute MI. In 1980 DeWood at. al.[101] published angiographic findings on 322 patients who had undergone an angiogram within the first 24 hours from the onset of symptoms of transmural MI. They found total coronary occlusion in 110 of 126 patients (87%) who were evaluated in the first 4 hours of onset of symptoms; this proportion decreased significantly to 37 of 57 (65%) when patients were studied 12to 24 hours after the onset of symptoms. Overall these results highlighted that total coronary occlusion is frequent during the early hours of a transmural MI and finally established that coronary thrombosis was the proximate cause of infarction.

Animal studies have also confirmed that platelet aggregates develop if the coronary vessel intima is damaged[102], again linking plaque rupture, platelet thrombus and acute MI.

More recently the development of intra-vascular ultrasound has allowed a more sophisticated analysis by providing transluminal tomographic images of coronary arteries in vivo. Fukuda et. al. in 2001[103] used the technique to show plaque morphology and composition in the setting of an acute MI (59 subjects) and stable angina (59 subjects). Low echoic thrombus (acute MI 15% Vs. stable angina 0%), subtle dissection (37% Vs. 4%), echolucent areas (31% Vs. 0%) and bright speckled echo material (90% Vs. 0%) were more common in the infarction group than in the stable angina group ($p<0.001$ for all). The findings demonstrated the morphological characteristics associated with plaque at the time of acute MI and corresponded pathologically to ruptured plaque.

In the last decade our understanding of the pathophysiology of an acute MI has continued to advance and most of the developments have been focused on the

pathogenesis of atherosclerosis at the molecular level[104] as well as the determinants of plaque rupture[105]. These are described in detail in section 1.2.1.

1.5.2 Anti-Platelet Drugs and Myocardial Infarction

Perhaps the most convincing evidence of the importance of platelets in myocardial infarction is indirect in the form of antiplatelet agents and reduction of subsequent cardiovascular events. The last 5 years has seen the emergence of a number of novel antiplatelet agents and in the setting of increasing percutaneous intervention their use is increasing dramatically.

No single treatment has been studied more than aspirin. Its main mechanism of action is by inhibiting cyclo-oxygenase by irreversible acetylation and thus inhibiting thromboxane A_2 production. However, not all the anti-thrombotic effects of aspirin are fully understood, including its anti-inflammatory role. Nevertheless, small doses of aspirin, sufficient to modify platelet aggregation, are associated with a 25-35% reduction in IHD incidence[106,107], in both primary and secondary prevention, and also reduction in infarct size.

The Persantine-Aspirin Reinfartion Studies (PARIS) reported reductions in non-fatal MI and cardiac death in patients receiving aspirin and dipyridamole[108]. Dipyridamole has several actions (I) it is a phosphodiesterase inhibitor, which increases platelet cAMP levels thereby reducing platelet reactivity; (II) it is a coronary vasodilator; (III) it inhibits adenosine uptake and adenosine deaminase, potentiating the effects of adenosine; and (IV) it antagonises the proaggregatory effects of ADP on platelets.

The thienopyrdins, ticlodipine and clopidogrel, are inhibitors of ADP resulting in reduced aggregation. Ticlodipine was analysed in a study of 652 patients with unstable angina or non-q wave infarction, and compared to standard treatment (without aspirin or heparin) and showed a significant reduction in death and MI at 6 month follow up: 13.6% vs. 7.3% ($p=0.01$)[109]. The CURE trial compared clopidogrel to placebo in 12,562 patients with acute coronary syndromes without ST-segment elevation with a mean treatment duration of 9 months. All subjects were on aspirin. There was significant reduction in cardiovascular death, MI and stroke in the active treatment arm[110], (relative

risk (with clopidogrel), 0.80 (0.72-0.90) p<0.001). A sub-study of CURE highlighted the benefits of pre-treatment (6 days) and prolonged treatment (8 months) with clopidogrel compared to conventional treatments in patients receiving stent insertion at angioplasty (relative risk 0.70 (0.50-0.97), p=0.03)[111]. The benefits of short-term treatment (4 weeks) after percutaneous intervention with clopidogrel had already been confirmed in a number of studies[112,113].

Activated GPIIbIIIa receptors connect with fibrinogen to form bridges between activated platelets, leading to the formation of platelet thrombi. This is the final common pathway for all methods of platelet activation and therefore is an ideal therapeutic target for anti-platelet treatment. The GPIIbIIIa inhibitors have been shown to reduce cardiac complications following percutaneous coronary intervention[114]. In patients admitted with acute coronary syndromes, systemic use of GPIIbIIIa receptor blockers in addition to aspirin and heparin has been studied in six large-scale randomised trials (PRISM, PRISM-PLUS, PUTSUIT, PARAGON-A, PARAGON-B and CAPTURE). The results are very consistent and have been elegantly summarised in a meta-analysis[115]. In total 31,402 subjects enrolled and at 30 days there was a 9% reduction of death or MI in the GPIIbIIIa group, 10.8% vs. 11.8%, odds ratio 0.91 (0.84-0.98); p=0.015.

In summary, current anti-platelet strategies in acute coronary syndromes, based on evidence-based practice, include aspirin, ADP antagonists and glycoprotein IIbIIIa receptor blockers. These strategies serve to highlight the importance of platelets in the aetiology and risk stratification of MI.

What is clear from the offset is that the platelet is central to an acute MI and also plays a major role in the pathogenesis of atherosclerosis. It is not surprising therefore that for nearly half a century clinicians have tried to correlate increased platelet activation to an increased risk of myocardial infarction and tried to dissect the determinants of platelet activation. The rest of section 1.5 will describe the key studies on platelet activation and MI as well as the major studies demonstrating the heterogeneity of platelet function and its determininants.

1.5.3 Studies on platelet activation and myocardial infarction

Increased platelet aggregability was the first described measure of heightened platelet response in the setting of an acute evolving MI[116] or in stable angina with a history of previous MI[117]. McDonald et al. in 1957[116] carried out the first case-control study comparing a variety of coagulation tests between a group of 48 patients with stable angina (some of which had previously suffered a MI) to age –sex matched controls. They demonstrated significant differences ($p<0.001$) in thromboplastin generation and platelet stickiness (a coarse measure of platelet adhesion) between the groups. However, they were cautious to point out that the increased coagulability of the blood could be the result, rather than the cause of the disease. Mueller et al. demonstrated similar results of heightened platelet reactivity (using plasma β thromboglobulin and platelet aggregation studies), in the peripheral and coronary circulation[118] of 59 subjects during an evolving MI. In 1973, Dreyfuss and Zahavi showed increased platelet aggregation in response to ADP in the acute setting, within 3 days of MI, in 66 male MI patients compared to 20 controls (all p-values <0.001). They also found that the MI subjects continued to demonstrate an increased aggregability up to 24 months from the initial event and that this was most pronounced in the 11 subjects who suffered a further MI during follow up (all p-values <0.01). Trip et al.[119] tested unstimulated platelet aggregation as a predictor of subsequent events in 149 survivors of MI with five-year follow up. Subjects who were positive for spontaneous platelet aggregation (>20% decrease in light transmission within the aggregometer within 10 minutes) had a relative risk of 5.4 (2.2-13.4) of a subsequent event compared to the individuals negative for this test (<20% decrease in light transmission in 1 hour), $p = 0.001$.

Increased mean platelet volume, a marker of platelet reactivity (see next section), has been associated with acute MI[54] and similarly predicts subsequent increased risk for further events[120]. Martin et al.[54] initially measured mean platelet volume (MPV) in 15 MI subjects within the first 12 hours of symptoms and found significantly larger platelets (+ 0.98 fl, $p<0.001$) compared to the 22 controls. The same group subsequently carried out a prospective study in 1716 men with MI with 2 year follow up. MPV measured at 6 months post MI was an independent predictor for a further ischaemic event (fatal and non-fatal) and all cause mortality (all p-values < 0.001).

Various markers of platelet secretion and activation (p-selectin[121-123], β-thromboglobulin[118], thromboxane[124]) have also shown to be increased in acute MI, or following coronary angioplasty and may even predict adverse outcome following percutaneous intervention. In the setting of elective angioplasty in 102 subjects, Tschoepe et al.[121] compared pre-procedural platelet p-selectin and thrombospondin, determined by flow cytometry, to the risk of acute ischaemic events (first 24 hours) post procedure. All events occurred in patients classified as 'activated' (six of 46 or 13%), whilst there were no clinical events in the non-activated group (0 of 56), giving a significant difference (p=0.007). Flow cytometry determined p-selectin expression was also employed by Itoh et al.[122] in a case-control study using 48 subjects with acute coronary syndromes (ACS) and 30 controls. There were significant differences between the controls (0.11 ± 0.2%) and ACS subjects with (2.1 ± 2.3%) or without a definitive MI (0.3 ± 0.4%), all p-values < 0.05. Similar significant results were obtained for p-selectin expression by Gawas et al.[123] comparing 15 acute MI subjects post angioplasty to 15 stable angina subjects. undergoing an elective procedure. Fitzgerald et al.[124], in a study of 16 unstable angina, 14 MI and 6 controls, quantified thromboxane synthesis by measuring plasma and urinary metabolites. Episodic increases in thromboxane production were demonstrated correlating to chest pain, and therefore ischaemia, and this was highest in the unstable angina group (p-value < 0.001). Increased thromboxane and β-thromboglobulin production in the setting of an acute evolving MI was also described in 59 subjects by Mueller at al.[118].

Increased platelet fibrinogen receptor activity (glycoprotein IIbIIIa), quantified using specific monoclonal antibodies and flow cytometric analysis, has been demonstrated in acute MI[123]. Kabbani et al.[125] also employed similar methods and demonstrated that increased GPIIbIIIa activation to ADP was associated with an increased 90 day complication rate (MI and revascularisation) in 112 subjects undergoing angioplasty (relative risk of high Vs. low responders = 3.78 (1.10-13.01), p = 0.035).

The complicated mechanisms involved in thrombosis and the multifactorial aetiology of myocardial infarction is highlighted by the diversity of the studies and the fact that the two largest prospective studies (Meade et al.[126] 740 subjects with 16 year follow up,

Elwood et al.[127] 1812 subjects with 5 year follow up) both failed to show a significant correlation between increased platelet aggregation and subsequent cardiovascular events.

1.5.4. Studies on the heterogeneity of platelet function

It has long been appreciated that platelet function is heterogeneous with significant intra and particularly inter-individual variations. The first case-control study of blood coagulability, in 48 stable angina patients and matched controls, by McDonald et al.[116] in 1957 demonstrated large inter-individual variations in all the platelet and coagulation tests measured. This included an overlap of the ranges, between the two groups of all the parameters studied (prothrombin time, fibrinogen, platelet count, contact clotting, factor VII, thromboplastin generation, platelet 'stickiness'). All subsequent studies employing platelet function tests have shown similar large variations. Understanding the mechanisms controlling or influencing this variation would greatly enhance our knowledge of platelet and coagulation physiology and the pathological diseases in which they play an aetiological role.

1.5.4.1 Influence of platelet volume and count

In 1967, Mannucci and Sharp[128], employing a Coulter counter for platelet volume measurements, presented indirect evidence that larger platelets were preferentially aggregated following the addition of ADP, thrombin, collagen or adrenalin and that unaggregated platelets were smaller platelets. This work was confirmed by Karpatkin[129], who in 1968[130] separated human platelets into heavy and light platelet populations and demonstrated that heavy platelets (which are enriched with larger platelets) function better than lighter platelets (enriched with smaller platelets) in their ability to aggregate with ADP, thrombin or epinephrine. It was suggested that the larger-heavy platelets are young platelets, which become smaller-lighter platelets with age. A considerable body of evidence supported this hypothesis[131,132]. Animal studies carried out by Hirsch at al.[133] and Kraytman[132] confirmed that younger or larger platelets demonstrated increased ability to bind to collagen or form a blood clot respectively. In 1977, Karpatkin[129], employing Coulter counter volume measurements and platelet aggregometry, demonstrated a direct proportionality between large platelet

volume with the velocity of platelet aggregation induced by ADP, collagen and epinephrine with correlation coefficients of r = 0.62, 0.59, and 0.53 respectively.

Normal platelet counts range from 150-400 000 / mm^3 and higher counts have been associated with increased ADP-induced aggregation[134]. Variations in platelet count and platelet volume[134,135] have shown an inverse relationship suggesting the total platelet volume or mass may remain stable.

1.5.4.2 Influence of circadian rhythm

Observational studies have reported that nonfatal MI[136] and sudden cardiac death[137] are more likely to occur between 6am and noon than during other times of the day. Tofler et al.[138] sought an explanation for this by measuring platelet activity at 3 hour intervals for 24 hours in 15 healthy men. In vitro platelet responsiveness (measured by the aggregation response) to either ADP or epinephrine was lower at 6 a.m. (before the subjects arose) than at 9 a.m. (60 minutes after they arose). The lowest concentration of these agents required to produce biphasic platelet aggregation decreased (i.e. aggregability increased) from a mean ± SEM of 4.7 ± 0.6 to 3.7 ± 0.6μM ($p<0.01$) for ADP and from 3.7 ± 0.8 to 1.8 ± 0.5μM ($p<0.01$) for epinephrine. Thus, despite the large inter-individual variations seen in these subjects, significant intra-individual variations were also demonstrated, a significant proportion of which was related to circadian rhythm and early morning activity.

1.5.4.3 Influence of epidemiological characteristics

The epidemiological characteristics of platelet function were established in 958 participants in the Northwick Park Heart Study[139]. The main analyses were based on the dose of ADP at which primary aggregation occurred at half its maximum velocity. Aggregability increased with age in both sexes, was greater in whites than blacks (particularly among men), and tended to decrease with the level of habitual alcohol consumption. Aggregability was, however, greater in women than men and in non-smokers than smokers. In a smaller study of 113 healthy men, Terres et al.[140] found age

to be the only significant determinant of platelet ADP and collagen induced aggregability.

1.5.4.4 Influence of other haemostatic risk factors

The extent of the thrombotic response following a plaque event is a result of complex interplay between platelets and the coagulation and fibrinolytic systems. A number of individual components of the coagulation and fibrinolytic systems have also been proposed as MI risk factors and may also indirectly influence platelet function.

The coagulation system is a cascade of inter-related biochemical reactions resulting in thrombin generation, fibrin formation and platelet activation. Increased plasma levels or activity of any of the molecules involved in the cascade may potentially predispose to thrombosis. The most important coagulation component is fibrinogen, which is both an acute phase reactant and the precursor to fibrin in the thrombus. The Northwick Park Heart Study[139] showed a strong association between the plasma fibrinogen concentration and platelet aggregability. Prospective studies support plasma fibrinogen as an independent risk for MI[141,142].

Factor VII plays a key role in coagulation. An overview of the six prospective epidemiological studies to date[143] testing the hypothesis that increased factor VIIc is associated with CHD incidence, does not support the original positive findings in the Northwick Park cohort[144].

Plasma von Willebrand factor (i) mediates platelet adhesion to damaged arterial walls, (ii) mediates platelet aggregation at high shear stress and (iii) binds and stabilizes factor VIIc[145]. There is inconsistent but suggestive evidence that von Willebrand factor concentration is an independent risk factor for cardiovascular disease[143]. The large prospective ARIC study[146] showed a 40% increase of CHD incidence in the highest compared to lowest quartile of von Willebrand factor (RR = 1.4 (95% CI 1.1-1.8), p = 0.006).

Factor VIII and von Willebrand factor are bound in plasma, and are correlated, and therefore association of these factors with CHD is similar[143]. Factor VIII also serves as

a cofactor for factor IXa. Both factor VIII and IXa on the surface of activated platelets and in the presence of calcium catalyse the conversion of factor X to Xa, which in turn catalyses the formation of thrombin. It is therefore conceivable that increased factor VIII level may accelerate thrombin generation.

Fibrinolysis involves the action of tissue plasminogen activator (t-PA) on plasminogen to produce plasmin, which in turn degrades the cross-linked fibrin of a thrombus. The major inhibitors of fibrinolysis are plasminogen activator inhibitor-1 (PAI-1), which inhibits t-PA, α_2-antiplasmin, which neutralizes plasmin, and thrombin activatable fibrinolysis inhibitor (TAFI)[147]. Animal models have shown increased thromboses in plasminogen or t-PA deficient mice as well as up regulation of PAI-1 and t-PA at the site of atherosclerotic plaques[146]. Various components or markers of the fibrinolytic system have been associated with thrombosis risk.

Cell lysis is a global measure of fibrinolysis and increased fibrin lysis time was associated with increased CHD incidence in men from the Northwick Park cohort[144] (relative risk = 1.67 per standard deviation greater lysis time, $p < 0.05$). t-PA antigen (reflecting decreased fibrinolytic activity)[148] and PAI-1 levels have been positively and consistently associated with CHD in prospective epidemiological studies[146]. The D-dimer molecule reflects fibrin turnover and has uniformly been strongly positively associated with increased CHD incidence[146]. In the ARIC study the relative risk was 4.21 ($p < 0.05$) for the highest compared to lowest quartile of D-dimer.

1.6 Platelet Polymorphisms

Polymorphisms (genetic variation within a gene) in the genes of various functional platelet proteins could also influence platelet reactivity and add to their heterogeneity and therefore, could modify the thrombogenicity of platelets. The platelet GPIaIIa (Section 1.3.5.3) and the GPIIbIIIa receptor complexes (Section 1.3.5.4) are highly polymorphic (i.e. display genetic variation) and can be recognised as alloantigens or autoantigens[149]. These variants have been implicated in the autoimmune thrombocytopenias, due to incompatibility of epitopes on the various platelet-surface glycoproteins. Their discovery has also led to a number of studies testing for functional

effects or association with thrombotic events and disease. To date the results have been conflicting. The main studies are summarised below and discussed in more detail in chapter 5.

1.6.1 The GPIaIIa C807T/G873A polymorphisms

Following the characterisation of the platelet GPIaIIa receptor[150], high inter-individual variability of its expression on the platelet surface was soon recognised. Kunicki et al[151] first noted this using flow cytometry in a study of 27 subjects in whom the GPIaIIa receptor expression ranged between 968-2,874 molecules per platelet. This was in direct contrast to the GPIb-IX-V and GPIIbIIIa (range 48,644-52,868) receptors, which showed some inter-individual variation in expression but these never exceeded a fraction of the mean population level. In 14 of these individuals (7 male, 7 female) a more detailed analysis of platelet attachment to collagen types I and III, fibronectin and fibrinogen was carried out every 3-5 days for 90 days (thus quantifying GPIaIIa, GPIb-IX-V and GPIIbIIIa functional activity). The results showed large variations between individuals for the mean number of platelets attached to type I (range 1.1-24.2 x 10^5 adherent platelets per well) and type III (range 5.4-28.4 x 10^5 adherent platelets per well) collagen. Individual subject's attachment rates, whether low or high, remained reproducible throughout the study suggesting inter rather than intra-individual variation. Again these differences were in marked contrast to the fibronectin and fibrinogen attachment rates (12.3-15.6 x 10^5 adherent platelets per well and 18.7-23.8 x 10^5 adherent platelets per well respectively), which showed little functional variability between subjects. A final analysis in 60 individuals revealed a four-fold variation in GPIaIIa receptor expression and this correlated significantly to the twenty fold and five fold variation in platelet attachment rates to type I and type III collagen ($r = 0.742$; $p<0.01$ and $r = 0.636$; $p<0.01$)[151].

The same group went on to show that a significant proportion of this variation was genetically mediated[66]. They identified a total of eight DNA sequence variants (polymorphisms) leading to conservative changes in the amino acid coding region of the α_2 subunit located on chromosome 5. The most influential of these were positioned

at nucleotides 807 (TTT/TTC at codon Phe^{224}) within exon 7, and 873 (AGA/ACG at codon Thr^{246}) within exon 8 of the cDNA sequence. Although these variants did not

change the amino acid sequence of the α_2 protein, there was a significant correlation between the DNA sequences and expression levels of $\alpha_2\beta_1$. To date the mechanism of how these alleles alter receptor expression has not been characterised. The alleles were in complete linkage disequilibrium (i.e. allele 807C was inherited with allele 873G and allele 807T inherited with 873A in all cases). The 807T/873A sequences were associated with higher levels of $\alpha_2\beta_1$ expression which corresponded to increased rates of platelet attachment to type I collagen at high shear flow rates[152,153], mimicking the role of the GPIaIIa receptor in vivo. These findings were confirmed further in family studies[152].

Subsequent studies on normal Caucasian populations estimated the allele frequencies of the 807C/873G and 807T/873A at 0.60 and 0.40 respectively. The percentage of the population homozygous (carrying two identical alleles) for the 807C/873G allele was estimated at 15%. Given the functional consequences of this genetic trait, such individuals could have an increased genetic predisposition to thrombosis and therefore MI. Other polymorphisms have been identified for the $\alpha_2\beta_1$ receptor but these seem to have little or no influence on expression or function[152].

Table 3 ***Association studies of the GPIaIIa G873A polymorphism and MI***

Study	Population size (age±SD)	Genotype frequency G allele	Genotype frequency A allele	Odds ratio (95% CI) p-value A/A Vs. G/A and G/G
Moshfegh et al.[154]	177 cases (57(32-72))*	-	-	3.3 (1.2-8.8), p=0.022 unadjusted
	89 controls (57(32-74))*	0.68	0.32	6.2 (1.8-21.9) p = 0.005 adjusted
Santoso et al.[155]	1050 cases (62.2±9.5)	0.59	0.41	
Overall	1187 controls (61.4±9.9)	0.61	0.39	1.13 (0.91-1.40) p = 0.25 adjusted
		G/G	**G/A and A/A**	
<62 years	481 cases	0.32	0.68	1.57 (1.14-2.13) p= 0.004 adjusted
	576 controls	0.39	0.61	
<49 years	91 cases	0.30	0.70	2.61 (1.26-5.41) p = 0.009 adjusted
	132 controls	0.42	0.58	
Roest et al.[156]	480 cases (59.1±4.1)	0.62	0.38	1.2 (0.8-1.7) p > 0.05 adjusted
Overall	496 controls (56.8±4.2)	0.62	0.38	
MI	217 cases	-	-	1.3 (0.8-2.1) p>0.05 adjusted
smokers	159 cases	-	-	2.2 (1.1-4.4) p < 0.05 adjusted
Croft et al.[157]	546 cases (61.9±9.2)	0.60	0.40	0.88 (0.74-1.05) p = 0.17
Overall	507 controls (58.6±10.6	0.57	0.43	unadjusted
<55 years	-		-	1.00 (0.53-1.90) p = 0.99 unadjusted
Morita et al.[158]	210 cases (60.9±8.5)	0.63	0.37	0.84 (0.59-1.21) p= 0.35 adjusted
	420 controls (60.9±8.5)	0.65	0.35	
< 55 years	35 cases	0.61	0.39	0.94, p = 0.89 unadjusted
	70 controls	0.61	0.39	
Benze et al.[159]		**G/G**	**G/A and A/A**	
	287 cases (40.2±2.8)	0.45	0.55	0.73 (0.47-1.12) p> 0.05 unadjusted
	138 controls (40.5±3.4)	0.38	0.62	

* median age and (range) given

A number of association studies have followed (Table 3) which have failed to give a definitive answer as to whether the polymorphisms represented an independent risk factor for MI. They will be discussed in more detail in chapter 5. These studies tended to be positive in the younger cohorts[155,160] although the youngest group of MI patients

studied by Benze et al.[161] failed to show a significant result. Only one prospective analysis has been carried out, by Roest et al., in the form of a nested case-control study[156]. This study did not show an association between the polymorphism and coronary artery disease but was positive for MI in women who smoked or had other risk factors for endothelial dysfunction such as diabetes or microalbuminuria (odds ratio = 2.2 (1.1-4.4) $p < 0.05$). No study to date has assessed both the genetic distribution and functional consequence of the C807T/G873A polymorphisms in a group of MI patients and matched controls.

1.6.2 The GPIIbIIIa C196T (leu33pro, Pl^{A1}/Pl^{A2}) polymorphism

The alloantigen referred to as Pl^A is one of the most frequently implicated in the syndromes of immune–mediated platelet destruction. Kunicki et al. demonstrated that anti- Pl^A antiserum from Pl^A-negative persons reacted with platelet GPIIIa. Subsequently, Newman et al.[162] identified the molecular basis of this polymorphism within the GPIIIa receptor gene on chromosome 17q: individuals positive for Pl^{A1} have a leucine at position 33 of mature GPIIIa; individuals positive for Pl^{A2} have a proline at this position, which is the result of the substitution of cytosine for thymidine at position 196 in exon 2 of the GPIIIa gene. This single nucleotide substitution results in the creation of a unique restriction enzyme cleavage site, recognised by the enzyme Nci I, therefore allowing conventional genotyping of the polymorphism. Subsequent studies in normal Caucasian populations showed the gene frequencies for the two alleles to be 85% C196 (Pl^{A1}): 15% 196T (Pl^{A2}).

The presence of the 196T allele has been proposed as a risk factor for MI. A detailed review of all relevant association (retrospective and prospective) and functional studies is given in chapter 5. Tables 4(a) and 4(b) list the most relevant studies carried out on the C196T polymorphism and its association with MI and procedural risk.

Table 4(a) ***Glycoprotein IIbIIIa receptor C196T polymorphism and association with myocardial infarction***

Retrospective studies

Study	Population size (age±SD)	Genotype frequency		Odds ratio (95% CI) p-value C/T and T/T Vs. C/C
		C allele	T allele	
Weiss et al.[163]	71 MI subjects (56.3±12.8)	0.77	0.23	2.8(1.2-6.4), p < 0.05
	68 controls (58.3±14.3)	0.90	0.10	
Marian et al.[164]	104 MI	0.89	0.11	
	164 controls	0.84	0.16	1.5(0.9-2.6), p = 0.13
Herrmann et al.[165]	619 MI (54.0±8.1)	0.84	0.16	
	699 controls (53.2±8.4)	0.85	0.15	Non significant
<55 years	287 MI	0.85	0.15	
	354 Controls	0.85	0.15	Non significant
Carter et al.[166]	156 MI (59.7±1.6)	0.82	0.18	
	216 controls (58.0±1.8)	0.85	0.15	1.66 (1.15-2.39), p = 0.007
Zotz et al. [167]	124 MI (55.7±9.6)	0.88	0.12	0.9(0.7-1.4), p = 0.75
	91 controls (55.7±9.6)	0.87	0.13	
< 60 years	70 MI	0.84	0.16	
	66 controls	0.89	0.11	1.3(0.9-2.0), p = 0.19
Scaglione et al.[168]	98 MI (40±4)	0.86	0.14	
	98 controls (41±4)	0.85	0.15	0.8(0.4-1.4), p > 0.05
Ardissino et al.[169]	200 MI (40.7±4.1)	0.84	0.16	
	200 controls (41.3±5.1)	0.90	0.10	1.84(1.12-3.03), p < 0.05
Benze et al.[159]	287 MI (40.2±2.8)	**C/C**	**C/T and T/T**	
		73.5%	26.5%	1.65(1.09-2.54), p = 0.01
	138 controls (40.5±3.4)	84.8%	15.2%	
Anderson et al.[170]	225 MI (63±11)	0.82	0.18	
	276 controls (62±11)	0.85	0.15	1.39(0.95-2.04), p = 0.09
Bottiger et al.[171]	793 MI (62.6±11.6)	0.84	0.16	
	340 controls (63.4±10.3)	0.85	0.15	1.06(0.79-1.42), p > 0.05
Grove et al.[172]	1019 cases, 529 MI (60.2±10.1)	0.83	0.17	
	1191 controls (59.6±9.6)	0.85	0.15	1.40(1.13-1.75), p = 0.002

Table 4(a) continued ***Glycoprotein IIbIIIa receptor C196T polymorphism and association with myocardial infarction***

Prospective studies

Study	Population size (age±SD)	Genotype frequency C allele	T allele	Odds ratio (95% CI) p-value C/T and T/T Vs. C/C
Ridker et al.[173]	374 MI (60.3)	0.87	0.13	0.93(0.7-1.2), p = 0.40
	704 controls (60.3)	0.85	0.15	
<60 years	-	-	-	0.81(0.5-1.2), p = 0.30
Bray et al.[174]	385 cases (59.2±9.7)	0.84	0.16	1.38(1.04-1.83), p = 0.028
	382 controls 59.2±9.6)	0.86	0.14	
Bojesen et al.[175]	9149 subjects (4082 men, entry age<50) 22 year follow up	0.84	0.16	T/T Vs. C/C (men) <40 years RR=5.2(1.5-18), p<0.05 40-50 years RR=3.5(1.6-7.5), p<0.05 >50years RR=0.5(0.1-1.5), p>0.05

Table 4(b) ***Glycoprotein IIbIIIa receptor C196T polymorphism and association with procedural risk***

Intervention studies

Study	Population size (age±SD)	Genotype frequency C allele	T allele	Odds ratio (95% CI) p-value C/T and T/T Vs. C/C
Walter et al.[176]	318 subjects (62.9) 224 C/C 54 C/T	0.90	0.10	5 (1.9%) stent thrombosis 6 (9.5%) stent thrombosis odds ratio 5.26(1.55-17.85)
Laule et al.[177]	653 subjects (60.6)	0.85	0.15	Composite endpoint (revascularisation, MI, death) Odds ratio=1.36(0.70-2.66),p=0.37
Kastrati et al.[178]	1150 stent subjects (62.8±10.4)	0.85	0.15	Restenosis at 6 months Odds ratio=1.42(1.09-1.84), p=0.009.
Zotz et al.[179]	251 CABG cases (64.4±8.2)	0.86	0.14	1 year post-op complications (graft occlusion, MI, death) Odds ratio=4.73(1.3-17.4),p=0.011

Weiss et al.[163] first described this in a cohort of 71 MI/unstable angina cases and 68 controls. They reported an odds ratio of 2.8 (CI 1.2-6.4, p<0.05) for risk of a coronary event in 196T allele carriers compared to C196 homozygous individuals. Once again, these studies tended to be more positive in the younger cohorts[163,167] including the

youngest group (mean age 40.2 years) of MI patients (287 cases and 138 controls) studied by Benze et al.[161], who reported an odds ratio 1.65(CI 1.09-2.54, p = 0.01) for risk of MI in 196T allele carriers. At the initiation of the work in thesis only one prospective analysis had been carried out, by Ridker et al.[173], in the form of a nested case-control study, and this failed to show an association with MI. Therefore, despite numerous studies controversy remains as to whether the 196T allele represents an independent risk factor to MI and post procedural risk after percutaneous[176,178] or surgical intervention[180] .

There is also controversy as to the mechanism of the proposed increased thrombotic risk associated with the 196T allele (Table 5). Proposed functional consequences of the 196T allele include increased fibrinogen binding to the GPIIbIIIa receptor[181-183], altered sensitivities to anti-platelet agents[183-185] and increased platelet reactivity[182,183]. Table 6 summarise the main functional studies carried out on the C196T polymorphism to date. The studies have applied a number of the methods available to assess platelet function and have yielded inconsistent results. They are also discussed in greater detail in chapter 5.

As with the GPIaIIa G873A polymorphism, the association and functional studies on the C196T polymorphism have varied in population size and the phenotype studied. Again, no study has yet looked at the association with MI and quantified platelet function in the same cohort.

Table 5 ***Functional study results on the C196T polymorphism and platelet function***

Study	Population and platelet function	Allele frequency		Gene affect on platelet function			
		C allele	T allele	C/C	C/T	T/T	Overall
Feng et al.[182]	1422 normal subjects	0.84	0.16	n=1017	n=369	n=36	
	Aggregation threshold						
	Epinephrine	(μmol/l)		0.9(0.9-1.0)	0.7(0.7-0.9)	0.6(0.4-1.0)	p=0.007
	ADP	(μmol/l)		3.1(3.0-3.2)	3.0(2.9-3.2)	2.8(2.4-3.3)	p=190
Lasne et al.[186]	102 normal subjects	0.87	0.13				
	Aggregation response			Whole group Vs. T allele carriers =			
	TRAP	(μmol/l)		8.2 ± 3.5 Vs. 5.9 ± 1.5			p = 0.0012
	ADP	(μmol/l)		1.6 ± 0.3 Vs. 2.7 ± 1.1			p = 0.023.
Frey et al.[187]	150 normal subjects	0.84	0.16	n=102		n=48	
	Aggregation response			C/C Vs. T allele carriers =			
	ADP, Trap, epinephrine, thromboxane analogue			No significant differences between genotype groups for any of the agonists			-
Goodall et al.[181]	70 stable angina patients	0.85	0.15	n=50	n=20		
	Flow cytometry			C/C Vs. T allele carriers =			
	fibrinogen binding, ADP			Significant differences between groups at			P <0.0001
	TRAP			all ADP concentrations but not TRAP			
Huang et al.[188]	54 healthy subjects	0.86	0.14	n=40		n=14	
	Flow cytometry						
	GPIIbIIIa expression						p=0.54
	p-selectin expression	(Mean fluorescence intensity)		70.3±1.8		68.1±3.5	p=0.89
				0.4±0.1		0.4±0.2	
Meiklejohn et al.[189]	70 healthy volunteers	-	-	n=35		n=35	
	Flow cytometry						
	fibrinogen binding, ADP	(% fibrinogen binding)		64.3±14.7		62.2±15.3	p=0.60
	Baseline	(% fibrinogen binding)		0.98±0.62		1.01±1.0	p=0.90
Bennett et al.[190]	100 healthy volunteers	0.84	0.16	n=10	n=11	n=5	
	ADP induced:						
	Dissociation constant	(mol/l x 10^{-7})		1.36±0.22	1.28±0.13	0.73±0.08	ns*
	Max. fibrinogen binding	(mol/l x 10^{-7})		2.65±0.15	2.90±0.14	2.17±0.11	ns*
Cadroy et al[191]	40 healthy volunteers	0.76	0.24	n=21		n=19	
	Collagen induced						
	thrombus: Normal shear	Platelet deposition		0.97 ± 0.42		0.94 ± 0.58	ns
	High shear	x10^{-7}/cm^2		4.23±1.82		4.08±1.77	ns
Cooke et al.[184]	26 healthy volunteers	-	-	n=15	n=11	0	
	Aspirin [ ] for 50%						
	aggregation inhibition	(μmol/l)		22.8±5.8	2.3±1.2		p=0.005
Undas et al.[185]	40 healthy volunteers	-	-	n=25		n=15	
	Thrombin generation	(nmol/l x 10^3)		14.6± 10.0		12.6± 7.9	
	baseline and after aspirin	(nmol/l x 10^3)		to 5.1± 3.1		to 8.8± 7.61	ns
Michelson et al.[183]	56 healthy subjects	-	-	n=20	n=20	n=14	
	Aggregation IC_{50} aspirin	(μmol/l)		13.1±3.7	7.4±2.5	14.0±2.1	p=0.024
	IC_{50} abciximab	(μmol/l)		2.27±0.19	1.90±0.21	2.13±0.14	p=0.099
Andrioli et al.[192]	63 healthy subjects	0.87	0.13	n=49	n=12	n=2	
	Aggregation IC_{50} aspirin						p<0.005
		(μmol/l)		23.4±3.3		2.7±0.6	

* C/C Vs. C/T =ns, C/C Vs. T/T < 0.05, overall affect non significant

1.7 Aims of study

Given the importance of platelets in the pathogenesis of MI and the huge burden this disease imposes on the UK from its morbidity, mortality and financial consequences, even modest mediators of risk, in the form of platelet reactivity including genetic influences, could have a large impact on targeting primary and secondary prevention strategies. Understanding the mechanisms involved at the molecular level could also lead to the development of novel therapeutic agents and more precise diagnostic capabilities.

The objectives of this study were:

1. To recruit young individuals to minimize environmental influences and enhance identification of any genetic predisposition to risk of MI
2. To study genotype and phenotype effects of the platelet polymorphisms in the same cohort (i.e. influence on risk of MI and effect on platelet function)
3. To use a platelet function test (flow cytometry) that allowed direct quantification of receptor expression and binding at the molecular level, allowing a more robust and accurate analysis of the consequences of genetic variation.

The specific aims of this study were:

- To determine whether there was a significant variability in the platelet function of normal individuals (inter-individual variation) and if so to quantify the degree of variability and the possible determinants of this variability (Chapter 4)

- To determine whether platelet function and variability is significantly different between normal individuals and those with a history of premature MI (Chapter 4)

- To determine whether platelet glycoprotein G873A and C196T polymorphisms contribute to variability in platelet function (Chapter 5)

- To determine whether in the same cohort platelet glycoprotein G873A and C196T polymorphisms contribute to the risk of a premature MI i.e. a genotype-phenotype correlation in the same population rather than looking for association alone where a causal pathway is lacking (Chapter 5)

Given the fact the study involved the recruitment of a large cohort of premature MI subjects and matched controls, a number of subsidiary aims were also included:

- Emerging biochemical risk factors (Lp(a), homocysteine, vitamin B12, folate, C-reactive protein) were analysed for their impact on MI risk (Chapter 6)

- A number of polymorphisms thought to influence intermediate phenotypes (Lp(a), fibrinogen and homocysteine) were also genotyped and correlated to the risk of MI (Chapter 6)

Chapter 2

Methods

2.1 Design of study

In this study the primary aim was to accurately quantify platelet reactivity in a large cohort of normal individuals, looking for inter-individual variations in platelet function. A similar detailed analysis was also performed in a cohort that has demonstrated a tendency to thrombosis, i.e. individuals that have suffered a premature MI.

More specifically the aim was to characterise the components, including genetic, that may influence platelet reactivity, in particular the role of the platelet glycoprotein receptor polymorphisms; G873A for the GPIa receptor and the C196T (leu33pro, PLA1/PLA2) for the GPIIbIIIa receptor.

2.1.1 Type of study

A case-control study was carried out, recruiting a group of premature MI patients, representing individuals with potentially high thrombotic risk and given the young age, more likely to be genetic mediated risk. Age and sex matched controls were recruited to represent the normal population. This allowed comparison between the two populations in terms of platelet function, conventional and emerging risk factors for MI and in particular genetic mediated risk factors (polymorphisms), not only for their influence on MI risk but also on platelet function.

2.1.2 Power calculation

Relevant platelet polymorphism frequencies vary between populations[193]. The key variant in this study was the platelet C196T polymorphism which has an allele distribution of around 85% C196: 15% 196T (see Table 4). In 200 subjects, this would give approximately 140 subjects with the 196C/C genotype and 60 subjects that express at least one 196T allele. This would provide sufficient power to detect a 1.5-fold increase in risk of MI associated with 196T allele. This is a far more conservative estimate compared to the initial study by Weiss et al.[163] which detected a 2.8 fold increase in risk associated with the T allele.

Previous studies[181] that have looked at the functional effect of this polymorphisms on platelet function have reported statistically significant results with N < 15 per genotype group. Therefore, there should be more than adequate power to reliably detect any functional effects.

2.1.3 Selection criteria for cases and controls

To avoid genetic heterogeneity only Caucasian subjects who themselves and whose both parents were born within Europe were approached. Exclusion criteria included any history of thrombophilic disorders and study visits were only completed if there were no concurrent acute illnesses to avoid influence on platelet function and measurements of the acute phase reactants (fibrinogen, C-reactive protein).

Cases

The study set out to recruit 200 cases that had suffered a heart attack before the age of 50 and were the age of 55 years or younger at time of recruitment. All cases had their diagnosis of MI verified using World Health Organisation criteria[194] from their hospital records at the time of event admission. The subjects were at least 3 months from their acute MI or any unstable angina symptoms. All case subjects had to be on aspirin and no other anti-platelet or anticoagulation agents.

Controls

Age and sex matched controls were recruited from two primary care practices based in Leicester and therefore from the same geographical region as the cases. The controls had no history of coronary artery disease.

2.1.4 Ethical approval

The Leicestershire Health Authority Research Ethics Committee granted full approval for the Platelet Reactivity and Polymorphisms in Myocardial Infarction (PRAMIS) Study. Ethics reference 5506, project number RFL 472. This included approval to recruit from the three Leicester Hospitals, local primary care groups and from the Midlands Family Heart Study. Permission to recruit subjects from June 1999 until May 2002 was given.

2.2 Recruitment and characterisation

2.2.1 Identification procedures for case subjects

Once ethical approval for the study was granted potential case subjects were identified from the Midlands Family Heart Study and the three Leicester Hospitals CCU databases.

Within the department we were already recruiting families with two or more living siblings with a history of coronary heart disease as part of the Midlands Family Heart Study. Some of the individuals who came forward voluntarily, following a media campaign, did not meet the criteria to participate in this study, but did fulfil the requirements for the PRAMIS Study. Such individuals were invited to participate.

The three Leicester Hospitals CCU databases (Leicester Royal Infirmary, Glenfield General Hospital and Leicester General Hospital) were used to identify cases that had suffered a MI under the age of 50 years in the period January 1998 to December 1999. The General Practitioners of these individuals were sent simple tick box questionnaires, from the admitting CCU cardiologist, to confirm patient details, mortality status and for permission for further contact with regard to the study. Only after favourable replies were received, were individuals contacted by post asking them to fill in a single page questionnaire regarding their medical history, place of birth, parental place of birth and willingness to participate in the study. Individuals that responded and met the correct demographic characteristics were contacted by telephone to confirm details and book an appointment for study participation.

2.2.2 Identification Procedure for control subjects

Shortly after the case population recruitment was started, an age and sex matched control population was sought. Two primary care practices within Leicestershire were used (after obtaining consent from all partners within the practice). The primary care databases were used to target appropriately matched subjects by age, sex and no history of coronary heart disease. The appropriate General Practitioner always made initial

subject contact. This was by post asking individuals to complete a single page questionnaire verifying their medical history, place of birth, parental place of birth and willingness to participate in the study.

2.2.3 Subject phenotyping

Accurate phenotyping and characterisation of the subjects was central to this study. All subject contact including initial recruitment processes and particularly the study visits were carried out either by myself or one of two experienced research nurses, both of whom had been trained specifically for the duties.

Once a suitable subject had been identified and provided written consent for further contact, an appointment for the study visit was given. All study visits were made in the mornings only to avoid potential circadian effects on measurements[136-138]. Clear written instructions were given for the subjects to be fasting from midnight on the visit day and for all current smokers to abstain from smoking for at least 12 hours prior to their appointment to minimise the acute effects of smoking on platelet function and various plasma thrombotic factors being measured. The latter was verified using a carbon monoxide breathalyser.

The study visit had the following main parts:

1. Obtaining a written consent.
2. The following data was recorded and stored in hard copy format (all details were anonymised before entering on the computerized database).

- Full contact details, age, sex, marital status, occupation
- Age of coronary event (unless control), including details of any hospital admissions.
- Symptoms of coronary heart disease prior to event and current status
- History of risk factors – Hypertension
  Hyperlipidaemia,
  Diabetes mellitus

Only taken to be positive if reliable history and on appropriate medication.

Detailed smoking history,

Socio-economic background,
History of regular exercise undertaken

- Family pedigree- A detailed family tree was obtained which included parental and sibling cardiovascular history and history of grandparents if possible.
- Any other medical history
- Drug history.

3. The following measurements were taken:

A. Blood pressure – 3 readings taken after subject has been supine for 15 minutes (an Omron HEM-705CP portable semi-automated oscillometric device (Omron Healthcare). The average of the 2 most consistent readings was recorded.
B. Height (in meters) and weight (in kilograms) to obtain body mass index.

4. Approximately 50ml of blood was taken from the anti-cubital fossa for the following investigations:

A. Lipid profile-HDL, LDL, triglyceride and lipoprotein a (LPa).
B. Factors which may influence thrombosis:
Plasma fibrinogen,
Plasma homocysteine, folate and vitamin B12
Platelet count, platelet volume, white cell count, haemoglobin,
C reactive protein (CRP)
C. Platelet function- Flow cytometry analysis
D. DNA extraction and genotyping.

2.2.4 Verification of MI in case subjects

All recruited cases were only formally entered into the study after verification from hospital records of the event defining myocardial infarction. Our verification criteria were based on the World Health Organisation definition of a myocardial infarction[194] which states that a diagnosis of MI can be made in the presence of two out of the following three criteria:

- the presence of unequivocal ECG changes (ST elevation and/or pathological q waves)
- unequivocal enzyme changes (CK or LDL) > twice upper limit of normal
- the history may be typical or untypical.

We were also able to obtain angiography data in the majority of the recruited cases, some of which were performed acutely at the event defining MI. It should be noted that the recruitment time for this study preceded the routine use of troponin as a plasma marker for myocardial necrosis. The development of this biochemical marker has had a major impact on the definition and diagnosis of myocardial infarction, mainly because of the greater sensitivity and specificity of the assay allowing more subtle clinical events to be defined as a MI. Therefore, in this study a definition of MI represented a far greater amount of necrosis and with the additional angiographic data a much more robust phenotype.

2.3 Phlebotomy and sample collection

The nature of platelet function tests and analysis of potential thrombotic risk factors dictate that all phlebotomy has to be strictly controlled to minimise artificial platelet and thrombotic factor activation. Also, samples have to be processed quickly to minimise degradation and leakage of intra-cellular components.

Samples were obtained through a 21-gauge butterfly needle using clean venepuncture without a tourniquet with any repeat venepuncture being made distal to the initial site. All samples were taken from the opposite arm to blood pressure readings and only after 30 minutes rest and in the supine position.

A strict order of sample collection was followed (Table 6). All samples were collected into Monovette tubes (Becton Dickinson). Due to the laborious nature of flow cytometric analysis of platelet function and the number of samples being collected, some of these were spun down to either plasma or serum and frozen for analysis in batches at the end of the study. An order of collection with some of the special

considerations is listed below in Table 6. A more detailed description of the methods used follows.

Table 6 ***Order of sample collection***

Sample Number	Analysis	Quantity	Type of Sample	Special Considerations
1.	Full blood count. Platelet volume.	5ml.	EDTA	Analysis of platelet volume should only be carried out after a minimum of 2 hours to control swelling. Therefore leave at room temperature for 2 hours. Processed within the department.
2.	Platelet function by: -Flow cytometry	5ml	Citrate	Process samples within 10 minutes. Further processing using the flow cytometer should be carried out within the subsequent 2 hours.
3.	Plasma markers: - Homocysteine - C-RP	10ml	Lithium/ Heparin	Process sample within 10 minutes. Centrifuge at 3000 rpm, 4°c, for 30 minutes to remove all cells to produce citrated plasma. Transfer plasma using a 1ml Gilson pipette into 500µl aliquots. Store at -70 °c.
4.	Serum folate Serum lipids: LDL, HDL, TG, LPa	10ml	Serum	Process sample after 10 minutes at room temperature. Centrifuge at 3000 rpm, 4°c, for 30 minutes to remove all cells to produce serum. Transfer serum using a 1ml Gilson pipette into 500µl aliquots. Store at -70 °c.
5.	Fibrinogen	5ml	Citrate	Fresh assay required. Analyse on day of collection.
6.	DNA Extraction	10ml	EDTA	Initial steps of DNA extraction carried out on day of collection to produce higher DNA yields. Samples stable at room temperature and extraction completed in batches.

2.4 Flow cytometry

The principle platelet function assessment technique employed in this study was flow cytometry (Section 1.4). The flow cytometric assay used was quantification of the amount of fibrinogen bound to the platelet surface under basal conditions and after agonist stimulation. Specific monoclonal antibodies were also used to quantify activated GPIIbIIIa receptors and GPIaIIa expression. Since the GPIIbIIIa activation and fibrinogen binding is the common final pathway for all forms of platelet activation, this was deemed to be a robust assay.

2.4.1 Reagents

2.4.1.1 Antibodies

Platelet bound fibrinogen was detected using a rabbit anti-human fibrinogen antibody coupled to FITC (R alpha fibrinogen-FITC), purchased from Dako Limited, UK. Monoclonal antibodies (Mab) were used to identify and quantify GPIa (CD49b) and GPIIbIIIa (RFGP56) receptor expression (Table 7). All antibodies were used at their optimal concentrations to achieve maximum fluorescence with minimum non-specific binding as determined by titration.

Table 7 ***Properties of antibodies used in platelet flow cytometric assay***

Antibody	Antigen Recognised	Fluorochrome	Volume per tube	Manufacturer	Batch number
Fibrinogen	Platelet GPIIbIIIa	FITC	2µl	DAKO	048(301)
RPGP56	Platelet GPIIbIIIa	FITC	2µl	Cyntos Biotechnology	-
CD49b	Platelet GPIa	FITC	2µl	Pharmingen	M040452

2.4.1.2 Agonists

The agonists used in this study were ADP, purchased from Sigma UK Limited, and thrombin receptor agonist peptide (TRAP), synthesized by the Protein and Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester. The agonists were titrated to determine various levels of platelet activation and appropriate concentrations of ADP and TRAP were prepared to give low, intermediate and maximum levels of platelet response. Platelet function was quantified as (i) platelet fibrinogen binding (that is the actual amount of fibrinogen occupying the GPIIbIIIa receptors on the platelet surface and therefore these are activated GPIIbIIIa receptors) in resting and stimulated samples (ADP and TRAP); (ii) GPIIbIIIa receptor expression both resting and after stimulation and (iii) GPIaIIa receptor expression in resting samples only.

2.4.2 Assay procedure

Blood samples were prepared for flow cytometric analysis using the standard whole blood method described by Janes et al.[83] and Michelson[86]. Particular measures were taken to avoid artificial or ex vivo platelet activation. These included discarding the first 2ml of drawn blood; collection without the use of a tourniquet into polypropylene tubes containing an anticoagulant; using a method which involve no washing, gel filtration, vortexing or stirring steps; gentle mixing with agonists; reducing platelet count by dilution of the sample and preparing all reagents in advance and avoiding delays in the procedure after sample collection.

The procedure involved transferring 5µl of whole blood from citrated tubes, within 10 minutes of collection, into LP3 tubes containing 50µl of HEPES-buffered saline, pH 7.4, 2µl of the appropriate antibody and 5µl of the appropriate concentration of agonist if used. After gentle mixing, the samples were incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 500µl of 0.2% (v/v) formyl saline, before being diluted 1/10 by the same concentration of formyl saline into LP4 tubes. Samples were prepared in duplicate, apart from the control and washes, giving a total of 25 tubes per subject. The exact details of antibodies and agonists used are detailed in Table 8.

The samples were analysed within 2 hours using a Profile EPICS-XL MCL flow cytometer (Coulter Electronics Limited, UK). This is an automated machine, which employs a 15mW laser, which emits a 488nm wavelength beam. The prepared samples were placed on a carousel in the correct running order (Table 9) and a pre-determined programme was used to analyse the samples. The flow cytometer was aligned daily with 10 µm "Immunocheck" and "Standard Brite" beads (Coulter Immunology), to calibrate the light scatter and fluorescence parameters respectively. Platelets were discriminated from the other cells by their specific forward scatter (FS) and side scatter (SS) characteristics, and the relevant cell population was live gated in an electronic bit map (Figure 11). The events were subjected to a single colour (FITC-fibrinogen, FITC-GPIIbIIIa, FITC-GPIa) analysis to obtain the positive percentage and median fluorescence of fibrinogen binding and GPIIbIIIa or GPIa expression in the platelet

population. For each measurement 5000 platelets were analysed and the mean of duplicate samples calculated.

Table 8 ***Running order and contents of flow cytometry samples***

Tube number	Agonists	Marker of platelet activation being quantified
1	-	Fibrinogen binding (control)
2/3	-	Fibrinogen binding (resting)
4/5	1 x 10^{-7} mol/l ADP	Fibrinogen binding
6/7	1 x 10^{-6} mol/l ADP	Fibrinogen binding
8/9	1 x 10^{-5} mol/l ADP	Fibrinogen binding
10	wash	wash
11/12	3 x 10^{-6} mol/l TRAP	Fibrinogen binding
13/14	1 x 10^{-6} mol/l TRAP	Fibrinogen binding
15/16	3 x 10^{-5} mol/l TRAP	Fibrinogen binding
17	wash	wash
18/19	-	GPIIbIIIa expression (resting)
20/21	1 x 10^{-5} mol/l ADP	GPIIbIIIa expression
22/23	3 x 10^{-5} mol/l TRAP	GPIIbIIIa expression
24/25	-	GPIa expression (resting)

2.5 Platelet count and mean platelet volume (MPV)

To complement the flow cytometric analysis of platelet function, platelet count and volume were determined. Although these have both previously been described as independent risk factors for MI, the main purpose for this analysis was to control for extreme values of each parameter, which may alter the flow cytometric results.

EDTA samples were stored at room temperature for two hours to allow maximum platelet swelling and therefore avoid differences in MPV due to differences in time taken to analysis. Measurement of the number of red blood and white blood cells, haemoglobin concentration, platelet count and platelet volume were then carried out using a Coulter® $A^{C.}T$ diff™ analyser within the department.

2.6 Genetic analysis

Genotyping of the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms was achieved by whole blood genomic DNA extraction followed by use of a novel multilocus genotyping assay, a gift donated by Roche Molecular Systems, Alameda, USA.

2.6.1 DNA extraction

10ml of blood collected in EDTA was used to extract genomic DNA using a commercially available DNA isolation kit (Puregene DNA isolation kit, Flowgen, UK). In brief, this method involved initial red cell lysis (ammonium chloride, ethylenediaminetetraacetic acid) and centrifugation to produce a white cell pellet, which was resuspended in a cell lysis solution (tris(hydroxymethyl)aminomethane) to release the white cell contents. Subsequent treatment with RNAse and protein precipitation solution (ammonium acetate) was followed by further centrifugation. The supernatant containing the DNA was poured into 100% isopropanol to precipitate the DNA that was then washed with 70% ethanol and suspended in the hydration solution (TRIS-EDTA buffer solution). All samples were checked for yield and quality by 260/280nm optical density (OD) readings on a spectrophotometer.

The samples were used to create master and working 96 well plates with the appropriate dilution of the stock DNA.

2.6.2 The multilocus genotyping assay

2.6.2.1 Background

Conventional methods of genotyping the GPIIbIIIa C196T polymorphism was first described by Newman et al.[162], using polymerase chain reaction (PCR) of the target sequence followed by allele specific endonuclease restriction digestion, and running of the products on agarose gels. Similar approaches for genotyping of the GPIa C807T/G873T polymorphisms were described by Santoso et al.[155].

Table 9 ***Candidate genes genotyped using the Roche multilocus assay***

Factors effecting thrombosis	
Clotting factors	**Platelet receptors**
Factor II *(G20210A)*	Glycoprotein Ia *(G873A)*
Factor V *(arg506gln)*	Glycoprotein IIIa *(C196T)*
Factor VII *(del -323ins)*	**Fibrinolytic factors**
Factor VII *(arg353glu)*	Plasminogen activator inhibitor type 1 *(5g/4g)*
Fibrinogen *(G455A)*	Plasminogen activator inhibitor type 1 *(G11053T)*
Factors affecting inflammation	
Proinflammatory cytokines	**Leukocyte adhesion**
Tumour necrosis factor (TNF) α *(G-376A)*	E-Selectin (ELAM) *(ser128arg)*
Tumour necrosis factor (TNF) α *(G-308A)*	E-Selectin (ELAM) *(leu554phe)*
Tumour necrosis factor (TNF) β *(thr26asn)*	Intracellular adhesion molecule 1 (ICAM1) *(gly214arg)*
Homocysteine metabolism	**Renin-angiotensin system**
Cystathione β synthatase (CBS) *(ile278thr)*	Angiotensin converting enzyme (ACE) *(ins/del)*
Methyltetrahydrofolate reductase (MTFHR) *(C677T)*	Angiotensinogen (AGT) *(met235thr)*
Lipid metabolism	
Apolipoprotein (Apo)	**Peroxisome proliferator receptors**
Apolipoprotein (Apo) A *(C93T) (G121A)*	Peroxisome proliferator activated receptors y (PPARγ) *(pro12ala)*
Apolipoprotein (Apo) A-IV *(thr347ser)*	**Hepatic lipase**
Apolipoprotein (Apo) A-IV *(glu360his)*	Hepatic lipase (LIPH) *(C-480T)*
Apolipoprotein (Apo) B *(thr71ile)*	**Lipoprotein lipase**
Apolipoprotein (Apo) B *(arg3500gln)*	Lipoprotein lipase (LPL) *(T-93G)*
Apolipoprotein (Apo) CIII *(C-641A) (C-482T)*	Lipoprotein lipase (LPL) *(asp9asn)*
Apolipoprotein (Apo) E *(E2/E3/E4)*	**Low density lipoprotein**
Paraoxanase 1	Low density lipoprotein (LDLR) *(Nco+/ Nco-)*
Paraoxanase 1 (PON1) *(met55leu)*	**Cholesterol ester transfer protein**
Paraoxanase 1 (PON1) *(gln192arg)*	Cholesterol ester transfer protein (CETP) *(C-630A)*
Paraoxanase 2	
Paraoxanase 2 (PON2) *(ser311cys)*	
Sympathetic system	**Vessel wall**
β2 adrenergic receptor	**Atrial natriuretic peptide**
β2 adrenergic receptor (ADRb2) *(arg16gly)*	Atrial natriuretic peptide (ANP) *(val7met)*
G protein b3 subunit	Atrial natriuretic peptide (ANP) *(T2238C)*
G protein b3 subunit (GNB3) *(C825T)*	**Nitric oxide synthase**
	Nitric oxide synthase 3 (NOS3) *(A-948G)*
	Nitric oxide synthase 3 (NOS3) *(glu298asp)*

We had the advantage of using a novel multilocus genotyping assay[195] developed by Roche Molecular Systems. This prototype assay was developed with the multifactorial genetic risk component of cardiovascular disease in mind (Chapter 1, Table 3) and allowed the genotyping of 63 polymorphisms within 35 candidate genes implicated in cardiovascular disease[195] (Table 9), in only two sets of multiplex reactions. The platelet

polymorphisms genotyped by the assay included the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms.

The multilocus assay (CVD35) uses pooled PCR primer pairs to co amplify the targets from genomic DNA in two sets of reactions. The amplified PCR products are then detected colorimetrically with sequence-specific oligonucleotide probes immobilised in a linear array on nylon membranes[196] (the test strips). The allele specific probe sequences have been developed to permit genotyping of all sites under a single assay condition. Therefore, large cohorts, such as the PRAMIS population, can be typed rapidly for the 63-biallelic polymorphisms, which were represented on 4 separate test strips (2 strips for each set of reactions). An example of some actual test strips used is shown in Figure 27(a), Chapter 5. The main advantages of this method are the higher rates of throughput which can be achieved and the ease with which genotyping can be carried out from the strips using a template to read each individual polymorphism (Figure 27(b), chapter 5).

2.6.2.2 Methods

20 to 25 ng of the extracted genomic DNA was amplified by two separate multiplex PCR reactions with biotinylated primers, in 96-well plates. The master mix for these reactions contained 3.3X buffer containing Tris-HCl, KCl, Mg-acetate, DMSO, dNTPs, and AmpliTaq GoId™ DNA Polymerase. Primer blend A contained biotinylated primers for amplification of targets in Multiplex A: LPA, APOA4, APOB, APOC3, APOE, ADRB3, CETP, LDLR, LIPC, LPL, PON1, PON2, and PPARG genes (Abbreviations according to LOCUSLINK). Primer blend B contained biotinylated primers for amplification of targets in Multiplex B: ACE, ADD1, ADRB2, AGT, NPPA, AGTR1, SELE, SCNN1A, F2, F5, F7, GPIA, GPIIIA, FGB, GNB3, ITGA2, ITGB3, ICAM-1, MTHFR, NOS3, SERPINE1, MMP3, TNF, and LTA genes. The majority of the primers were developed by Roche molecular systems[195]. The 96-well plates were placed into the Perkin-Elmer GeneAmp® System 9600 thermal cycler. The PCR program is given below and completed in ~2.5 hr.

PCR Program:

HOLD: 94°C for 12 min. This step is critical for enzyme activation.

CYCL: 96°C for 15 sec, 60°C for 1.0 min, 72°C for 1.25 min X 33 cycles

HOLD: 68°C for 5 min.

HOLD: 10°C until tubes are removed.

2.7 Biochemical studies

A number of extra blood samples in addition to those for the platelet function and genetic analysis tests were taken. This was to test for factors that may influence platelet function (fibrinogen) and those which may influence thrombosis (homocysteine in conjunction with folate and vitamin B12). The opportunity was also taken to measure a number of other established cardiovascular risk factors (LDL, HDL, triglyceride) as well as emerging risk factors (lipoprotein a [Lp(a)], C-reactive protein). Fibrinogen measurements were carried out at the Department of Haematology, Glenfield Hospital. Lipid, homocysteine, C-reactive protein, folate, vitamin B12 and Lp(a) were all carried out in batches at the Department of Biochemistry, Leicester Royal Infirmary. There were full internal quality controls for all assays, along with external quality assurance and full CPA accreditation of both laboratories.

2.7.1 Fibrinogen levels

Fibrinogen measurements were performed on fresh citrated plasma samples in an automated coagulation analyser, Sysmex CA 1000(Sysmex, UK), using the Clauss method[197].

2.7.2 Lipids

Plasma cholesterol, LDL, HDL and triglyceride were measured based on enzyme assays[198,199] in conjunction with chromogens to produce a colour reaction proportional to the concentration of lipid present. Standard reagents (7D62-01, 7D67-01, 7D74-01) on the AEROSET™ system (Abbott Laboratories, IL, USA) were used.

2.7.3 C-reactive protein

CRP levels were measured using the Olympus CRP Ultra-Assay (Olympus Diagnostic Systems Group, Olympus America Inc.), a latex particle enhanced immunoturbidimetric assay for the quantitative determination of human CRP in serum by an ELISA technique[200], on the Olympus AU 400 Analyser. This ultra sensitive assay allows detection and quantification of CRP levels much lower (in the normal range) than conventional assays that have historically been used as a general test for inflammation. This is necessary because even marginally elevated CRP levels have been found to be independent predictors for cardiac events in apparently healthy men[201].

2.7.4 Homocysteine

Plasma total homocysteine levels were measured using a commercially available kit (Chromesystems Limited, UK) by high-performance liquid chromatography (HPLC) with fluorescence detection based on the methods described by Araki et al[202]. Plasma specimens obtained from subjects were refrigerated immediately after phlebotomy and stored at or below -20°C. The stability of plasma homocysteine in samples stored at temperatures below -20°C has been reported previously[203].

2.7.5 Lipoprotein a

Lp(a) measurements were performed by a commercially available ELISA (Sigma-Aldritch, UK), on the Olympus AU 400 Analyser. In brief, this is an automated immunoprecipitation procedure that uses a monoclonal antibody against apo(a) that does not cross-react against plasminogen and a second polyclonal antibody directed against the apo(a) portion of Lp(a)[204].

2.7.6 Folate and vitamin B12

Folate and vitamin B_{12} levels were measured using a competitive chemiluminescent immunoassay on an Advia Centaur Analyser using commercially available assays supplied by Bayer Diagnostics, UK. In brief, folate in the patient sample competes with

acridinium ester-labelled folate in the Lite Reagent for a limited amount of biotin-labelled folate binding protein. Biotin labelled folate binding protein binds to avidin that is covalently coupled to paramagnetic particles in the Solid phase, which are then detected by the Advia Centaur Analyser.

2.8 Statistical analysis

All the collected data, including history, measurements, biochemical, platelet and genetic analyses were entered on Excel spreadsheets (Microsoft office professional, 2000). These were used to create a single master database containing all the information on one large spreadsheet.

All subsequent analyses were carried out using the Excel 2000 statistics package, SPSS version 12 or Prism 3. These programmes were also used to generate all graphs and histograms included in this thesis. Professor John Thompson and Dr. Martin Tobin from the University of Leicester Department of Statistics provided guidance and help with the data analysis.

Chapter 3

Population characteristics

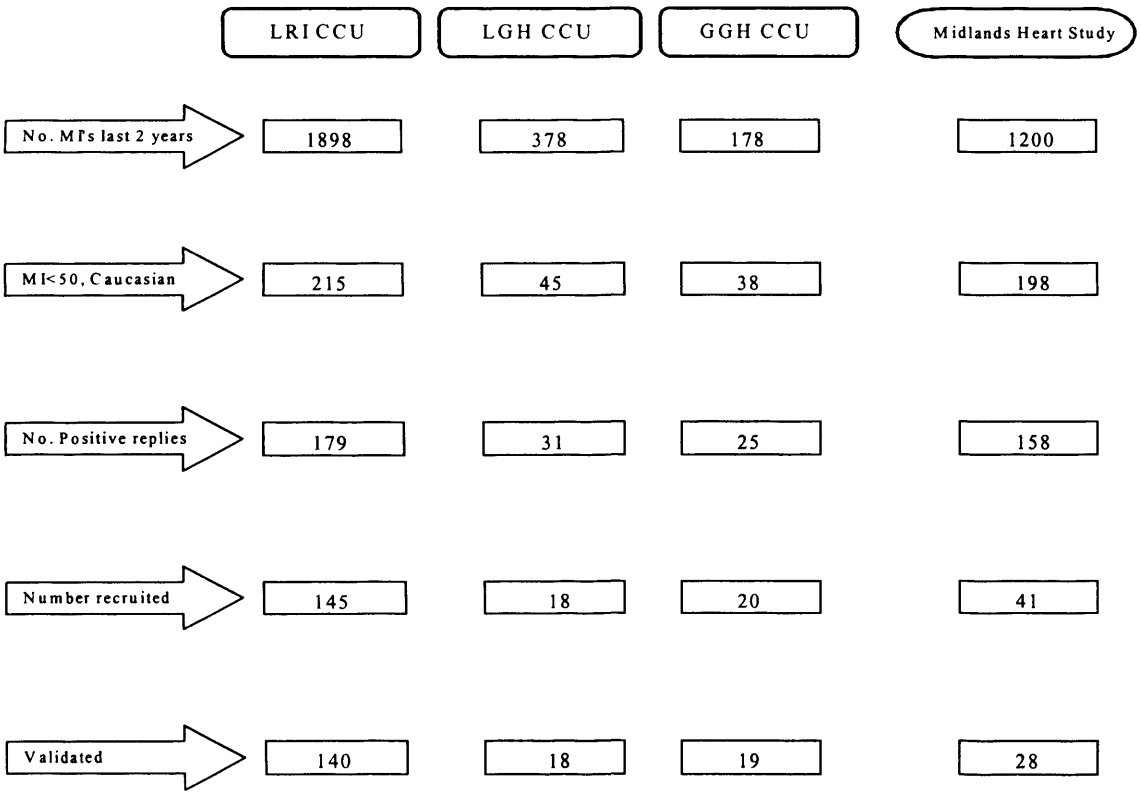
3.1 Population characteristics

In this chapter details of subject recruitment, population demographics and characteristics are described. Demographic details and conventional risk factors are compared between the case and control populations. By using the angiographic data available in the majority of the case population, comparisons between low and high coronary disease burden subjects are made. The main strengths and weaknesses of the recruited population and its relevance to this type of study are discussed.

3.1.1 Population recruitment and validation

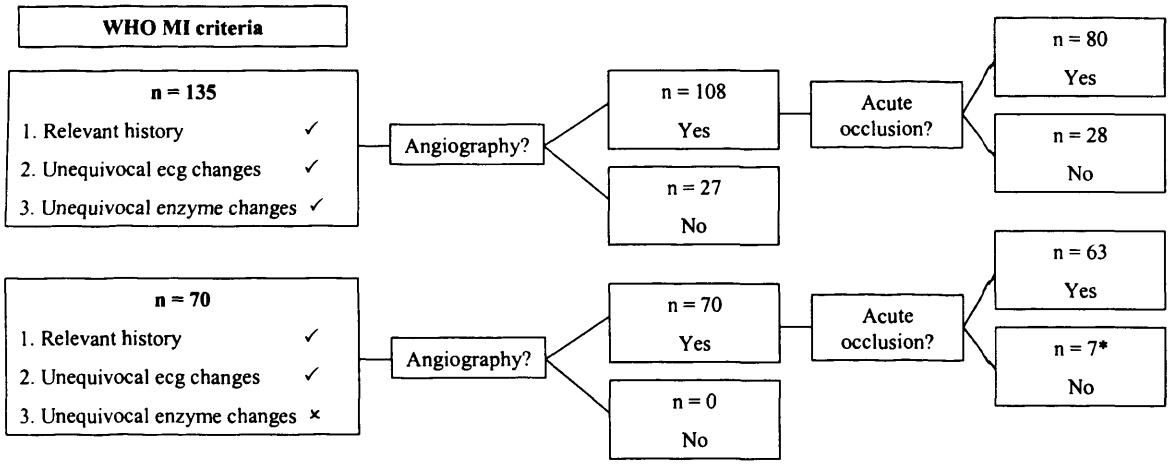
Cases were recruited through the 1998-1999 databases of the three Leicester coronary care units and via the Midlands Family Heart Study. The sources of the cases are summarised in figure 13.

Figure 13 ***Case recruitment processes***



In brief, initially the registered general practitioners of the subjects were contacted to confirm address details, whether the subject was still alive and gain permission to contact. In total 496 potential case subjects were invited to participate in the study. Of these, 393 returned questionnaires that met the recruitment criteria and were willing to participate in the study. These subjects were then contacted in the chronological order of their reply to arrange a study visit appointment. In total 224 subjects were recruited as cases into the study and underwent all the investigations. 12 subjects were excluded from the final analysis due to insufficient data from hospital records to validate their MI. A further 9 subjects were excluded due to incomplete flow cytometry data as a result of technical complications with the machine and unwillingness of the subjects to a return visit. This gave a total of 205 validated cases with a full set of investigative results (Figure 14).

Figure 14 ***Validation of the case population phenotype***



* Although in these seven cases an unequivocal enzyme rise or acute coronary occlusion could not be verified, a relevant history with acute ST elevation and subsequent angiography showed at least 1 occluded artery was enough to satisfy MI criteria.

This figure reflects the detailed MI phenotype verified from hospital records in the 205 recruited cases

Controls were easier to recruit and achieved using two large primary care groups in Leicester. With the consent of all the practice partners, a database of subjects without a

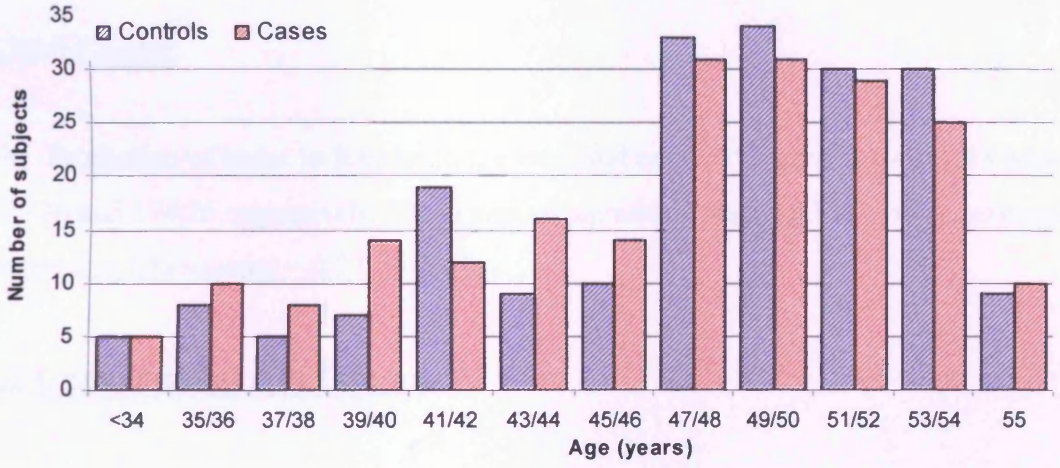
history of coronary heart disease was constructed. Appropriate aged and sex-matched subjects were then invited to take part in the study by a questionnaire. Approximately 500 questionnaires were sent in total, giving 350 willing replies. 200 controls were subsequently recruited in total with no dropouts.

3.1.2 Population demographics

3.1.2.1 Age

The mean ages (± SD) of the case and control populations were 46.8 (± 6.1) and 47.3 (± 6.0) years respectively. There was no significant difference in age between the groups, p=0.18. A graphical representation of the age distribution is given in Figure 15.

Figure 15 ***Age at recruitment of cases and controls***



3.1.2.2 Event age

The mean event age of MI in the case population was 42.3 (± 5.7) with an event range of 23 to 50 years. The median and mode were 43 and 49 respectively. The event age distribution is represented graphically in Figure 16.

Figure 16 ***Distribution of age at MI in case population***

3.1.2.3 Gender

The distribution of males to females in the recruited case and control populations were 175:30 and 174:26 respectively. There was no significant statistical difference between the groups, Chi squared = 0.23, p=0.63.

3.2 Conventional risk factors

A detailed history of the conventional risk factors was obtained for all recruited subjects. A summary of the basic characteristics and distribution of the conventional risk factors are shown in Table 10.

Table 10 ***Basic characteristics of case and control population***

	Cases	Controls	p-value
Number recruited	205	200	
Male/female	175/30	174/26	ns*
Mean age	47.8 ± 6.1	47.3 ± 6.0	ns*
Event age	42.3 ± 5.7	-	-
Hypertension history	58/205 (28.3%)	18/200 (9%)	<0.001
Diabetes mellitus	22/205 (10.7%)	2/200 (1%)	<0.001
Current smokers	42/205 (20.5%)	36/200 (18%)	ns*
Total cholesterol (mmol/l)	4.93 ± 1.27	5.21 ±1.04	p=0.007
LDL cholesterol (mmol/l)	2.93 ± 1.05	3.18 ± 0.92	p=0.005
HDL cholesterol (mmol/l)	1.13 ± 0.35	1.35 ± 0.34	p<0.001
Triglyceride (mmol/l)	1.93 ± 0.99	1.50 ± 0.84	p<0.001
Body mass index (kg/m2)	29.8 ± 5.3	26.9 ± 3.9	p<0.001
Systolic BP (mmHg)	128 ± 18	133 ± 14	0.002
Diastolic BP (mmHg)	82 ± 12	86 ± 10	0.001

*ns = non significant

3.2.1 Hypertension

A positive history of hypertension was accepted only if the subjects were actually treated for the condition. There was a significant difference between the groups, with a higher history of hypertension in the case group, 58/205 (28.3%) compared to the controls, 18/200(9%), Chi squared = 27.72, p<0.001.

Blood pressure readings were taken at the study visit in a controlled setting (Section 2.2.3). It should be noted that 179 (87%) of the cases were on medication that would lower blood pressure (mainly for secondary MI prophylaxis) at the study visit compared to only 9 (5%) of the controls. Therefore, the cases had a significantly lower mean reading for both systolic (p=0.002) and diastolic (p=0.001) blood pressure

3.2.2 Diabetes Mellitus

A history of diabetes was only accepted if individuals had been formally diagnosed and/or were on diabetic medication. Once again there was a higher proportion of diabetics in the case population, 22/205 (10.7%) compared to the controls, 2/200 (1%), Chi squared = 17.20, $p<0.001$.

3.2.3 Smoking

A detailed smoking history was taken, including number of years, age of initiation, age of termination if relevant and number of cigarettes smoked per day. For simplification of analysis, individuals were categorised into smokers, ex-smokers or non-smokers. To allow a more appropriate association with risk of MI, smoking history in the cases at the time of MI was used. There was a clear statistically significant association between smoking and risk of MI, $p<0.001$. However, it should be noted that there was no significant difference in current smoking status between the groups at the time of recruitment (20.5% Vs. 18%), which is relevant in comparing platelet function tests between the groups (Table 11).

Table 11 ***Smoking histories in cases and controls***

	Cases at MI	Cases at recruitment	Controls
Non-smoker (%)	36 (17.6)	36 (17.6)	100 (50)
Ex-smoker (%)	33 (16.1)	127 (61.9)	64 (32)
Smoker (%)	136 (66.3)	42 (20.5)	36 (18)
Total	205	205	200

3.2.4 Cholesterol

No direct comparison could be made using serum cholesterol levels at the time of recruitment, as they would be heavily influenced by the fact that 158 (77%) of the cases were on a statin compared to none of the controls. The serum results of cholesterol,

LDL and HDL are shown in Table 10. As expected the total cholesterol and LDL levels are significantly lower in the cases as a result of the statin treatment. However, HDL cholesterol and triglyceride levels, which are less affected by statin treatment, remain as persistent risk factors in the case group (lower HDL levels associated with MI).

3.2.5 Family history

A true comparison of family history of coronary artery disease or myocardial infarction could not be made between the cases and controls, as one of the exclusion criteria for the controls was the presence of a positive family history. We used two separate definitions of a positive family history. The first (FH1) definition was a history of angina, MI or stroke in a first-degree relative under the age of 65 years. The second (FH2) was a stricter definition of a MI in a first-degree relative under the age of 50.

Of the 205 recruited cases, 123 subjects (60%) were positive for the softer (FH1) definition. 61 subjects (29.8%) of the cases actually gave a positive family history of MI using the stricter definition (FH2). These results in particular highlight the genetic contribution to MI risk, especially in a young cohort with premature disease.

3.2.6 Body mass index

Body mass index (BMI) was calculated by dividing the weight of the subject in kilograms by the squared height of the subject in meters. There was a significantly higher mean BMI in the cases compared to the controls, 29.8 ± 5.3 vs. 26.9 ± 3.9, $p<0.001$

3.3 Non-conventional risk factors

A number of emerging biochemical risk factors were also assessed. These were fibrinogen, homocysteine, vitamin B12, folate, C-RP and Lp(a). The results are presented and discussed in Chapter 6.

3.4 Angiographic data

There was angiographic data on 175 out of the 205 cases. A coronary artery was considered significantly diseased if a 50% or greater stenosis was present. Angiography data showed 0, 1, 2, or 3-vessel disease in 8, 67, 50 and 50 individuals respectively. Figure 17 compares the prevalence of the conventional risk factors in these individuals in relation to disease burden by using the number of diseased vessels as a surrogate of this.

Figure 17 ***Prevalence of the conventional risk factors (%) and burden of coronary disease (number of diseased vessels), n=175.***

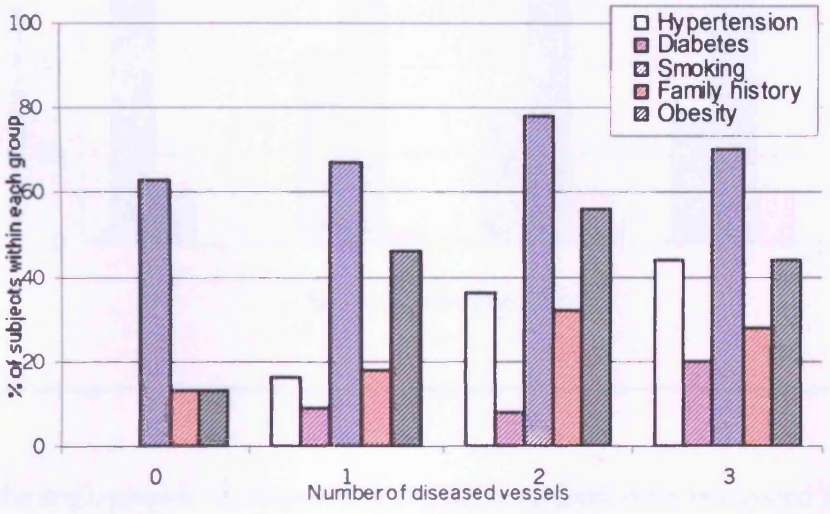
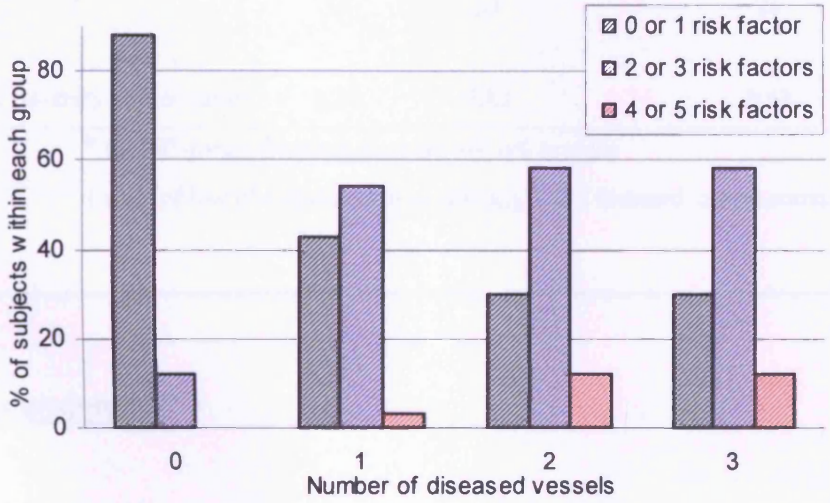


Figure 18 compares the total number of risk factors to coronary burden. Smoking was a ubiquitous risk factor at all levels of coronary burden underlining its causal relationship with atherosclerosis and thrombosis. A history of 20 or greater pack years of smoking (1 pack year = smoking 20 cigarettes per day for 1 year) more than doubled the odds of developing a higher coronary burden (2-3 vessel disease), odds ratio 2.10 (1.14-3.88), p=0.018. Of the conventional risk factors hypertension was significantly more prevalent in the higher coronary burden individuals, Chi squared = 13.32, $p<0.001$ (Figure 17), and also was the only significant predictor of higher coronary burden compared to low burden (0-1 vessel disease) individuals (odds ratio=3.88 (1.82-8.25),

p<0.001). There was a significant relationship between higher disease burden and total number of risk factors, Chi squared = 14.96, p = 0.021 (Figure 18). Having three or more conventional risk factors significantly increased the odds of having a higher coronary disease burden, odds ratio=2.59 (1.22-5.45), p=0.012.

Figure 18 ***Prevalence of number of conventional risk factors (%) and burden of coronary disease (number of diseased vessels), n=175.***



Finally, the angiographic findings in the recruited subjects were compared to previous MI cohorts with similar angiographic data available. Table 12 highlights the increasing coronary burden seen in more elderly groups and the importance of selecting young MI subjects to study the direct role of thrombosis.

Table 12 ***Angiographic coronary disease in the PRAMIS and four other MI cohorts***

	Glover et al[205]	Benze et al[159]	PRAMIS	Bigi et al[206]	Laster et al[207]
Number	114	274	175	159	55
Mean age*	**32.1 ± 3.0**	**40.2 ± 2.8**	**47.8 ± 6.1**	**58 ± 10.0**	**83.3 ± 2.3**
Male : female (%)	93 : 7	100 : 0	85 : 15	87 : 13	36 : 64
Coronary burden (%)					
0	6	18	5	3	0
1	51	27	38	35	31
2	27	21	29	35	42
3	15	34	29	26	27
Low:high coronary burden ratio†	**1.36**	**0.82**	**0.74**	**0.62**	**0.45**

* the MI groups increase in mean age left to right

† ratio of low (0-1 diseased artery) to high (2-3 diseased artery) coronary burden.

3.5 Discussion

As planned, I was able to recruit a large young Caucasian cohort with detailed phenotypic information with all MIs verified from hospital records using strict criteria[194] to avoid ambiguity. The basic characteristics of the cases and controls are summarised in Table 10. The cases and controls are comparable by age and gender. As expected[208], conventional risk factors (hypertension, diabetes mellitus, smoking, BMI and family history) were significantly increased in the cases. Recruiting controls only if there was no family history of coronary disease would have further amplified these differences. It would have been ideal to try and match the populations for the conventional risk factors but this would require a ten-fold increase in the size of the control population being screened. Also, it would increase the chances of recruiting controls who may have sub clinical coronary heart disease. Finally, as all the cases are on various treatments, their systolic BP, diastolic BP and total cholesterol were all actually significantly lower than the controls.

The main strengths of this study population included:

The low mean age of the population and in particular the low mean event age in the cases, reflecting a cohort with a history of a significantly premature MI. Looking at such an extreme phenotype would increase the chances of obtaining significant results with regard to thrombotic and genetic risk factors. The former point is reinforced by Table 12, which compares angiographic data from 5 separate aged cohorts and shows increasing coronary burden with age. The genetic susceptibility is highlighted by the fact that nearly two-thirds of the cases had a first degree relative with a history of coronary or cerebrovascular disease under the age of 65, and that nearly one-third had a first degree relative with a MI under the age of 50. The Swedish twin study[48] showed that inherited risk factors for CHD are relevant only for individuals with premature heart disease and that the genetic effect decreases at older ages. Indeed, as the discussion sections in Chapter 5 highlight most previous positive results for the G873A and C196T polymorphisms have been in premature MI cohorts.

Detailed phenotyping not only of classical cardiovascular risk factors and lifestyle (Chapter 3) but also a number of established and emerging biochemical risk factors (Chapter 6). This information led to a robust logistic regression analyses taking into account a number of relevant biological factors, not only to the risk of MI but also platelet function. This type of analysis is also more appropriate to the multifactorial nature of MI pathophysiology and platelet function determinants.

Detailed platelet functional analysis (Chapter 4). This phenotype in particular was extremely important in allowing the quantification of directly relevant platelet receptors and function, to the polymorphisms being studied.

The main limitations to this study were:

Fatal cases of MI were unable to be recruited and may represent the most extreme phenotype in terms of thrombotic risk and platelet reactivity. Due to the nature of platelet function this study could not have been done in the acute setting, however, previous studies[117] have shown increased platelet activation in the acute setting and that this continues to be demonstrated up to 24 months after the acute event.

Recruiting only Caucasians minimised racial genetic heterogeneity but may also have underestimated important genetic differences more prominent in other racial groups.

A single visit strategy allowed the recruitment and planning of such a study (over 500 patient visits in total) to remain manageable. However, platelet function in particular shows tremendous heterogeneity and more than one visit if practical would have been desirable. Nevertheless, the intra and inter-assay variability of platelet flow cytometry showed good reproducibility (Chapter 4).

Population size, although of adequate power to detect a 1.5-fold increase in risk of MI with the polymorphisms being studied, may have been too small to detect a more modest effect. Especially as the pathophysiology of MI and thrombosis are multi-factorial processes (Chapter 1). However, this study represents the largest study undertaken to date, of platelet flow cytometric analysis in a MI cohort.

Chapter 4

Tests of platelet function

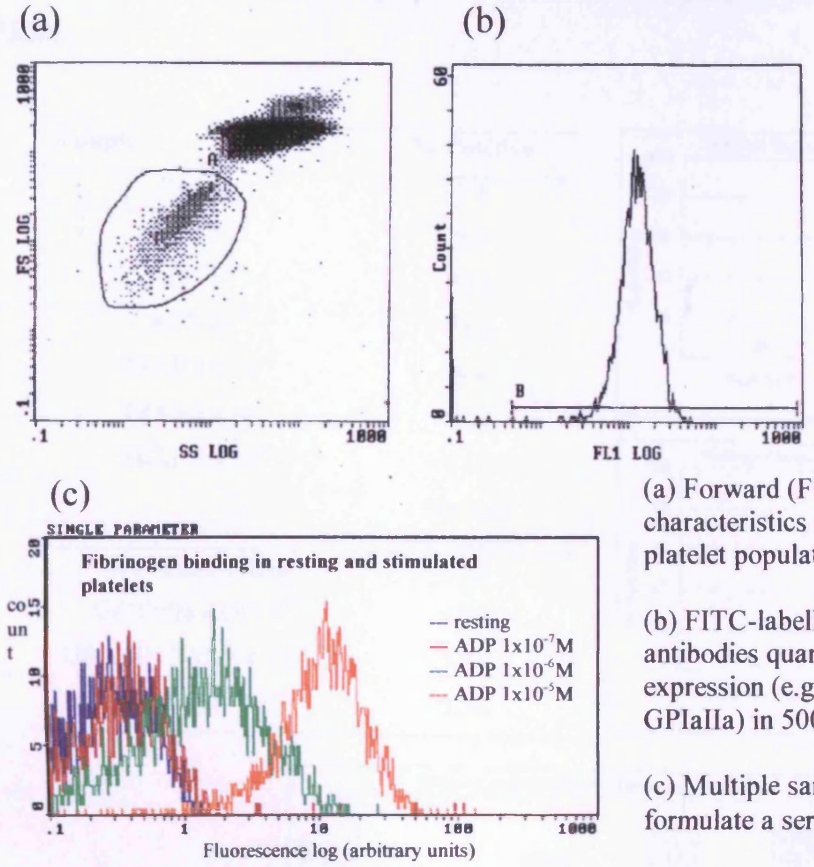
This chapter includes (i) explanation of the flow cytometry results and analysis; (ii) measures undertaken to optimise the flow cytometry assay including description of the effect of unstable angina on platelet function and the effect of prolonged time delay from sample preparation to analysis; (iii) the assessment of the repeatability of the method by measuring inter and intra-assay variation; (iv) platelet function in the control and case subjects including inter-individual variations and identification of demographic parameters that influence platelet function; (v) comparison of platelet function assays between the control and case populations.

4.1 Flow cytometry results and analysis

The principles and methods used in flow cytometry have been described in Chapters 1 and 2 respectively. A brief explanation of the analyses undertaken follows. Whole blood flow cytometry using FITC-labelled antibodies were used to detect (i) platelet activation, by quantifying platelet surface fibrinogen binding (unstimulated and stimulated samples); (ii) expression of the GPIIbIIIa receptor (unstimulated and stimulated samples) and (iii) expression of the GPIa receptor (unstimulated samples only).

The flow cytometer initially differentiates particles passing across its laser by their forward scatter and side scatter profiles (Figure 19(a)), thus allowing the platelet population to be identified (gated). For the assessment of platelet function 5000 separate particles (platelets), are analysed for each individual sample. Specific FITC-labelled antibodies are detected within the population in the form of emitted fluorescence (Figure 19(b)). Two principle results are obtained from each sample; (i) the percentage of the particles (platelets) that emit fluorescence (% positive) and (ii) the average amount of fluorescence emitted per particle per sample, this is taken as the median rather than the mean value due to the skewed distribution of the results.

Figure 19 ***Analysis of platelet function using flow cytometry***



(a) Forward (FS) and side scatter (SS) characteristics are used to identify the platelet population

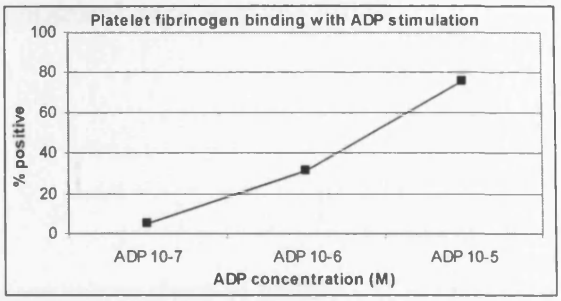
(b) FITC-labelled monoclonal antibodies quantify specific antigen expression (e.g. fibrinogen, GPIIbIIIa, GPIaIIa) in 5000 platelets

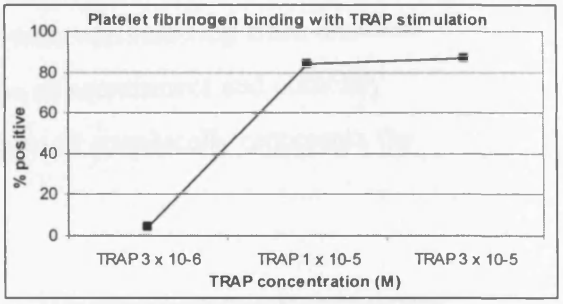
(c) Multiple samples are assessed to formulate a series of results

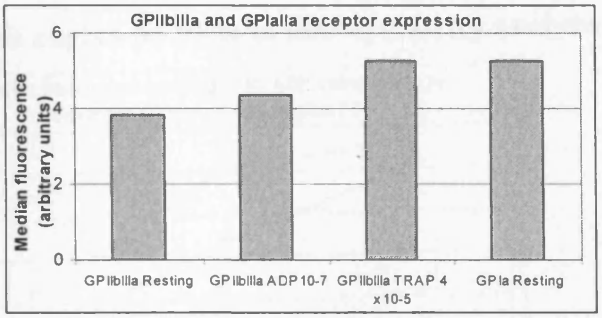
Therefore, for each subject, 11 separate samples in duplicate (Figure 20) were analysed and the average % positive and median fluorescence of the paired samples tabulated on an excel worksheet for further analysis.

Figure 20 ***Example of flow cytometry assessment of platelet function (individual P502)***

Sample	% Positive
Resting	2.1
ADP 10^{-7}	5.0
ADP 10^{-6}	31.5
ADP 10^{-5}	75.6
TRAP 3 x 10^{-6}	4.4
TRAP 1 x 10^{-5}	84.2
TRAP 3 x 10^{-5}	87.1
	Median florescence
GPIIbIIIa Resting	3.84
GPIIbIIIa ADP10^{-7}	4.35
GPIIbIIIa TRAP 4 x 10^{-5}	5.26
GPIa	5.26







Platelet surface fibrinogen binding will only occur upon the activation of the platelet leading to the conformational exposure of the epitope within the GPIIbIIIa receptor[209]. Quantification of the percentage (%) of the platelet population expressing surface bound fibrinogen, by flow cytometry, is a sensitive and standardised method of detecting activated platelets[85]. The method allows the detection of much lower levels of platelet activation (<2% of the platelet population) compared to alternatives such as p-selectin or CD63 expression[85] and even allows resting samples to be analysed.

In quantification of receptor expression the measured antigen is present on the surface of all platelets and the % positive result would not be discriminatory and therefore it is

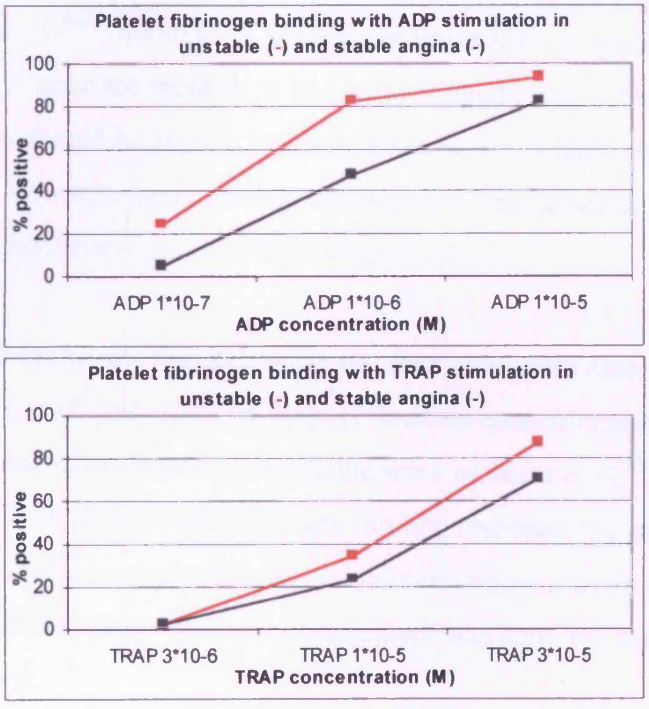
the median fluorescence that differentiates between levels of receptor expression. Median fluorescence levels have no specified units and therefore the results are expressed in arbitrary units.

The flow cytometry analysis generated a detailed set of platelet function results for all 405 subjects (200 controls, 205 cases) recruited.

4.2 Effects of unstable angina

To investigate the effect of unstable angina on flow cytometric analysis of platelet activation, a comparison was made on a single subject who was suffering from unstable angina (history and ECG evidence) on the first occasion of recruitment and clinically stable on the second visit some three months later. Figure 21 graphically represents the main results.

Figure 21 **Effect of unstable angina on platelet flow cytometry analysis**



The results, in a single individual, demonstrate that upon stimulation (with ADP and TRAP) there is an increase in the number of platelets binding fibrinogen when the subject is clinically unstable with angina. Unstable angina did not have a noticeable effect on resting fibrinogen binding (2.1% Vs. 2.3% in stable and unstable setting respectively) or the expression of the GPIIbIIIa receptor on the platelet surface. There was a small increase in the GPIa expression associated with unstable angina. These results correlate to previous findings that have demonstrated increased platelet activation in the setting of an acute MI or unstable angina[117,118,122,124,210,211]. Possible explanations for the hyper-reactive platelet function include inherent hyper-reactivity predisposing to unstable angina; secondary to the actual clinical event (plaque rupture) and secondary to drug therapy such as heparin[77].

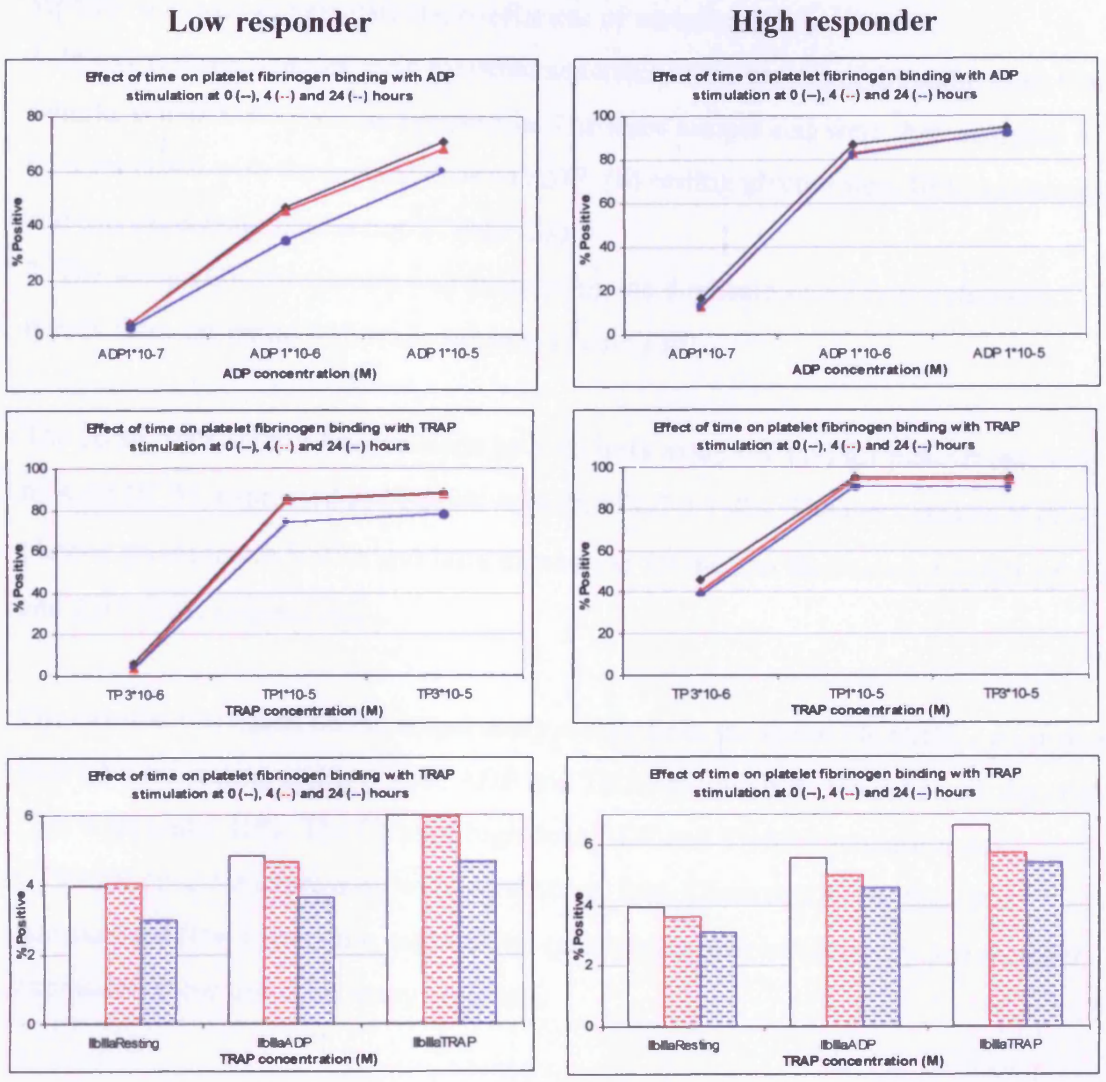
To minimise these confounding factors, all case subjects recruited into the study were at least 3 months post any unstable symptoms or ischaemic events.

4.3 Effect of delay in flow cytometry assessment

Standard protocols[85,86] quote ideal assessment times from preparation of samples for flow cytometry (once the incubation has been stopped using 0.2% formylsaline) to actual analysis, should be kept to less than 2 hours. To evaluate the potential effects of time delays in flow cytometry assessment, samples were assessed at 0, 4 and 24 hours in 1 low and 1 high response individual (Figure 22).

These results demonstrate that a delay in the flow cytometry assessment from 0 to 4 or 24 hours has a significant effect on platelet function quantification in both low response and high response subjects and confirms the work of Janes et al.[83]. This was evident in the ADP and TRAP stimulated fibrinogen binding response and expression of GPIIbIIIa receptors as a result of sample and antibody deterioration with time. The time from sample preparation to flow cytometry assessment was kept less than 2 hours in all the recruited subjects.

Figure 22 ***Effect of time delay on flow cytometry assessment on a low and high responder individual***



4.4 Reproducibility of the platelet reactivity flow cytometric assay

The single visit strategy planned in this study would require these results to accurately represent the platelet function of an individual. It was therefore essential that the assays used were standardised and had low intra and inter assay variability.

4.4.1 Intra-assay variability

The intra-assay variability of the flow cytometry assay was quantified by using two separate methods to calculate the coefficient of variation (CV):

1. Initially, three samples were prepared separately 10 times using the same fresh blood sample, standard reagents and protocols. The three sample sets were then analysed for (i) stimulation with the highest dose of ADP, (ii) resting glycoprotein IIbIIIa expression and (iii) Ia receptor expression (Table 13a).
2. The second CV calculation was made using the duplicate set of flow cytometry results from the entire 405 study subjects (Table 13b).

The 10 separate sample preparations gave an intra assay CV (%) for platelet reactivity to ADP 10^{-5}M, expressed as % positive cells, of 2.7 ± 1.4%. The intra assay CV (%) for platelet glycoprotein IIbIIIa and IaIIa expression for median fluorescence is 0.9 ± 0.7% and 2.0 ± 0.9% respectively.

The calculations based on the actual study results from the entire PRAMIS cohort show high CVs for resting and low dose ADP and TRAP stimulation. However, all the other CVs were under 10%. The CVs for high dose ADP and TRAP stimulation and GPIIbIIIa receptor expression were particularly low. These results confirm that standardised flow cytometric methods for quantifying platelet reactivity and receptor expression show low intra assay variation.

Table 13 ***Measurement of flow cytometry intra-assay variation***

(a) Based on analyses of 10 separately prepared samples

	(i) ADP 10^{-5}M response	(ii) GPIIbIIIa expression	(iii) GPIa expression
Sample no.	% Positive	Median fluorescence	Median fluorescence
1	83.0	3.42	7.69
2	78.0	3.43	7.71
3	81.3	3.46	7.75
4	80.1	3.40	8.04
5	85.3	3.42	7.85
6	83.8	3.42	7.85
7	83.3	3.41	8.11
8	80.1	3.46	8.12
9	83.7	3.48	7.77
10	82.0	3.44	7.87
Mean	82.1	3.4	7.9
SD	2.20	0.03	0.16
CV (%) (mean ± SD)	**2.7** ± 1.4	**0.9** ± 0.7	**2.0** ± 0.9

Three sets of samples were prepared using standard protocols for (i) stimulation with the highest dose of ADP; (ii) resting glycoprotein IIbIIIa expression and (iii) Ia receptor expression were. Each of the three samples was prepared 10 times and the results used to quantify the intra-assay variability.

(b) Based on variation between paired sample results from entire PRAMIS cohort (n=405)

Sample	Result analysed	Coefficient of variation (mean ± SD)
Fibrinogen binding Resting	% Positive	14.7 ± 12.8
Fibrinogen binding ADP 10^{-7}	% Positive	16.8 ± 21.8
Fibrinogen binding ADP 10^{-6}	% Positive	6.7 ± 8.0
Fibrinogen binding ADP 10^{-5}	% Positive	3.8 ± 8.0
Fibrinogen binding TRAP 3 x 10^{-6}	% Positive	26.3 ± 26.3
Fibrinogen binding TRAP 1 x 10^{-5}	% Positive	7.2 ± 14.8
Fibrinogen binding TRAP 3 x 10^{-5}	% Positive	2.3 ± 3.2
GPIIbIIIa Resting	Median fluorescence	1.6 ± 1.8
GPIIbIIIa ADP10^{-7}	Median fluorescence	1.5 ± 1.9
GPIIbIIIa TRAP 4 x 10^{-5}	Median fluorescence	2.9 ± 11.0
GPIa	Median fluorescence	6.6 ± 8.7

4.4.2 Inter-assay variability

To quantify the inter-assay variability of the flow cytometry assay 10 subjects (4 controls and 6 cases) were tested on 2 separate occasions at least 1 month apart. The comparison of the two sets of results would depend on the inter and intra assay variability. Measures were taken to avoid changes in the clinical situation in subjects between the 2 visits i.e. change in angina status or medications. Samples were taken and prepared using the standard protocols on both occasions. The panel of tests carried out included resting and stimulated platelets with ADP ($1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$M) and TRAP ($3x10^{-6}$, $1x10^{-5}$, $3x10^{-5}$M).

The coefficients of variation for the samples are summarised in Table 14. The results represent the mean and standard deviation for 10 subjects on two separate visits. Coefficients of variations were calculated for the % positive results. The appropriateness of the statistical methods adopted for this purpose has been described in greater detail by Bland and Altman [212].

Table 14 ***Coefficient of variation results for resting and stimulated platelet samples using flow cytometry in 10 subjects on 2 separate visits***

	% Positive		
	Mean	**SD**	**CV (%)**
Fibrinogen binding Resting	2.46	0.43	**6.0 ± 20.3**
Fibrinogen binding ADP 10^{-7}	15.22	7.07	**46 ± 24.6**
Fibrinogen binding ADP 10^{-6}	61.59	7.64	**17 ± 17.4**
Fibrinogen binding ADP 10^{-5}	80.27	3.29	**6.9 ± 5.4**
Fibrinogen binding TRAP 3 x 10^{-6}	15.82	3.67	**27.9 ± 13.0**
Fibrinogen binding TRAP 1 x 10^{-5}	70.51	9.87	**25.3 ± 23.8**
Fibrinogen binding TRAP 3 x 10^{-5}	80.74	2.55	**3.2 ± 3.0**

The above results suggest that maximum stimulation with either ADP or TRAP lead to the most reproducible results and are in keeping with the intra assay variability results.

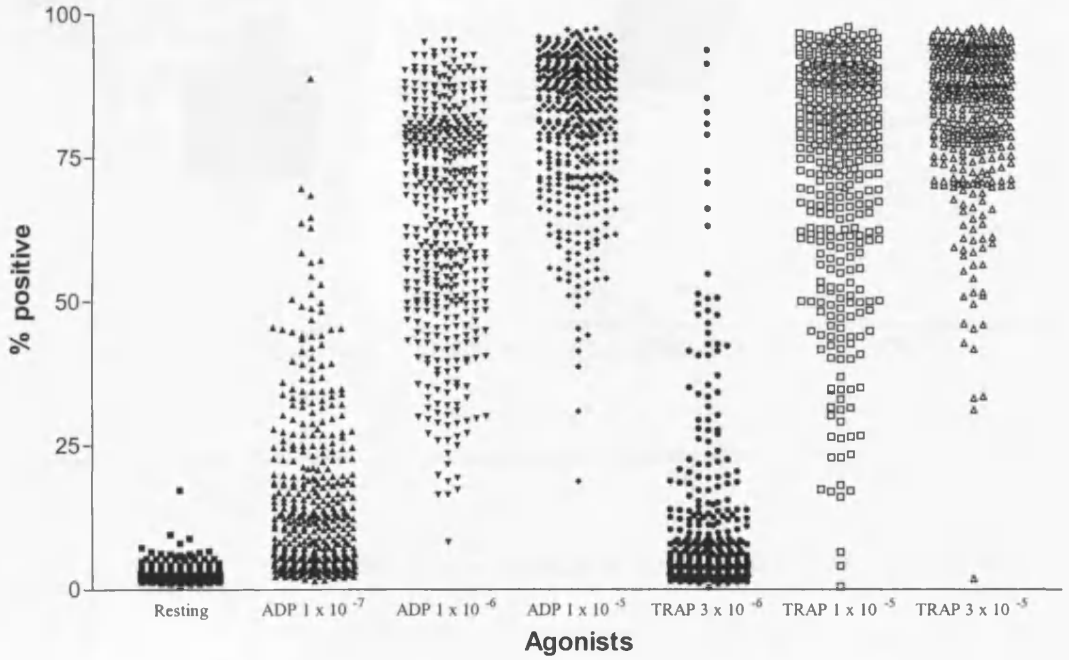
It is also evident that the resting and low stimulation samples, which tend to have lower values for means lead to the higher variations.

4.5 Platelet function variation and characterisation

4.5.1 Variation of platelet function

The platelet flow cytometry results of the entire cohort are represented in Figure 23. The ranges of values for each of the parameters measured indicate large inter-individual variation in platelet function. This is true for fibrinogen binding in stimulated platelet samples at all concentrations of ADP and TRAP. Although not obvious from Figure 23 even the resting platelet samples showed greater than a10-fold inter individual difference in fibrinogen binding.

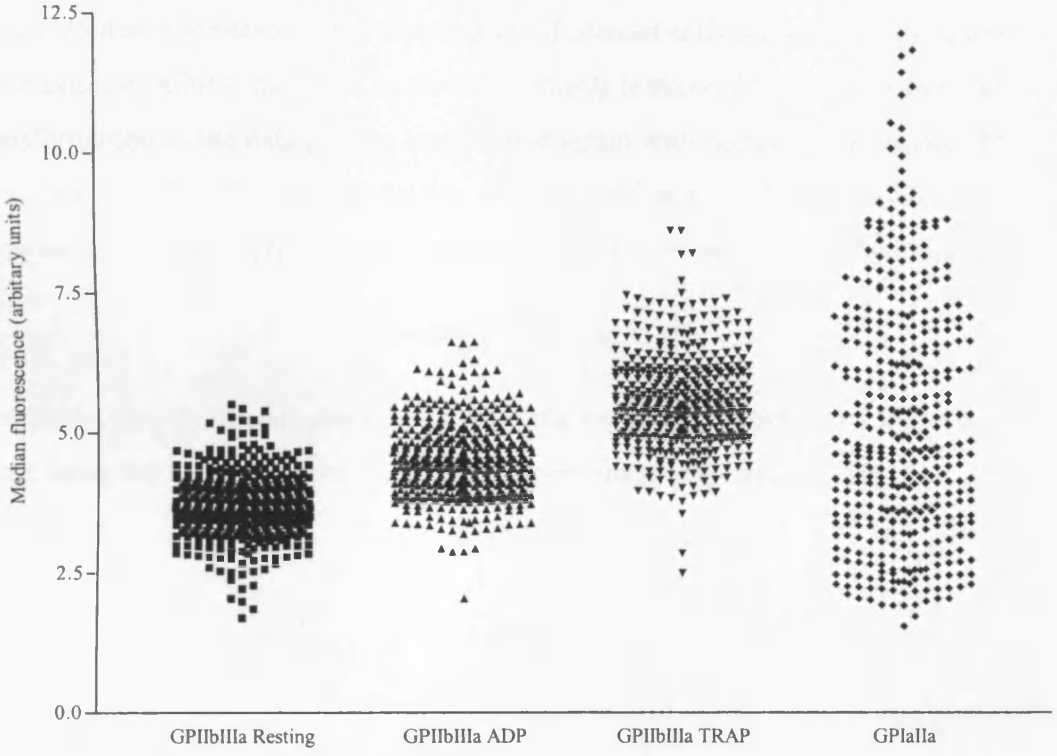
Figure 23 ***Variation in platelet activation (% positive platelets with fibrinogen binding) in 405 subjects in resting and stimulated sample***



Similarly, GPIIbIIIa receptor expression varied by approximately two-fold between the subjects not only in the resting state but also after stimulation with maximum

concentration ADP and TRAP, Figure 24. However, by far the largest overall variation in platelet function was demonstrated by the expression of the GPIaIIa receptor. There was almost a seven-fold variation in the median fluorescence between the subjects and a more genuine spread of the receptor expression values was demonstrated (Figure 24). Separate analyses by either control or case status gave very similar results, for brevity the data is not shown separately.

Figure 24 ***Variation in glycoprotein receptor expression (median fluorescence) in 405 subjects in resting and stimulated samples***



4.5.2 Normal and non-normal distribution of platelet function tests

Figure 23 demonstrates that the platelet activation results, resting or with stimulation, are not normally distributed. As the responses must lie within 0 to 100% there is extreme skewness at low and high response. The resting and low concentration stimulation with ADP and TRAP show the majority of the results are towards the lower

values of the range (skewed to the left). The intermediate and high dose stimulation results are more towards the higher values of the range (skewed to the right). To model the response into a normally distributed data set the following equation was used to transform the data:

The Kolmogorov-Smirnov test for the raw data in the control subjects confirmed a non-

$$Z = \log\left(\frac{\text{response}}{100\text{-response}}\right)$$

normal distribution in all the platelet reactivity tests and that transformation was able to give normal distributions in all but the resting and low dose TRAP samples (Table 15). Figure 25 demonstrates that the distribution of platelet activation results (% positive) for maximum ADP stimulation in the 200 controls is skewed to the right and that transformation of the data gives a centred histogram with normal distribution. The transformation therefore allows the use of parametric statistical analysis and gives more accurate confidence intervals and p-values (Table 15). However, after calculations were

$$\text{Response} = 100\left(\frac{e^z}{1 + e^z}\right)$$

complete using the transformed data the results were back transformed to the original scale using the following formula to give a more understandable result:

Table 15 ***Platelet function test results (raw and transformed) in the controls***

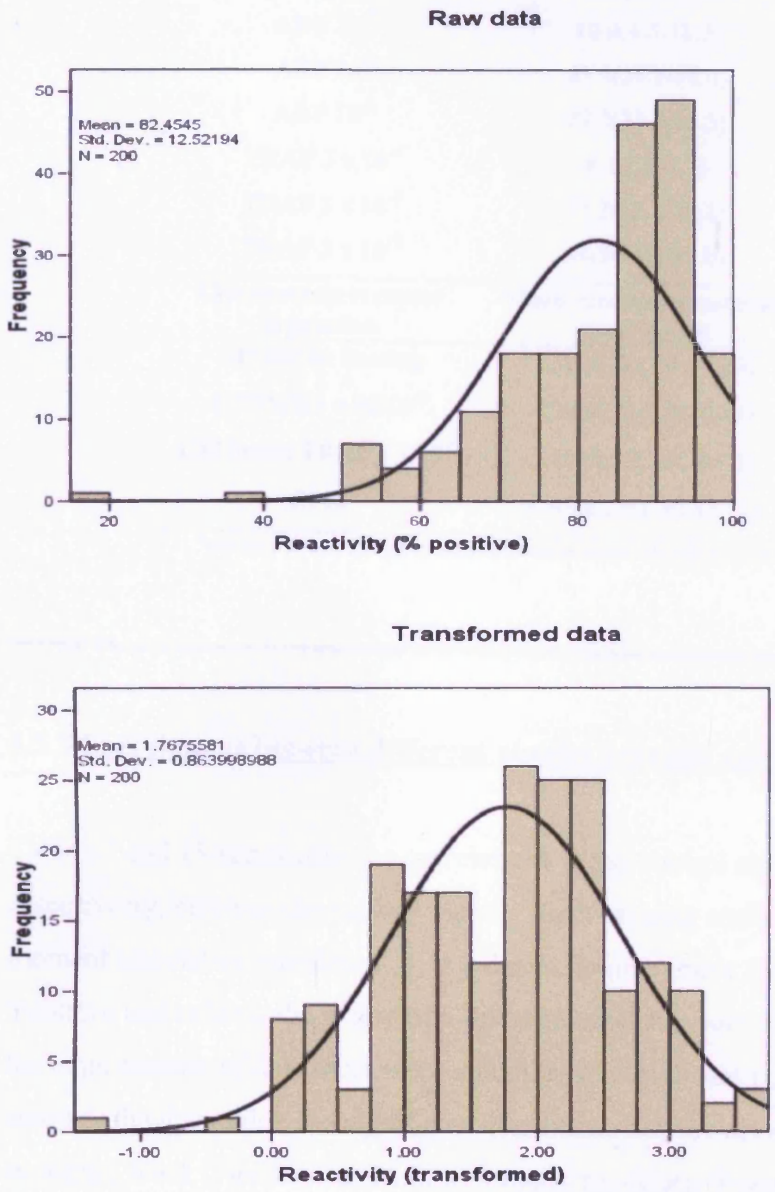
	Raw data		Transformed data	
Fibrinogen binding	**Mean % positive ± SD (range)**	**K-S test for normal distribution***	**Mean % positive (95% CI for mean)**	**K-S test for normal distribution***
Resting	2.8±1.5(1.0-17.3)	Z=2.49,p<0.001	2.6 (2.5-2.7)	Z=1.45,p=0.030
ADP 10^{-7}	17.8±14.6(1.5-68.6)	Z=1.97,p=0.001	13.5 (11.9-15.3)	Z=1.09,p=0.183
ADP 10^{-6}	67.1±18.5(8.3-95.4)	Z=1.59,p=0.013	69.9 (67.0-72.6)	Z=0.77,p=0.594
ADP 10^{-5}	82.4±12.5(18.8-97.4)	Z=2.20,p<0.001	85.4 (83.9-86.9)	Z=1.04,p=0.232
TRAP 3 x 10^{-6}	10.7±13.9(1.2-85.6)	Z=3.62,p<0.001	6.6 (5.7-7.7)	Z=1.81,p=0.003
TRAP 1 x 10^{-5}	76.1±19.7(16.1-97.1)	Z=2.27,p<0.001	80.8 (78.2-83.2)	Z=0.98.p=0.294
TRAP 3 x 10^{-5}	85.7±10.7(33.2-97.7)	Z=2.17,p<0.001	88.3 (87.1-89.5)	Z=0.77,p=0.591

	Raw data	
Glycoprotein receptor expression	**Mean median florescence ± SD (range)**	**K-S test for normal distribution***
GPIIbIIIa Resting	3.66±0.60(1.61-5.32)	Z=0.68,p=0.741
GPIIbIIIa ADP10^{-7}	4.42±0.68(1.82-6.49)	Z=0.52,p=0.946
GPIIbIIIa TRAP 4 x 10^{-5}	5.45±0.87(2.26-8.47)	Z=0.58,p=0.894
GPIa	5.31±2.13(1.71-11.70)	Z=0.99,p=0.284

* A significant p-value for the Kolmogorov-Smirnov test indicates that the data set is NOT normally distributed. Therefore non-significant values indicate a normal distribution

All the glycoprotein receptor expression distributions (Figure 24) were normal and this was confirmed using the Kolmogorov-Smirnov test, Table 15. Therefore, all subsequent calculations for this data used the raw results.

Figure 25 ***Distribution of platelet reactivity (% positive) with maximum ADP stimulation using the raw and transformed data***



Similarly, the results in the cases also demonstrated non normal distributions of the fibrinogen binding data requiring transformation. The receptor expression data demonstrated a normal distribution. Table 16 shows the mean results with inter-quartile ranges for the tests and the results of the Kolmogorov-Smirnov test (raw data not shown).

Table 16 ***Platelet function tests in the cases***

Fibrinogen binding	Mean % positive (Inter-quartile range)*	K-S test for normal distribution
Resting	2.5(2.0-2.7)	2.25 p<0.001
ADP 10^{-7}	10.0(4.3-18.0)	1.06 p=0.213
ADP 10^{-6}	63.8(39.1-78.0)	0.59 p=0.877
ADP 10^{-5}	82.2(71.6-89.5)	0.64 p=0.811
TRAP 3 x 10^{-6}	6.1(2.8-8.7)	2.28 p<0.001
TRAP 1 x 10^{-5}	77.3(62.2-79.2)	0.99 p=0.280
TRAP 3 x 10^{-5}	84.3(74.9-91.0)	2.31 p=0.684
Glycoprotein receptor expression	**Mean median florescence ±SD (range)**	**K-S test for normal distribution**
GPIIbIIIa Resting	3.50±0.60(1.49-5.39)	0.61 p=0.854
GPIIbIIIa ADP10^{-5}	4.28±0.71(2.84-6.49)	1.00 p=0.276
GPIIbIIIa TRAP 4 x 10^{-5}	5.29±0.87(2.63-8.02)	1.04 p=0.232
GPIa	4.80±2.25(1.30-12.11)	1.47 p=0.027

*statistics obtained using transformed data in similar manner to controls

4.5.3 Correlations between different platelet function assays

Table 17 and 18 summarise the correlations in the control and case populations respectively, between the various platelet function tests analysed. The Pearson product moment correlation coefficient, r, is a dimensionless index that ranges from -1.0 to 1.0 inclusive and reflects the extent of a linear relationship between two data sets. Due to the large amount of data there were a number of significant results. Therefore, to simplify the discussion the significant correlation may be divided into 3 arbitrary strengths; weak (Pearson correlations < 0.40), moderate (Pearson correlations 0.40 – 0.60) or strong (Pearson correlations > 0.60). The results may be summarised into the following points:

(i) The resting platelet fibrinogen binding showed a weak to moderate correlation to fibrinogen binding with ADP and TRAP stimulations in both the controls (Pearson correlation range 0.307-0.467) and cases (Pearson correlation range 0.306-0.548).

(ii) There were strong correlations between ADP responses at different doses (Pearson correlation range 0.857-0.961) and between TRAP responses at different doses (Pearson correlation range 0.611-0.898) in both the controls and cases. There was also a moderate to (predominantly) strong correlation between the ADP and TRAP responses in both the controls and cases (Pearson correlation range 0.486-0.839).

(iii) There was no correlation between GPIIbIIIa and GPIaIIa receptor expression and resting platelet fibrinogen binding in the controls but the cases demonstrated a very weak association (Pearson correlation range 0.138-0.255).

(iv) There was a predominantly weak correlation between GPIIbIIIa and GPIaIIa expression and responses to both ADP and TRAP stimulation in both the controls and cases (Pearson correlation range 0.145-0.436).

(v) The strengths of the various correlations were similar in the control and case groups.

These results suggest that fibrinogen binding in response to ADP and TRAP are strongly correlated, especially with intermediate and high concentration stimulation. Receptor expression (GPIIbIIIa and GPIaIIa) and resting levels of fibrinogen binding do not significantly influence subsequent platelet response to ADP and TRAP stimulation.

Table 17 ***Correlations between platelet function assessments in the control subjects***

		Resting	ADP 1 x 10-7	ADP 1 x 10-6	ADP 1 x 10-5	TRAP 3 x 10-6	TRAP 1 x 10-5	TRAP 3 x 10-5	GPIIbIIIa Resting	GPIIbIIIa ADP 1x 10-5	GPIIbIIIa TRAP 3 x 10-5	GPIa Resting
Resting	Pearson Correlation	1	.467**	.413**	.373**	.423**	.307**	.354**	.015	.035	-.016	.058
	Sig. (2-tailed)	.	.000	.000	.000	.000	.000	.000	.828	.628	.818	.413
	N	200	200	200	200	200	200	200	200	200	200	200
ADP 1 x 10-7	Pearson Correlation	.467**	1	.913**	.857**	.597**	.671**	.739**	.145*	.230**	.149*	.309**
	Sig. (2-tailed)	.000	.	.000	.000	.000	.000	.000	.040	.001	.035	.000
	N	200	200	200	200	200	200	200	200	200	200	200
ADP 1 x 10-6	Pearson Correlation	.413**	.913**	1	.942**	.550**	.730**	.830**	.207**	.299**	.204**	.318**
	Sig. (2-tailed)	.000	.000	.	.000	.000	.000	.000	.003	.000	.004	.000
	N	200	200	200	200	200	200	200	200	200	200	200
ADP 1 x 10-5	Pearson Correlation	.373**	.857**	.942**	1	.506**	.720**	.839**	.260**	.353**	.255**	.304**
	Sig. (2-tailed)	.000	.000	.000	.	.000	.000	.000	.000	.000	.000	.000
	N	200	200	200	200	200	200	200	200	200	200	200
TRAP 3 x 10-6	Pearson Correlation	.423**	.597**	.550**	.506**	1	.693**	.621**	.109	.144*	.074	.041
	Sig. (2-tailed)	.000	.000	.000	.000	.	.000	.000	.123	.042	.297	.563
	N	200	200	200	200	200	200	200	200	200	200	200
TRAP 1 x 10-5	Pearson Correlation	.307**	.671**	.730**	.720**	.693**	1	.869**	.273**	.341**	.306**	.237**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.	.000	.000	.000	.000	.001
	N	200	200	200	200	200	200	200	200	200	200	200
TRAP 3 x 10-5	Pearson Correlation	.354**	.739**	.830**	.839**	.621**	.869**	1	.391**	.440**	.389**	.318**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.	.000	.000	.000	.000
	N	200	200	200	200	200	200	200	200	200	200	200
GPIIbIIIa Resting	Pearson Correlation	.015	.145*	.207**	.260**	.109	.273**	.391**	1	.927**	.853**	.366**
	Sig. (2-tailed)	.828	.040	.003	.000	.123	.000	.000	.	.000	.000	.000
	N	200	200	200	200	200	200	200	200	200	200	200
GPIIbIIIa ADP 1x 10-5	Pearson Correlation	.035	.230**	.299**	.353**	.144*	.341**	.440**	.927**	1	.923**	.370**
	Sig. (2-tailed)	.628	.001	.000	.000	.042	.000	.000	.000	.	.000	.000
	N	200	200	200	200	200	200	200	200	200	200	200
GPIIbIIIa TRAP 3 x 10-5	Pearson Correlation	-.016	.149*	.204**	.255**	.074	.306**	.389**	.853**	.923**	1	.378**
	Sig. (2-tailed)	.818	.035	.004	.000	.297	.000	.000	.000	.000	.	.000
	N	200	200	200	200	200	200	200	200	200	200	200
GPIa Resting	Pearson Correlation	.058	.309**	.318**	.304**	.041	.237**	.318**	.366**	.370**	.378**	1
	Sig. (2-tailed)	.413	.000	.000	.000	.563	.001	.000	.000	.000	.000	.
	N	200	200	200	200	200	200	200	200	200	200	200

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 18 ***Correlations between platelet function assessments in the case subject***

		Resting	ADP 1 x 10-7	ADP 1 x 10-6	ADP 1 x 10-5	TRAP 3 x 10-6	TRAP 1 x 10-5	TRAP 3 x 10-5	GPIIbIIIa Resting	GPIIbIIIa ADP 1 x 10-7	GPIIbIIIa TRAP 3 x 10-5	GPIa Resting
Resting	Pearson Correlation	1	.548**	.461**	.430**	.414**	.306**	.350**	.214**	.209**	.138*	.255**
	Sig. (2-tailed)	.	.000	.000	.000	.000	.000	.000	.002	.003	.048	.000
	N	210	204	204	210	204	204	204	204	204	204	202
ADP 1 x 10-7	Pearson Correlation	.548**	1	.914**	.857**	.486**	.602**	.674**	.272**	.370**	.320**	.388**
	Sig. (2-tailed)	.000	.	.000	.000	.000	.000	.000	.000	.000	.000	.000
	N	204	204	204	204	204	204	204	204	204	204	202
ADP 1 x 10-6	Pearson Correlation	.461**	.914**	1	.961**	.548**	.707**	.764**	.258**	.408**	.348**	.321**
	Sig. (2-tailed)	.000	.000	.	.000	.000	.000	.000	.000	.000	.000	.000
	N	204	204	204	204	204	204	204	204	204	204	202
ADP 1 x 10-5	Pearson Correlation	.430**	.857**	.961**	1	.534**	.696**	.773**	.284**	.436**	.375**	.305**
	Sig. (2-tailed)	.000	.000	.000	.	.000	.000	.000	.000	.000	.000	.000
	N	210	204	204	212	204	204	204	204	204	204	202
TRAP 3 x 10-6	Pearson Correlation	.414**	.486**	.548**	.534**	1	.632**	.611**	.160*	.226**	.204**	.059
	Sig. (2-tailed)	.000	.000	.000	.000	.	.000	.000	.022	.001	.003	.407
	N	204	204	204	204	204	204	204	204	204	204	202
TRAP 1 x 10-5	Pearson Correlation	.306**	.602**	.707**	.696**	.632**	1	.898**	.283**	.368**	.399**	.310**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.	.000	.000	.000	.000	.000
	N	204	204	204	204	204	204	204	204	204	204	202
TRAP 3 x 10-5	Pearson Correlation	.350**	.674**	.764**	.773**	.611**	.898**	1	.311**	.403**	.430**	.345**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.	.000	.000	.000	.000
	N	204	204	204	204	204	204	204	204	204	204	202
GPIIbIIIa Resting	Pearson Correlation	.214**	.272**	.258**	.284**	.160*	.283**	.311**	1	.923**	.853**	.421**
	Sig. (2-tailed)	.002	.000	.000	.000	.022	.000	.000	.	.000	.000	.000
	N	204	204	204	204	204	204	204	205	205	205	203
GPIIbIIIa ADP 1 x 10-7	Pearson Correlation	.209**	.370**	.408**	.436**	.226**	.368**	.403**	.923**	1	.927**	.424**
	Sig. (2-tailed)	.003	.000	.000	.000	.001	.000	.000	.000	.	.000	.000
	N	204	204	204	204	204	204	204	205	205	205	203
GPIIbIIIa TRAP 3 x 10-5	Pearson Correlation	.138*	.320**	.348**	.375**	.204**	.399**	.430**	.853**	.927**	1	.430**
	Sig. (2-tailed)	*.048*	*.000*	*.000*	*.000*	*.003*	*.000*	*.000*	*.000*	*.000*	.	*.000*
	N	204	204	204	204	204	204	204	205	205	205	203
GPIa Resting	Pearson Correlation	.255**	.388**	.321**	.305**	.059	.310**	.345**	.421**	.424**	.430**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.407	.000	000	.000	.000	.000	.
	N	202	202	202	202	202	202	202	203	203	203	203

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.5.4 Characterisation of the correlation between the platelet ADP and TRAP responses

Further characterisation of the strong correlation between the platelet ADP and TRAP responses was made by using proteins that break down or block the response of either ADP or thrombin, therefore allowing the study of the specific effects of a single agonist pathway.

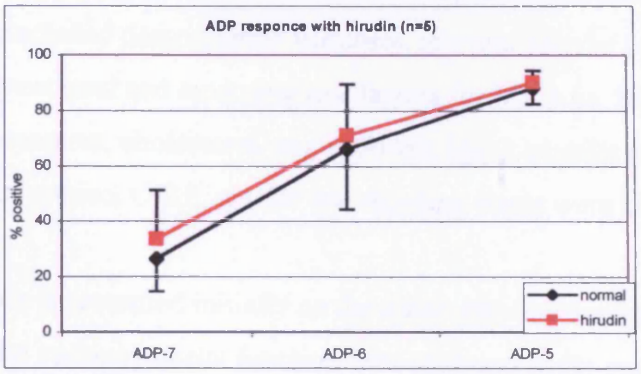
Apyrase is a naturally occurring enzyme extracted from the potato (Solanum tuberosum). It contains the two isoenzymes adenosine 5'-triphospatase (ATPase) and adenosine 5'-diphospatase (ADPase). It therefore breaks down ATP to ADP + Pi and ADP to AMP + 2Pi. Hirudin is a naturally occurring protein (now recombinantly engineered) found in leeches. It directly neutralizes thrombin and therefore is expressed in anti-thrombin units (ATU).

Standard protocol titration experiments using hirudin found that 1μl of 100units/ml was sufficient to block the maximum concentration TRAP response and similarly apyrase 2μl of 200units/ml was sufficient to block the maximum concentration ADP response (all % positive for fibrinogen binding< 5%). Fresh whole blood samples were stimulated, in duplicate, with 5μl of ADP ($1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$M) or TRAP ($3x10^{-6}$, $1x10^{-5}$, $3x10^{-5}$M). In addition identical samples were repeated containing either hirudin (1μl of 100units/ml) or apyrase (2μl of 200units/ml) to block the TRAP or ADP response respectively. Platelet activation was determined by flow cytometry analysis. The experiment was carried out using 5 separate subjects.

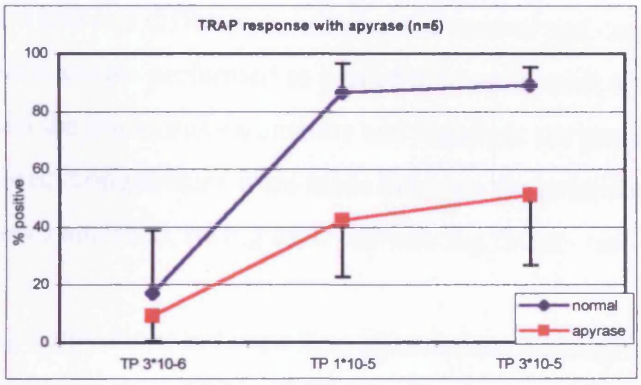
The results showed that the ADP response was not affected by the addition of hirudin, Figure 26(a). Therefore ADP mediated platelet activation occurs independent of thrombin. The TRAP response was significantly reduced with the addition of apyrase at the intermediate and high concentrations (p-values =0.005 and 0.001 respectively), figure 26(b). Therefore, a significant proportion of the TRAP mediated platelet response is dependent on ADP pathways. This suggests stimulation via the thrombin platelet surface receptors leads to the up-regulation of other agonists through

intracellular vesicular content release. These findings also help explain the strong correlation demonstrated between ADP and TRAP mediated platelet activation.

Figure26 (a) ***Effect of hirudin on the ADP mediated response***



(b) ***Effect of apyrase on the TRAP mediated response***



4.6 Factors influencing platelet reactivity and glycoprotein receptor expression

Given the marked inter individual variability of platelet responses demonstrated (Figures 23 and 24), a number of parameters that may influence platelet function were analysed. These included demographic variables, relevant platelet parameters as well as a number of conventional and emerging risk factors for MI. Age, BMI, systolic and diastolic blood pressures, cholesterol, triglycerides, Lp(a), platelet count, MPV, fibrinogen, homocysteine, C-RP, gender and smoking status were all analysed.

In this section data is presented initially on the direct correlations made between these parameters and the various platelet function tests analysed in the controls and cases separately. The analysis was divided into continuous (Table 19) and categorical variables (Table 20). This allows an insight on the direct influence of the various factors on platelet function and any differences between the control and case groups. A logistic regression analysis was also performed as part of this assessment to investigate the interaction between the numerous parameters and highlight the prominent influencing factors. Finally, direct comparisons were made between the controls and cases on all the platelet function tests analysed, taking other influencing factors into account.

As there are strong correlations between low, intermediate and high ADP and TRAP responses and between resting, ADP and TRAP stimulated GPIIbIIIa expression (Section 4.5.3) further analyses of platelet function in the PRAMIS study was simplified and restricted to the following 5 parameters:

(i) % positive for fibrinogen binding in resting platelets
(ii) % positive for fibrinogen binding in ADP 1 x 10^{-5} M stimulated platelets
(iii) % positive for fibrinogen binding in TRAP 3 x 10^{-5} M stimulated platelets
(iv) median fluorescence for GPIIbIIIa expression in resting platelets
(v) median fluorescence for GPIaIIa expression in resting platelets.

However, it should be noted that the % positive fibrinogen binding result for resting platelets had high coefficient of variations for both intra and inter assay variability due to the low values in the majority of subjects.

Table 19 ***Parameters tested for influence on platelet function assays – continuous variables***

		Platelet function assay									
		Fibrinogen binding Resting		**Fibrinogen binding ADP $1x10^{-5}$**		**Fibrinogen binding TRAP $3x10^{-5}$**		**GPIIbIIIa expression Resting**		**GPIaIIa expression Resting**	
		Pearson correlation	p-value	Pearson correlation	p-value	Pearson correlation	p-value	Pearson correlation	p-value	Pearson correlation	p-value
Age	Controls	0.043	0.547	**0.187**	**0.008**	**0.157**	**0.026**	0.003	0.969	**0.143**	**0.044**
	Cases	-0.109	0.120	-0.041	0.558	0.035	0.614	0.022	0.751	0.012	0.860
BMI	Controls	-0.011	0.881	-0.010	0.891	0.028	0.691	**-0.163**	**0.021**	0.098	0.169
	Cases	-0.044	0.531	0.067	0.339	0.017	0.810	0.115	0.101	0.094	0.182
Systolic BP	Controls	0.129	0.068	0.099	0.164	0.089	0.212	0.040	0.577	0.089	0.208
	Cases	-0.105	0.137	0.127	0.070	0.038	0.588	-0.033	0.692	0.046	0.512
Diastolic BP	Controls	0.012	0.867	0.099	0.162	0.060	0.399	-0.024	0.741	0.109	0.124
	Cases	-0.115	0.103	0.068	0.331	0.001	0.993	-0.027	0.700	0.074	0.297
Cholesterol	Controls	-0.070	0.325	-0.012	0.869	0.063	0.373	0.125	0.077	**0.152**	**0.031**
	Cases	-0.047	0.507	0.026	0.713	-0.009	0.900	0.040	0.572	**0.156**	**0.027**
Triglyceride	Controls	0.061	0.390	0.049	0.493	0.108	0.129	0.064	0.365	0.121	0.088
	Cases	-0.098	0.162	0.084	0.231	0.014	0.845	-0.133	0.058	**0.240**	**0.001**
LP(a)	Controls	-0.089	0.247	0.099	0.198	-0.003	0.972	0.070	0.364	0.065	0.399
	Cases	**-0.183**	**0.009**	-0.116	0.101	-0.111	0.116	-0.069	0.329	-0.117	0.100
Platelet count	Controls	0.063	0.391	-0.124	0.089	-0.051	0.485	**-0.160**	**0.028**	-0.038	0.602
	Cases	-0.033	0.646	**-0.190**	**0.007**	**-0.183**	**0.010**	**-0.145**	**0.039**	-0.068	0.342
MPV	Controls	-0.064	0.385	0.229	0.116	0.122	0.096	**0.283**	**<0.001**	-0.067	0.363
	Cases	**0.247**	**<0.001**	**0.429**	**<0.001**	**0.476**	**<0.001**	**0.385**	**<0.001**	**0.283**	**<0.001**
Fibrinogen	Controls	-0.071	0.328	**-0.207**	**0.004**	**-0.232**	**<0.001**	0.020	0.785	0.031	0.667
	Cases	-0.044	0.543	**-0.233**	**0.001**	**-0.228**	**0.001**	0.050	0.492	0.004	0.955
Homocysteine	Controls	0.096	0.178	0.118	0.096	**0.151**	**0.033**	0.078	0.276	**0.195**	**0.006**
	Cases	-0.115	0.103	-0.116	0.099	-0.102	0.147	-0.047	0.506	0.033	0.202
C-RP	Controls	0.040	0.571	0.022	0.758	-0.002	0.972	**-0.212**	**0.003**	0.086	0.225
	Cases	-0.059	0.409	-0.130	0.066	**-0.175**	**0.013**	-0.057	0.425	0.044	0.540

*Categorical data not represented in this table
(Statistically significant results in bold)

Table 20 ***Parameters tested for influence on platelet function assays – categorical variables***

			Platelet function assay									
			Fibrinogen binding Resting		**Fibrinogen binding ADP $1x10^{-5}$**		**Fibrinogen binding TRAP $3x10^{-5}$**		**GPIIbIIIa expression Resting**		**GPIaIIa expression Resting**	
			% positive	p-value	% positive	p-value	% positive	p-value	Median fluorescence	p-value	Median fluorescence	p-value
Gender	Controls	Male (n=174)	4.7	0.184	81.8	0.172	84.8	0.111	3.80 ± 0.59	0.227	**5.57 ± 2.01**	**<0.001**
		Female (n=26)	4.4		78.5		80.5		3.65 ± 0.54		**3.94 ± 1.49**	
	Cases	Male (n=175)	4.4	0.345	78.1	0.833	79.9	0.638	**3.62 ± 0.58**	**0.049**	4.95 ± 2.29	0.902
		Female (n=30)	4.7		77.7		78.8		**3.85 ± 0.65**		4.90 ± 1.98	
Hypertension	Controls	No (n=182)	4.7	0.980	81.5	0.698	84.5	0.927	3.78 ± 0.59	0.664	5.41 ± 2.14	0.443
		Yes (n=18)	4.7		80.4		84.2		3.72 ± 0.59		5.80 ± 1.85	
	Cases	No (n=146)	**4.6**	**0.036**	77.9	0.878	80.0	0.802	3.65 ± 0.60	0.906	4.88 ± 2.28	0.510
		Yes (n=58)	**4.2**		78.2		79.4		3.64 ± 0.58		5.11 ± 2.18	
Diabetes	Controls	No (n=198)	4.3	0.719	84.9	0.612	82.6	0.780	3.84 ± 0.47	0.883	5.31 ± 2.11	0.956
		Yes (n=2)	4.7		81.4		84.5		3.78 ± 0.59		5.44 ± 2.11	
	Cases	No (183)	4.4	0.470	77.7	0.254	79.7	0.727	3.64 ± 0.59	0.391	4.90 ± 2.21	0.449
		Yes (n=22)	4.7		80.5		80.6		3.75 ± 0.63		5.29 ± 2.56	
Current Smoking	Controls	No (n=164)	4.7	0.779	**86.7**	**0.037**	88.7	0.208	3.65 ± 0.60	0.667	5.35 ± 2.11	0.617
		Yes (n=36)	4.7		**81.7**		86.6		3.69 ± 0.58		5.15 ± 2.24	
	Cases	No (n=162)	4.4	0.351	77.7	0.435	79.6	0.586	3.65 ± 0.59	0.943	4.92 ± 2.31	0.800
		Yes (n=42)	4.6		79.3		80.8		3.65 ± 0.64		5.02 ± 1.98	

Statistically significant results in bold

4.6.1 Resting platelet activity

Very low levels of fibrinogen binding in resting platelets were seen in the vast majority of subjects (inter-quartile range = 2.1-2.9%). The control subjects did not display any significant influences in the 16 parameters analysed. In the cases there was a weak influence on resting platelet fibrinogen binding by Lp(a) and MPV (Pearson correlations = -0.183, and 0.247, p-values = 0.009 and 0.001 respectively). Having a history of hypertension was associated with lower fibrinogen binding on resting platelets (4.6 Vs. 4.2%, p = 0.036) in the cases, this is contrary to expected results and may reflect the effect of medication.

4.6.2 Agonist stimulated platelet activity

Age had a significant but weak effect on platelet fibrinogen binding to maximum ADP and TRAP stimulation (Pearson correlation = 0.187 and 0.157, p-values = 0.008 and 0.026 respectively) in the controls but not the cases. Gender, a history of hypertension or diabetes, systolic or diastolic blood pressure readings, serum cholesterol, serum triglyceride and Lp(a) levels all had no significant influence to maximum ADP and TRAP stimulation in either the controls and cases. Current smoking status showed significantly lower levels of fibrinogen binding in response to maximum ADP stimulation in the controls (Non Vs. current smokers = 86.7 Vs. 81.7%, p-value = 0.037) but not the cases. Platelet count had a negatively weak affect on ADP and TRAP stimulation in the cases (Pearson correlations = -0.190 and –0.183, p-values = 0.007 and 0.010 respectively) but not the controls. MPV had a moderate influence on both ADP and TRAP responses in the cases (Pearson correlations = 0.429 and 0.476, p-values <0.001) but again not the controls. Plasma fibrinogen levels had a significant negative affect on ADP and TRAP responses in both the controls (Pearson correlations = -0.207 and –0.232, p-values = 0.004 and <0.001 respectively) and cases (Pearson correlations = -0.233 and –0.228, p-values <0.001 respectively). Plasma homocysteine had a weak influence on TRAP stimulation in the controls only (Pearson correlation = 0.151, p-value = 0.033) and plasma C-RP levels had a weak negative correlation with TRAP stimulation in the cases only (Pearson correlation = -0.175, p-value = 0.013).

4.6.3 Glycoprotein IIbIIIa receptor expression

In the controls 4 separate parameters had a direct significant influence on GPIIbIIIa receptor expression. These were BMI (Pearson correlation = -0.163, p-value = 0.021), platelet count (Pearson correlation = -0.160, p-value = 0.028), MPV (Pearson correlation = 0.283, p-value <0.001) and C-RP (Pearson correlation = -0.212, p = 0.003). Platelet count and MPV values also showed significant correlations with GPIIbIIIa expression in the cases (Pearson correlations = -0.145 and 0.385, p-values <0.001 respectively). Unlike the controls, there was no significant influence by either BMI or C-RP. However, the cases did show a weak but significant influence on GPIIbIIIa expression by gender (Males Vs. females = 3.62 Vs. 3.85 median fluorescence, p-value = 0.049).

4.6.4 Glycoprotein IaIIa receptor expression

GPIaIIa receptor expression was influenced by 4 parameters in the controls. There were weak correlations with age (Pearson correlation = 0.143, p-value = 0.044), cholesterol (Pearson correlation = 0.152, p-value = 0.031) and homocysteine (Pearson correlation = 0.195, p-value = 0.006). Gender had a significant influence with females expressing less GPIaIIa receptors compared to males (3.94 Vs. 5.57 median fluorescence, p <0.001). In the cases significant correlations with cholesterol (Pearson correlation = 0.156, p-value = 0.027), triglyceride (Pearson correlation = 0.240, p = 0.001) and MPV (Pearson correlation = 0.283, P <0.001) were observed.

4.6.5 Stepwise linear regression analysis

A stepwise linear regression analysis was performed on the control and case populations to highlight the significant predictors of platelet fibrinogen binding and receptor expression in each group. All the above parameters (MPV, platelet count, homocysteine, fibrinogen, C-RP, LP(a), cholesterol, triglyceride, age, BMI, blood pressure, gender and history of diabetes, hypertension and smoking) were entered into the model. In addition for the fibrinogen binding analyses the GPIIbIIIa and GPIaIIa receptor expressions were also included in the model.

For resting platelet fibrinogen binding in the controls the stepwise linear regression showed that none the above factors had a significant influence. In the cases the significant predictors were GPIa receptor expression, MPV and diastolic blood pressure (standardized coefficient $\beta = 0.233$, 0.219 and –0.176, p-values = 0.001, 0.003, 0.012 respectively). The effect size by GPIa expression, MPV and diastolic blood pressure on resting fibrinogen binding were all modest (4.8, 3.9 and 2.4% respectively).

The significant predictors of fibrinogen binding after maximum ADP stimulation in the controls were GPIaIIa expression (standardized coefficient $\beta = 0.280$, $p<0.001$); plasma fibrinogen ($\beta = -0.258$, $p=0.001$); age ($\beta = 0.252$, $p<0.001$); and MPV ($\beta = 0.161$, $p=0.022$). A univariate analysis of variance calculated the estimated effect size on fibrinogen binding in response to maximum ADP stimulation were 8.8% for GPIaIIa expression, 7.7% for plasma fibrinogen, 7.2% for age and 3% for MPV. Stepwise regression analysis in the cases showed the significant predictors of fibrinogen binding after maximum ADP stimulation were MPV (standardized coefficient $\beta = 0.305$, $p<0.001$), GPIaIIa expression ($\beta = 0.258$, $p<0.001$) and plasma fibrinogen ($\beta = -0.259$, $p<0.001$). These were exactly the same predictors to those seen in the control population, apart from the lack of influence of age. The estimated size of effect on overall variation of fibrinogen binding in response to maximum ADP stimulation seen in the cases was 10.5% for MPV, 7.2% for GPIa expression and 6.6% for plasma fibrinogen level.

Stepwise linear regression analysis in the controls showed platelet fibrinogen binding in response to maximum TRAP stimulation was influenced by GPIIbIIIa expression (standardized coefficient $\beta = 0.424$, $p < 0.001$), plasma fibrinogen (standardized coefficient $\beta = -0.384$, $p < 0.001$), plasma C-RP (standardized coefficient $\beta = 0.259$, $p = 0.004$) and age (standardized coefficient $\beta = 0.149$, $p = 0.036$). The effect sizes of these were estimated at 18.7, 11.0, 4.6 and 4.5% respectively. In the cases the analysis showed the main influencers were MPV (standardized coefficient $\beta = 0.327$, $p < 0.001$), GPIa expression (standardized coefficient $\beta = 0.271$, $p < 0.001$) and plasma fibrinogen (standardized coefficient $\beta = -0.236$), these were identical to the main influencers of ADP stimulation. The estimated effects of these were 12.4, 7.9 and 6.2% respectively.

In the controls the predictors of resting GPIIbIIIa receptor expression were MPV (β = 0.256, p=0.001), a negative influence of CRP (β = -0.214, p=0.003) and gender (β = -0.194, p=0.009). The estimated effect size on GPIIbIIIa expression of these was 7.6% for MPV, 4.8% for C-RP and 3.9% for gender. In the cases, the only independent predictor of GPIIbIIIa expression was MPV (β = 0.392, p<0.001). The estimate effect size of MPV on GPIIbIIIa receptor expression in the cases was 17.1%.

Control GPIa IIa expression was influenced by gender (β = -0.294, p<0.001) and serum cholesterol (β = 0.156, p<0.045). The estimated effect size of the overall variation of GPIaIIa expression attributed to gender and cholesterol were only 6.6 and 2.2% respectively. In the cases GPIa IIa expression was independently influenced by MPV (β = 0.268, p<0.001) and plasma triglyceride (β = 0.262, p<0.001). The estimated effect size on the variation of GPIaIIa expression in the cases was 8.9% (p<0.001) for MPV and 6.2% (p<0.001) for serum triglyceride.

4.7 Platelet function comparison between controls and cases

The platelet flow cytometric results of the controls were compared to those of the case population, table 21. As can be seen from the raw (unadjusted) data a significant difference was observed for most platelet function parameters studied and these tended to show a lower platelet response or receptor expression in the cases compared to the controls.

After adjusting for the main parameters that had a significant influence on platelet function in the direct controls or cases analyses (age, MPV, homocysteine and fibrinogen), the results were less significant. This was achieved using a univariate analysis of variance by SPSS entering age, MPV, homocysteine and fibrinogen as fixed factors and platelet function as the dependent variable and case control status as a co-variate. Adjusted analyses showed non-significant difference in the resting (controls Vs. cases = 2.6 Vs. 2.5%, p = 0.242) and maximum ADP stimulated samples (controls Vs. cases = 84.9 Vs. 82.9%, p = 0.082). There were significant differences with maximum TRAP stimulation (controls Vs. cases = 87.7 Vs. 84.9%, p = 0.005), resting GPIIbIIIa receptor expression (controls Vs. cases = 3.66 Vs. 3.49 median fluorescence, p = 0.005)

Table 21 ***Comparison of flow cytometry results between controls and cases***

	RAW DATA			ADJUSTED DATA*		
	Controls	Cases		Controls	Cases	
Fibrinogen binding	**Mean % positive (Inter-quartile range)**	**Mean % positive (Inter-quartile range)**	**p-value**	**Mean % positive (95%CI of mean)**	**Mean % positive (95%CI of mean)**	**p-value**
Resting	2.6 (2.1-3.2)	2.5 (2.0-2.7)	0.156	2.6 (2.3-2.6)	2.5 (2.4-2.7)	0.242
ADP 10^{-7}	13.5 (6.0-26.3)	10.0(4.3-18.0)	0.005	12.9 (11.3-14.7)	10.4 (9.1-11.8)	0.022
ADP 10^{-6}	69.9 (53.8-80.1)	63.8(39.1-78.0)	0.002	68.9 (66.0-71.7)	64.9 (61.9-67.8)	0.060
ADP 10^{-5}	85.4 (76.2-91.1)	82.2(71.6-89.5)	0.011	84.9 (83.2-86.3)	82.9 (81.1-84.4)	0.082
TRAP 3 x 10^{-6}	6.6 (2.9-11.3)	6.1(2.8-8.7)	0.781	6.3 (5.4-7.5)	6.2 (5.3-7.3)	0.887
TRAP 1 x 10^{-5}	80.8 (66.6-91.1)	77.3(62.2-79.2)	0.105	79.7 (76.9-82.2)	77.7 (74.9-80.4)	0.320
TRAP 3 x 10^{-5}	88.3 (79.7-93.5)	84.3(74.9-91.0)	<0.001	87.7 (86.4-88.9)	84.9 (83.3-86.3)	0.005
Glycoprotein receptor expression	**Mean median florescence ±SD (range)**	**Mean median florescence ±SD (range)**	**p-value**	**Mean median florescence ±SE(95%CI of mean)**	**Mean median florescence ±SE(95%CI of mean)**	**p-value**
GPIIbIIIa Resting	3.66±0.60(1.61-5.32)	3.50±0.60(1.49-5.39)	0.006	3.66±0.04(3.58-3.75)	3.49±0.04(3.40-3.57)	0.005
GPIIbIIIa ADP10^{-5}	4.42±0.68(1.82-6.49)	4.28±0.71(2.84-6.49)	0.031	4.42±0.05(4.32-4.52)	4.29±0.05(4.17-4.36)	0.026
GPIIbIIIa TRAP 4 x 10^{-5}	5.45±0.87(2.26-8.47)	5.29±0.87(2.63-8.02)	0.031	5.45±0.06(5.32-5.59)	5.27±0.06(5.15-5.39)	0.039
GPIa	5.31±2.13(1.71-11.70)	4.80±2.25(1.30-12.11)	0.020	5.34±0.16(5.01-5.66)	4.74±0.16(4.43-5.06)	0.011

* adjusted for age, homocysteine, MPV, fibrinogen

and resting GPIaIIa receptor expression (controls Vs. cases = 5.34 Vs. 4.74 median fluorescence, p = 0.011).

4.8 Discussion

In this chapter I have presented a detailed description of the flow cytometric analysis of platelet function in 200 healthy controls and 205 premature MI survivors. This work is the largest flow cytometric analysis of a MI case control cohort ever undertaken. Optimisation of the methods, including controlling for the effects of unstable angina and prolonged running times, allowed an efficient and reproducible profile of platelet function with low intra and inter assay variability (All intra and inter assay coefficient of variations < 7% for maximum ADP and TRAP stimulation and GPIIbIIIa and GPIaIIa expression assays).

The platelet function results revealed large inter-individual variation for all the flow cytometric assays analysed in both the controls and cases. These results are in keeping with previous studies on platelet function heterogeneity discussed in detail in Chapter 1 (Section 1.5.4), which also showed large spreads of results and overlap between the control and case groups for the various platelet function assays.

In both the controls and cases there were strong correlations between low, intermediate and high dose stimulation responses to ADP and TRAP and between both types of agonist response. This suggests that although there are large variations between subjects, individual subjects are predisposed to various levels of platelet response and the degree of activation is consistent across concentrations and types of agonist stimulation for ADP and TRAP. Further characterisation of the responses using apyrase and hirudin showed that a significant proportion of the TRAP mediated platelet response is dependent on ADP pathways probably through intracellular vesicular content release. This result also highlights the more strategic targeting of ADP blockade by the thienopyrdins compared to thrombin receptor inhibitors in the clinical setting of the acute coronary syndromes.

A detailed direct analysis of sixteen parameters for influence on platelet function was made followed by a stepwise regression analysis.

The effect of age on platelet function showed contrasting results between the controls and cases. The controls demonstrated positive correlations between age and fibrinogen binding with ADP and TRAP stimulation. The stepwise regression analysis in the controls supported age as an independent influence on platelet reactivity (estimated size effect = 7.2% on maximum ADP fibrinogen binding), in keeping with the results of the Northwick Park Study[144] (section 1.5.4.3). Possible explanations for the lack of influence in the cases include the effects of medications[213] (aspirin, statins, β blockers, calcium antagonists and nitrates) on platelet function negating the influence of age or indeed the disease process itself[214] leading to altered mechanisms controlling platelet function (previous studies discussed later in this section).

There was a lack of significant influence on platelet function in the controls or cases for BMI, diastolic or systolic blood pressure, and history of diabetes or hypertension. This may be due to relatively small numbers of affected subjects in the control cohort, the overriding effect of medications in the cases and a more direct involvement of these risk factors to atherosclerosis rather than thrombosis.

The anomaly of current smokers demonstrating reduced fibrinogen binding to platelet ADP stimulation in the controls was unexpected but similar to the Northwick Park Heart Study[139]. They may be explained by the fact that chronic repetitive stimulation of platelets from smoking over a period of time actually leads to a dampening of platelet reactivity and the platelet response is only acutely heightened at or near the time of smoking as demonstrated in previous smaller studies[215,216]. In this study on the day of platelet function analysis all current smokers were asked to abstain from smoking for at least 12 hours prior to their visit (verified by carbon monoxide breath analyser) and therefore the immediate acute effects of smoking were avoided. The cases did not demonstrate any significant effects on platelet function by current smoking status, again suggesting altered influencing mechanisms either due to medication or the disease process itself.

The effect of gender was also different between the groups. Control males had an increased platelet response to low and intermediate concentration ADP stimulation and also expressed a greater number of GPIaIIa receptors despite having significantly smaller platelets compared to the females. These results support the fact that males have an increased risk to premature MI compared to females[1] but were in contrast to the Northwick Park Study[144] which showed increased platelet aggregation in women rather than men. In the cases the females had increased expression of the GPIIbIIIa receptor at rest compared to males but did not demonstrate a difference in platelet fibrinogen binding at rest or with stimulation.

There was a lack of correlation between platelet function and the lipid risk factors (Lp(a), total cholesterol and triglyceride) in both the controls and cases. The causal pathway to increased MI risk by these factors was conventially thought to be mediated through accelerated atherosclerosis rather than thrombosis. More recently evidence is accumulating that cholesterol-enriched membrane microdomains (lipd rafts) are part of a general process that contributes to the efficiency and the coordination of platelet activation mechanisms[217]. Therefore, the influence of total cholesterol on GPIaIIa expression in the controls and plasma triglyceride on GPIaIIa expression in the cases seen in the logistic regression analysis may have a plausible biological explanation. However, the size effects of these influences were relatively small, 2.2% and 6.2% respectively.

There was discrepancy in the correlations between MPV and agonist stimulated fibrinogen binding in the cases and controls (Table 19). However, the stepwise regression analysis results in the controls and all the results in the cases support the previous studies linking increased MPV to increased platelet reactivity[128-133]. These initial studies assessed platelet function mainly in the form of aggregation using ADP, collagen and epinephrine, which were all carried out in normal individuals (Chapter 1, Section 1.5.4.1). The largest of this type of study was from the Caerphilly Collaborative Heart Disease Study[134], which demonstrated a significant correlation between increasing MPV and decreasing ADP threshold dose for aggregation in 242 men with no known heart disease. The lack of significant correlations in the normal PRAMIS cohort may partially be explained by the fact that platelet aggregation and flow cytometric analysis are quite separate platelet function tests measuring gross platelet

aggregation and specific fibrinogen binding at the GPIIbIIIa receptor level respectively. The results in the cases support the previous observations. An interesting potential prospective study would be to follow up the PRAMIS cohort to analyse whether MPV is predictive of further coronary events, an analysis found to be positive by Martin et. al. in 1716 post MI men, where MPV taken 6 months post MI was predictive of MI and death in the 126 event cases at 2 year follow up ($p<0.001$)[120]. Large platelets are more reactive per unit volume than small platelets (to ADP and collagen), contain more dense granules and they produce more prothrombotic factors such as thromboxane A_2, serotonin and β-thromboglobulin[120]. The same group explained that as platelets do not change size during the duration of their lifespan, the increased risk in the form of larger platelets is determined at the platelet precursor (megakaryocytopoiesis and thrombopoiesis)) level[53]. The exact mechanisms controlling platelet production and therefore determining platelet size have yet to be clarified.

The negative correlations of plasma fibrinogen and ADP and TRAP response in both the controls and cases were in contrast to expected findings, as a raised plasma level of fibrinogen has been established as an independent risk factor for acute coronary events[141,144,218] (Chapter 6). Possible explanations for the lack of positive results may include the fact that only 8 out of the 200 controls had a fibrinogen level above the upper limit of the normal range (1.5-4 g/l), and this may represent too small a cohort to establish a meaningful statistical result. Also, in the cases, the anti-inflammatory effects of aspirin (99% of cases)[201 219]and statins (80% of cases)[220] may have overridden any inflammation mediated fibrinogen effects. Finally, high plasma fibrinogen levels lead to increased binding to the anti-fibrinogen antibodies during flow cytometry analysis, therefore less antibody is available to attach to the fibrinogen bound to the GPIIbIIIa receptors on the platelet surface.

C-RP levels did not show a positive direct correlation to any of the platelet function tests in the controls or cases. The study may have been underpowered to detect such differences. In the cases treatments with aspirin and statins would have an anti-inflammatory effect[201 219,220]. Also, the mechanisms by which C-RP influences MI risk may not involve alterations in platelet function.

Plasma homocysteine is an established thrombotic risk factor (Chapter 6) and levels did weakly correlate with a number of platelet function tests in the control but not the cases. A plausible pathological mechanism for the causal pathway has still to be universally accepted[221] but the control data supports the hypothesis of raised plasma homocysteine, through increased platelet reactivity, may lead to a predisposition to thrombosis. The stepwise regression analyses however, could not demonstrate an independent effect on the platelet parameters analysed in either the controls or cases.

The stepwise regression analysis highlighted the independent predictors of the platelet function tests analysed. The platelet fibrinogen binding to maximum ADP response is of significant biological importance as it represents the final common pathway of all mechanisms of platelet activation, and as this study has shown there are strong correlations between all concentration of TRAP and ADP stimulation. This study found similar influencing mechanisms in the controls and cases, with the main predictors being MPV (estimated effect =3.0 Vs. 10.5%), GPIaIIa expression (estimated effect = 8.8 Vs. 7.2%) and plasma fibrinogen (estimated effect = 7.7 Vs. 6.6%). The only discrepancy was of the significant effect of age in the controls (estimated effect = 7.2%), but not the cases. These results suggest that the main factors controlling the final common pathway of platelet reactivity are similar between a group of 200 healthy and 205 premature MI subjects. The predictors with maximum TRAP response were identical in the cases to the ADP response; these were MPV, GPIa expression and plasma fibrinogen (estimated effects = 12.4, 7.9 and 6.2% respectively). The control results for TRAP stimulation were slightly different to ADP stimulation with the main predictors being GPIIbIIIa expression, plasma fibrinogen, plasma C-RP and age (estimated effects = 18.7, 11.0, 4.6 and 4.5% respectively.

Stepwise regression analysis showed that the factors correlating with GPIIbIIIa expression in the controls were MPV, C-RP and gender (estimated effect = 7.6, 4.8, 3.9% respectively) and in the cases MPV only (estimated effect = 17.1%). The strongest influence on GPIIbIIIa expression was MPV in both groups, again suggesting that the factors influencing expression do not seem to be effected either by the pathological processes involved with MI or its treatments. The GPIaIIa receptor expression in the controls significantly correlated with gender and serum cholesterol (estimated effect = 6.6 and 2.2% respectively) and in the cases with MPV and plasma triglyceride levels

(estimated effect = 8.9 and 6.2% respectively). A further logistic regression analysis of the receptor expressions was carried out to include the GPIIbIIIa C196T and GPIaIIa G873A polymorphisms. The results are presented and discussed in Chapter 5.

The direct comparison of the platelet flow cytometric results (Table 21) tended to show a lower platelet response or receptor expression in the cases compared to the controls, even after controlling for the significant influencing factors. Previous studies have demonstrated that aspirin does not have a significant affect on agonist stimulated platelet fibrinogen binding or CD63 and P-selectin expression when assessed by flow cytometry analysis. Therefore, this was the basis of using flow cytometry in assessing comparable platelet function data between MI cases all (100%) on aspirin and controls most (99%) not on aspirin. Chronos et. al. [88] measured expression of P-selectin (evidence of α-granule degranulation), CD63 (evidence of lysosomal release) and platelet fibrinogen binding with agonist stimulation in 10 healthy individuals and found no significant change in any of these platelet functions before and after 4 days of aspirin 300mg ingestion. Rinder et. al.[89] also showed that aspirin failed to affect ADP-induced P-selectin expression, detected by flow cytometry, allowing meaningful platelet function quantification unaffected by aspirin therapy. However, a more recent study carried out within the department highlighted the significant affect of both aspirin and clopidogrel on platelet function[222]. As part of the study, seven healthy volunteers had platelet fibrinogen binding assessed by flow cytometry at baseline and 4 hours after 150mg aspirin ingestion. Aspirin gave a 21% reduction in fibrinogen binding in response to ADP (63.5 ± 24.4% to 50.3 ± 18.9%, $p<0.05$) and a 9% reduction in response to TRAP (non significant). This study highlights the difficulty in comparing platelet functional analyses in our cohort, where all the cases are on long-term aspirin and nearly all the controls are not.

Anti-anginal agents have also been shown to affect platelet function. In a study of 9 healthy volunteers, Knight et. al.[213], quantified platelet fibrinogen binding (resting and after ADP and thrombin stimulation), P-selectin expression and GPIIbIIIa and GPIb expression, before and after treatment with three different classes of anti-anginals (calcium antagonist, nitrate and β blocker). They found nitrates (intravenous GTN) caused significant inhibition of aggregation, quantified by a decrease in fibrinogen

binding at rest and after ADP stimulation, as well as decreased degranulation (p-selectin expression). Amlodipine, a calcium antagonist significantly increased degranulation and atenolol, a β blocker, enhanced aggregation in response to thrombin. Other pharmacological treatments such as statins and ace inhibitors, now widely used in coronary artery disease may also influence platelet behaviour and again as there was a marked difference in pharmacological treatments between the two groups this also could impact on the platelet function results. In keeping with the PRAMIS study results, Knight et. al.[214], in a study of 12 males with documented coronary disease and 12 age and sex matched controls, showed significantly less P-selectin expression in the cases ($p = 0.005$) in resting samples. Furthermore, fibrinogen binding in response to ADP and thrombin was significantly reduced in the cases ($p<0.03$), as was GPIb and GPIIbIIIa expression compared to the controls. The authors concluded that atherosclerosis impairs platelet aggregatory (fibrinogen binding) responses.

Flow cytometry analysis involves assessment of platelets in the peripheral circulation, whereas in atherosclerosis and thrombosis, the most activated platelets are situated at the site of the lesions within the vessel endothelium. This particular discrepancy could be overcome by the measurement of various platelet activation markers (β thromboglobulin, platelet factor 4, soluble P selectin, soluble glycoprotein V and thromboxane) either in the plasma or urine. As frozen plasma samples have been stored for the entire PRAMIS cohort, this may be a future study undertaken to complement this detailed analysis. Previous studies showing an increased platelet response in the MI setting (Chapter 1, section 1.5.2) have tended to be done in the acute setting, a situation in which a number of additional parameters can influence platelet function (thrombolysis, heparin). Therefore, in this study the decreased platelet fibrinogen binding to agonist stimulation and decreased receptor expression demonstrated in the cases may be a result of various medications, methods used to assess platelet function or secondary to the disease process itself.

Finally, the case-control comparison of platelet function could be interpreted to show a desirable reduction in platelet activation secondary to treatments (predominantly aspirin) in the case group as a whole. However, large inter-individual variations remained and a detailed logistic regression analysis of the platelet function assays using

demographic variables, relevant platelet parameters as well as a number of conventional and emerging risk factors for MI, could only account for a small proportion of this variability. In general the main influencing factors for the various platelet functions analysed were similar in the controls and cases. Platelet function is heterogeneous and there may be additional mechanisms that significantly influence and explain the unaccounted for variability. Better understanding of these factors could impact on primary and secondary prevention strategies. Two possible candidates being proposed as risk factors for MI through modulation of platelet function are the GPIIbIIIa C196T and GPIaIIa G873A polymorphisms. They are discussed in detail in the following chapter.

4.9 Conclusions

In this chapter, analysis of platelet function by flow cytometry in 200 normal individuals and 205 individuals with a history of a premature myocardial infarction showed:

- Optimisation of the flow cytometry assay protocol (Sections 4.3-4.5) allowed accurate and reproducible assessment of platelet function analysis with low intra and inter assay variability

- Large inter-individual variation in both the controls and cases (Section 4.6) for all the platelet parameters measured including fibrinogen binding at rest and in response to ADP and TRAP stimulation; expression of GPIIbIIIa at rest and in response to ADP and TRAP; and GPIaIIa expression at rest.

- There were strong significant correlations, in both the controls and cases, between the various platelet parameters analysed (Section 4.6), in particular the ADP and TRAP response. This was partly explained by the fact that a significant proportion of the TRAP response was mediated through ADP pathways therefore accounting for the correlation.

- Factors influencing platelet function significantly in the controls, although most of these were subtle highlighting the complex multifaceted aspect of thrombosis, included a number of known risk factors for MI. The main positive correlations included MPV, gender, age and GPIaIIa expression. The main negative correlations were with plasma fibrinogen and current smoking status.

- Factors influencing platelet function significantly in the cases included MPV, GPIaIIa expression and fibrinogen levels. Most of these influences although significant were subtle. There were some differences in the influencing factors between the controls and cases. Potential explanations for these differences include affects of various medications over-riding innate platelet behaviour (secondary to treatment), affect of the disease processes involving a premature MI (secondary to disease) or true biological differences between the two groups (primary to disease). However, on the whole the main factors controlling the platelet functional parameters analysed were similar in the controls and cases.

- Direct comparison of the platelet function parameters between the groups tended to show lower values in the cases compared to the controls. This was probably secondary to the effect of various medications, in particular aspirin.

Chapter 5

The GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms: Functional effects and association with myocardial infarction

The GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms have been associated with increased MI risk (Section 1.6). Functional platelet studies have highlighted potential causal mechanisms, through increased platelet response, that may account for this relationship. However, controversy remains in the field due to contrasting findings. Prior to this study, a large investigation of the role and effect of these polymorphisms on platelet function as well as MI risk in the same cohort had not been carried out.

This chapter describes (i) the results of genotyping the GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms in the control and case populations; (ii) assesses the association between the polymorphisms and risk of premature MI; (iii) assesses the correlation between the two polymorphisms and functional platelet parameters quantified by flow cytometry; (iv) discusses and compares the results with previous studies in detail.

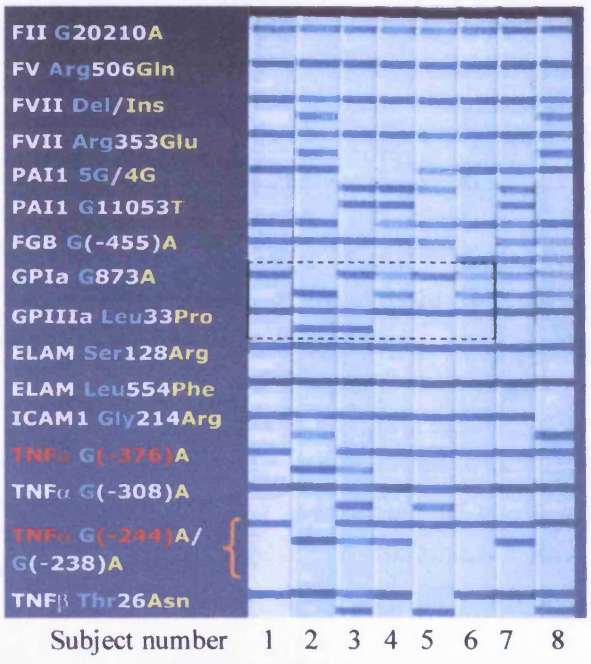
5.1 The GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms

5.1.1 Typing of the GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms

A novel multilocus genotyping assay[195] was used to genotype the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms, using PCR products from genomic DNA (Section 2.6.2). This allowed the genotyping of 63 polymorphisms within 35 candidate genes implicated in cardiovascular disease (Table 10). The 63-biallelic polymorphisms were represented on 4 separate test strips. An example of some actual test strips used is shown in figure 27(a). A template was used to read each individual polymorphism; examples of the allele identification processes for the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms are represented in figure 27(b). A more detailed description of the methods is described in Chapter 2.

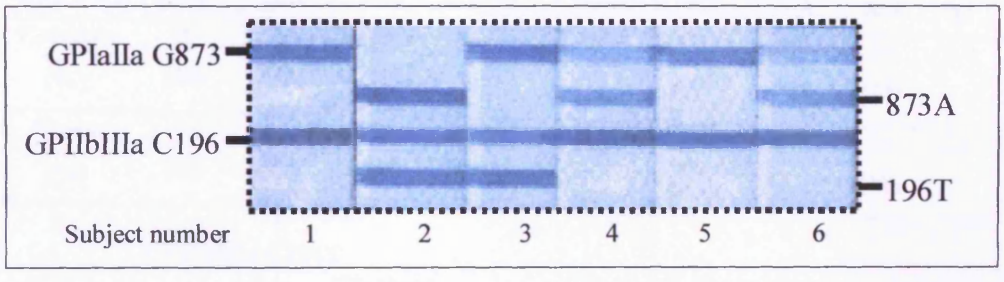
The accuracy of the multilocus assay has been assessed within our department in a case control MI cohort comprising of 1052 subjects. The genotyping results using the Roche strips[223] gave a 100% correlation to conventional method genotyping results for the angiotensin converting enzyme insertion/deletion[224] and methyltetrahydrofolate reductase (C677T)[225] polymorphisms previously published on the same cohort.

Figure 27(a) ***Multiplex PCR assay probe strip***



A representative probe strip demonstrating the identification of different alleles in 8 subjects is shown. For each vertical probe strip, the horizontal lines are positive probe signals; the guide on the left side identifies the allele detected by each probe.
These strips were 1 of a group of 4, which in combination allowed the genotyping of 63 biallelic sites on 35 different genes.
The area highlighted represents the GPIaIIa and GPIIbIIIa (leu33 = C196 and 33pro = 196T) genotypes of 8 subjects

Figure 27(b) ***Reading of the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms using the probe strips***



This figure demonstrates the genotyping of the GPIaIIa G873A and the GPIIbIIIa C196T polymorphisms. On each individual strip, for each polymorphism there is a pair of sequence specific immobilised probes. The presence of an allele specific product in the PCR product pool causes a colorimetric reaction, which allows detection. Therefore, in the figure above, for the GPIaIIa polymorphism, the G allele only is present in subjects 1, 3 and 5 (positive probe signal for higher band only) whilst subject 2 has only the A allele (positive probe signal for lower band only), making there genotype G/G and A/A respectively. Subject numbers 4 and 6 contain both alleles (positive probe signals for higher and lower bands) and therefore are heterozygotes, G/A. Using the same approach, the GPIIbIIIa genotypes for the 6 subjects are C/C, C/T, C/T, C/C, C/C, and C/C respectively.

5.1.2 Distribution of the GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms

The observed genotype distributions of the GPIaIIa G873A and the GPIIbIIIa C196T polymorphisms in the PRAMIS study are shown in table 22. The tables include the observed allele frequencies, the expected genotype distributions calculated using the Hardy-Weinberg equation ($a^2 + 2ab + b^2$) and the Chi squared test between the observed and expected values to test for equilibrium. Both in the controls and cases the distributions were in Hardy-Weinberg equilibrium.

Table 22 ***Genotype distributions of the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms***

GPIaIIa G873A

	Observed					Expected			Hardy-Weinberg test	
	Genotype			Allele frequency		Genotype				
	G/G	G/A	A/A	G allele	A allele	G/G	G/A	A/A	χ^2	p-value
Controls	80	85	35	0.613	0.387	75	95	30	6.0	0.199
(%)	(40)	(43)	(18)			(38)	(48)	(15)		
Cases	74	91	40	0.583	0.417	70	100	36	2.0	0.157
(%)	(36)	(44)	(20)			(34)	(49)	(18)		

GPIIbIIIa C196T

	Observed					Expected			Hardy-Weinberg test	
	Genotype			Allele frequency		Genotype				
	G/G	G/A	A/A	G allele	A allele	G/G	G/A	A/A	χ^2	p-value
Controls	142	52	6	0.840	0.160	141	54	5	2.0	0.157
(%)	(71)	(26)	(3)			(71)	(27)	(3)		
Cases	148	54	3	0.854	0.146	150	51	4	2.0	0.157
(%)	(72)	(26)	(1)			(73)	(25)	(2)		

5.2 The GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms and platelet function

A detailed assessment of the effects of the polymorphisms on the following parameters of platelet function were made:

(i) Platelet fibrinogen binding - resting,
- ADP 1 x 10^{-7}, 1 x 10^{-6}, 1 x 10^{-5} mol/l stimulation,
- TRAP 3 x 10^{-6}, 1 x 10^{-5}, 3 x 10^{-5} mol/l stimulation.

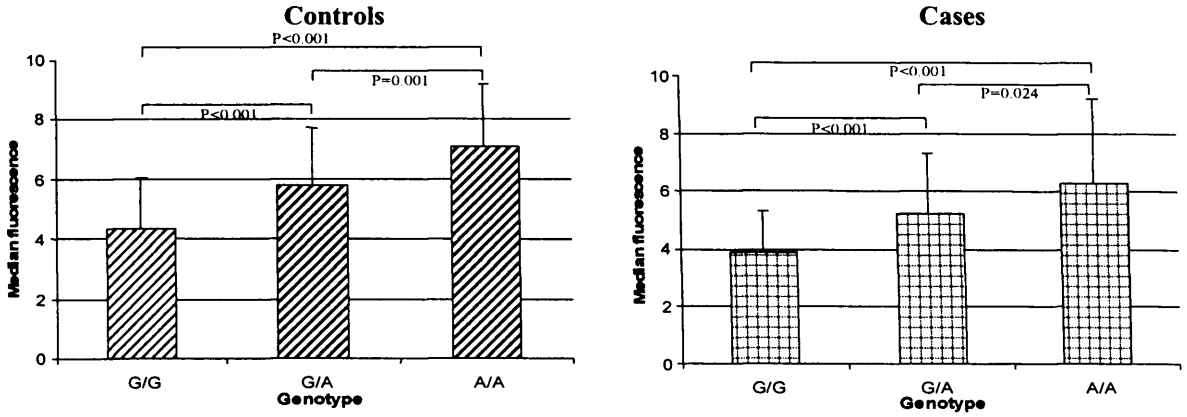
(ii) Platelet GPIIbIIIa receptor expression - resting,
- ADP 1 x 10^{-5} mol/l stimulation,
- TRAP 3 x 10^{-5} mol/l stimulation;

(iii) Platelet GPIaIIa receptor expression - resting;

5.2.1 The GPIaIIa G873A polymorphism

The G873A polymorphism had a significant influence on GPIaIIa receptor expression, in both the control and case populations (Figure 28). The median fluorescence for the G/G Vs G/A Vs A/A genotypes in the controls and cases were (mean ± SD) 4.37 ± 1.66, 5.77 ± 1.94, 7.10 ± 2.09 and 3.88 ± 1.42, 5.23 ± 2.07, 6.26 ± 2.92 respectively. A summary of the effects of the G873A polymorphism on other platelet parameters is shown in table 23.

Figure 28 ***The GPIaIIa G873A polymorphism and GPIaIIa receptor expression***



In the controls, stepwise linear regression analysis without the G873A polymorphism (Section 4.7.7 in chapter 4) found GPIa IIa expression was independently influenced by gender (β = -0.294, $p<0.001$) and serum cholesterol (β = 0.156, $p<0.045$) and that the estimated effect sizes were only 6.6 and 2.2% respectively. Once the polymorphism was entered into the model, the G873A polymorphism and gender were the only significant independent predictors of GPIaIIa expression (standardised β coefficient = 0.500, $p<0.001$ and -0.334, $p<0.001$ respectively). There was no significant interaction between GPIaIIa G873A genotype and gender, $p = 0.187$. Of the total variance in GPIaIIa expression, 27.5% was accounted for by the G873A polymorphism. Gender had an effect size of 16%.

In the cases, GPIa IIa expression stepwise linear regression analysis prior to the inclusion of the G873A polymorphism (Section 4.6.5 in chapter 4) showed independent influence of MPV (β = 0.268, $p<0.001$) and plasma triglyceride (β = 0.262, $p<0.001$) only. The estimated effect size on the variation of GPIaIIa expression was 8.9% for MPV and 6.2% for serum triglyceride.

Table 23 *The GPIaIIa G873A polymorphism and platelet function analyses in controls and cases*

	Controls				Cases			
Fibrinogen binding (% positive)	**G/G**	**G/A**	**A/A**	**p-value**	**G/G**	**G/A**	**A/A**	**p-value**
Resting	2.5(2.3-2.7)	2.7(2.4-2.9)	2.6(2.3-3.0)	0.566	2.5(2.3-2.6)	2.5(2.3-2.7)	2.4(2.1-2.7)	0.638
ADP 10^{-7}	12.3(10.1-15.0)	13.7(11.1-16.8)	15.9(11.7-21.1)	0.392	10.8(8.7-13.2)	9.6(7.7-11.9)	9.1(6.7-12.3)	0.615
ADP 10^{-6}	68.1(63.7-72.2)	70.0(65.0-74.4)	73.6(67.6-78.8)	0.384	65.2(60.2-69.9)	63.1(58.5-67.4)	61.3(54.1-68.0)	0.629
ADP 10^{-5}	84.3(81.7-86.5)	85.8(83.2-88.1)	86.9(83.3-89.9)	0.426	83.0(80.2-85.6)	81.7(79-84.0)	80.8(76.4-84.6)	0.602
TRAP 3 x 10^{-6}	6.2(4.8-7.8)	6.7(5.3-8.4)	7.6(5.0-11.3)	0.633	6.2(4.7-8.2)	5.5(4.5-6.9)	6.4(4.0-10.1)	0.741
TRAP 1 x 10^{-5}	80.5(76.5-83.9)	78.6(73.5-83.0)	85.9(82.1-89.0)	0.089	76.8(71.8-81.2)	74.4(68.9-79.2)	80.5(75.0-84.9)	0.295
TRAP 3 x 10^{-5}	87.9(85.9-89.6)	88.0(85.7-89.9)	90.1(87.7-92.1)	0.349	84.3(81.4-86.8)	83.0(79.6-85.8)	85.0(81.4-88.0)	0.636

	Controls				Cases			
Glycoprotein receptor expression (median fluorescence)	**G/G**	**G/A**	**A/A**	**p-value**	**G/G**	**G/A**	**A/A**	**p-value**
GPIIbIIIa Resting	3.75(3.62-3.89)	3.79(3.66-3.92)	3.82(3.65-4.00)	0.834	3.68(3.54-3.82)	3.60(3.46-3.72)	3.73(3.54-3.91)	0.456
GPIIbIIIa ADP10^{-5}	4.54(4.40-4.68)	4.58(4.43-4.74)	4.60(4.38-4.82)	0.872	4.56(4.40-4.72)	4.37(4.22-4.52)	4.45(4.21-4.69)	0.242
GPIIbIIIa TRAP 4 x 10^{-5}	5.70(5.51-5.89)	5.56(5.36-5.77)	5.69(5.42-5.95)	0.568	5.67(5.48-5.85)	5.37(5.18-5.57)	5.47(5.17-5.77)	0.106
GPIa	4.37(4.00-4.74)	5.77(5.35-6.18)	7.10(6.38-7.82)	<0.001	3.88(3.55-4.21)	5.24(4.80-5.67)	6.26(5.32-7.20)	<0.001

All results are expressed as mean (95% CI of mean)

Inclusion of the G873A polymorphism showed independent influence by GPIIbIIIa expression (($\beta = 0.432$, $p < 0.001$), the G873A polymorphism (($\beta = 0.341$, $p<0.001$) and plasma triglyceride ($\beta = 0.237$, $p<0.001$). The estimated effect size on the variation of GPIaIIa expression again became much larger and was 23.5% for GPIIbIIIa expression, 16.0% for the G873A polymorphism and 6.7% for serum triglyceride.

The GPIaIIa G873A polymorphism did not associate with any of the other platelet function parameters analysed (Table 23). Therefore, there was no direct effect of the G873A polymorphism on fibrinogen binding to the platelet at rest or with ADP and TRAP stimulation. Neither was there any significant correlation with GPIIbIIIa expression at rest, or with ADP or TRAP stimulation.

5.2.2 The GPIIbIIIa C196T polymorphism

The GPIIbIIIa C196T polymorphism did not correlate significantly with any of the platelet function parameters analysed. As the controls and cases contained only 6 and 3 subjects respectively that were homozygous for the T allele, they were combined with the T allele heterozygous groups for the majority of the analyses. The results are shown in table 24.

The resting GPIIbIIIa receptor expression for the C/C Vs C/T Vs T/T genotypes in the controls and cases were (mean ± standard deviation) 3.77 ± 0.58, 3.87 ± 0.56, 3.23 ± 0.78 (p=0.035) and 3.64 ± 0.59, 3.71 ± 0.58, 2.91 ± 0.96 (p=0.074) respectively. Comparisons between the C/C Vs C/T and T/T groups combined gave p-values of 0.662 and 0.779 in the controls and cases respectively. Similar comparisons in the ADP and TRAP stimulated samples in both the controls and cases also gave non-significant results (Table 24).

The significant predictors for resting GPIIbIIIa expression in the controls were MPV, C-RP and gender (p = < 0.001, 0.003 and 0.009 respectively). Adjusting for these independent predictors did not influence the lack of correlation between the C196T genotype and GPIIbIIIa expression, p=0.965. In the cases the only independent

predictor of GPIIbIIIa receptor expression was MPV ($p<0.001$). Adjusting for this gave a p-value of 0.472 between C/C Vs C/T and T/T groups.

Platelet fibrinogen binding at rest or with ADP and TRAP was not influenced by the C196T genotype in the controls or cases. The fibrinogen binding percent positive platelets with maximum ADP stimulation for the C/C Vs C/T Vs T/T genotypes in the controls and cases were (mean ± standard deviation) 84.66 ± 13.15, 87.37 ± 10.85, 84.90 ± 9.38 and 82.11 ± 12.76, 80.85 ± 12.98, 92.66 ± 2.18 respectively. There were no significant differences between the C/C Vs C/T and T/T groups in the controls and cases for maximum ADP stimulation, p= 0.130 and 0.836 respectively (table 24).

Table 24 ***The GPIIbIIIa C196T polymorphism and platelet function analyses in controls and cases***

	Controls			Cases		
Fibrinogen binding (% positive)	**C/C** n = 142 (71%)	**C/T and T/T** n = 58 (29%)	**p-value**	**C/C** n = 148 (72%)	**C/T and T/T** n = 57 (27%)	**p-value**
Resting	2.6(2.5-2.8)	2.6(2.3-2.8)	0.751	2.5(2.3-2.6)	2.5(2.3-2.7)	0.939
ADP 10^{-7}	13.1(11.2-15.3)	14.5(11.5-18.1)	0.474	10.4(8.8-12.1)	8.8(6.9-11.3)	0.285
ADP 10^{-6}	68.9(65.4-72.3)	72.1(67.3-76.5)	0.306	64.0(60.4-67.3)	62.3(56.4-67.9)	0.618
ADP 10^{-5}	84.7(82.7-86.5)	87.1(84.6-89.3)	0.130	82.1(80.1-84.0)	81.7(78.2-84.8)	0.836
TRAP 3 x 10^{-6}	6.2(5.2-7.4)	7.7(5.8-10.2)	0.191	6.2(5.1-7.6)	5.3(3.9-7.1)	0.386
TRAP 1 x 10^{-5}	79.4(75.9-82.6)	83.8(80.2-86.8)	0.105	76.8(73.1-80.1)	75.9(69.9-81.0)	0.794
TRAP 3 x 10^{-5}	87.8(86.2-89.3)	89.4(87.5-91.1)	0.234	83.9(81.7-86.0)	83.5(80.2-86.4)	0.835
Glycoprotein receptor expression (median fluorescence)	**C/C**	**C/T and T/T**	**p-value**	**C/C**	**C/T and T/T**	**p-value**
GPIIbIIIa Resting	3.77 ± 0.58	3.81 ± 0.61	0.662	3.64 ± 0.59	3.67 ± 0.62	0.779
GPIIbIIIa ADP10^{-5}	4.54 ±0.68	4.63 ± 0.64	0.394	4.46 ± 0.71	4.45 ± 0.71	0.930
GPIIbIIIa TRAP 4 x 10^{-5}	5.60 ± 0.89	5.73 ± 0.84	0.326	5.51 ±0.90	5.47 ± 0.89	0.824
GPIa	5.43 ± 2.08	5.46 ± 2.17	0.932	5.08 ± 2.34	4.58 ± 1.96	0.154

Results for receptor expression are mean fluorescence ± SD;
for fibrinogen binding are mean (95% CI of the mean).

In the controls the independent predictors for maximum ADP stimulation response were GPIaIIa receptor expression, plasma fibrinogen, age and MPV ($p<0.001$, 0.001, 0.001 and 0.022). Adjusting for these did not significantly influence the C196T genotype effect, p=0.489 for C/C Vs. C/T Vs. T/T and p = 0.274 for C/C Vs. C/T and T/T. Similarly, on adjusting for the independent predictors for fibrinogen binding with maximum ADP stimulation in the cases (MPV, GPIaIIa expression and fibrinogen, all p-values < 0.001), the influence of C196T genotype remained insignificant, p = 0.116 for C/C Vs. C/T Vs. T/T and p = 0.587 for C/C Vs. C/T and T/T.

5.3 The association of the GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms with risk of myocardial infarction

5.3.1 The GPIaIIa G873A polymorphism and risk of MI

There were no significant differences between the control and case populations for the G873A genotype distributions (Table 22), Chi squared=0.81, p=0.667. The A allele frequency was 0.39 and 0.42 in the controls and cases respectively, Chi squared=0.88, p=0.341. The odds ratio of the risk of MI associated with the A allele, using a co-dominant model, was 1.12 (95%CI 0.86-1.46), p=0.413. This did not change significantly after adjusting for age, gender, smoking, diabetes and hypertension (odds ratio=1.06(0.78-1.45), p=0.705). The results of recessive and dominant models are also shown in table 25, for unadjusted and adjusted data. For any of the models used, the GPIaIIa G873A polymorphism was not significantly associated with increased risk of MI. This was despite the study having enough power to detect a 1.5 fold increase in MI risk as indicated by the confidence interval in the co-dominant model (Table 25).

Table 25 ***The GPIaIIa G873A polymorphism and risk of MI***

	Co dominant	Dominant	Recessive
Unadjusted	1.12 (0.86-1.46) 0.413	1.18 (0.79-1.76) 0.419	1.14 (0.69-1.89) 0.602
Adjusted for age, sex, smoking, diabetes, hypertension	1.06 (0.78-1.45) 0.705	1.10 (0.69-1.75) 0.684	1.06 (0.59-1.90) 0.842

Results expressed as odds ratio (95% confidence intervals) and p-values, for risk of MI associated with the A allele.

5.3.2 The GPIIbIIIa C196T polymorphism and risk of MI

There were no significant differences between the controls and cases for the C196T genotype distributions (Table 22), Chi squared=1.46, p=0.760. The frequency of the T allele was 0.16 and 0.15 in the controls and cases respectively, Chi squared=0.29, p=0.590. The odds ratio for the risk of MI associated with the T allele, using a dominant model, was 0.94 (0.61-1.45), p=0.790. Once again the narrow confidence intervals indicate adequate power to detect a 1.5 fold increase in MI risk. Again adjusting for age, gender, smoking, diabetes and hypertension did not significantly affect the result (odds ratio=0.97(0.59-1.60), p=0.910). Results of recessive and dominant models are also shown in table 26.

Table 26 ***The GPIIbIIIa polymorphism and risk of MI***

	Co dominant	Dominant	Recessive
Unadjusted	0.90 (0.61-1.32) 0.588	0.94 (0.61-1.45) 0.790	0.48 (0.12-1.95) 0.304
Adjusted for age, sex, smoking, diabetes, hypertension	0.91 (0.59-1.43) 0.699	0.97 (0.59-1.60) 0.910	0.44 (0.09-2.23) 0.320

Results expressed as odds ratio (95% confidence intervals) and p-values, for risk of MI associated with the A allele.

5.4 The GPIaIIa G873A and GPIIbIIIa C196T platelet polymorphisms and coronary artery disease burden

Angiographic data was available on 175 of the cases. A number of previous studies have found a positive association between the T allele (PL^{A2}) of the C196T and the A allele of the G873A polymorphisms and coronary disease burden. For this analysis, a coronary stenosis of 50% or greater was considered significant and subjects were divided into low (0 or 1 stenosed coronary artery) or high (2 or 3 diseased arteries) coronary disease burden.

(i) GPIaIIa G873A polymorphism

There were no differences between the low and high burden groups for A allele carrier status, 26/75 (35%) and 34/100 (34%) respectively, Chi squared = 0.008, p = 0.927. Using a co-dominant model also did not reveal any significant differences between the groups, Chi squared = 1.173, p = 0.556.

(ii) GPIIbIIIa C196T polymorphism

Of the 75 subjects with low coronary burden, 19 (25%) were T allele carriers. In the high coronary burden group 27 out of 100 (27%) subjects possessed at least 1 T allele. The T allele did not correlate to a significant risk of increased coronary burden, Chi squared = 0.061, p = 0.804.

5.5 Discussion

In the following sections there is an in depth discussion of studies related to the GPIaIIa G873A and GPIIbIIIa C196T polymorphisms. The sections have been divided into functional and association studies. The review of the literature has been updated to include all relevant studies published since the completion of the PRAMIS study (up to July 2004) and therefore a number of recent publications not mentioned in the introduction section are discussed.

5.5.1 The GPIaIIa G873A polymorphism

5.5.1.1 Functional studies

In this study, the G873A polymorphism explained 27.5% and 16% effect of the total GPIaIIa receptor expression variance in the controls and cases respectively. This is clearly a striking biological effect made all the more important because the GPIaIIa expression is an independent predictor for fibrinogen binding with maximum ADP stimulation in both the controls and cases (estimated effect = 7.7% and 7.2% respectively). Therefore, the G873A polymorphism has a major direct influence on GPIaIIa expression, which in turn has a significant effect on fibrinogen binding to ADP stimulation, although, a direct correlation between the polymorphism and platelet fibrinogen binding was not observed.

The PRAMIS study is by far the largest investigation of the G873A GPIaIIa polymorphism and its functional impact on expression. It is also the only study to date that has looked at this consequence in a MI cohort, as most previous studies have been carried out on healthy subjects. The finding confirms the gene dose relationship on receptor expression in both a normal and high thrombotic risk group. There were no significant differences in the affect of A allele between the groups and all the correlations between the genotype groups and the phenotype (GPIaIIa expression) were highly significant even after adjusting for other independent predictors of GPIaIIa expression.

There have only been a small number of other functional studies on the G873A polymorphism. Kunicki et al.[151] were the first group to appreciate the large inter-individual variations of GPIaIIa receptor expression (between 968-2,874 molecules per platelet) on the platelet surface of 27 normal individuals. The expression variation results compare well to the PRAMIS cohort findings with the ranges in the controls and cases being 1.93-11.85 and 1.55-12.55 arbitrary units respectively. Figure 24 in chapter 4 graphically displays the increased variation of the GPIaIIa receptor compared to the GPIIbIIIa receptor. GPIaIIa expression correlated to a twenty fold and five fold variation in platelet attachment rates to type I and type III collagen ($r = 0.742$; $p<0.01$

and r = 0.636; p<0.01) including under experimental conditions mimicking high shear flow rates[152].

Kunicki et al. were also the first to describe the genetic influence, in 30 normal subjects, in the form of polymorphisms on GPIaIIa expression[66]. In particular the silent C807T and G873A polymorphisms, located in the α_2 subunit gene on chromosome 5, were in linkage disequilibrium and the T and A alleles respectively had a significant gene-dose affect on increased GPIaIIa expression, with the highest levels seen in the homozygous individuals and intermediate levels of expression in heterozygous subjects. A further 5 nucleotide polymorphisms were identified and linked to the original C807T/G873A polymorphisms and expression levels of GPIaIIa[152]. The group described 3 α_2 gene alleles incorporating the newly identified polymorphisms. Allele 1 (807T/837T/*873A*/Br^b) which was associated with increased levels of GPIaIIa expression; allele 2 (807C/837T/*873G*/Br^b) and allele 3 (807C/837C/*873G*/Br^a) which were associated with lower levels of GPIaIIa.

Corral et al.[153], in a cohort of 159 individuals comprising of subjects with a history of coronary heart disease, cerebrovascular disease, deep vein thrombosis or being blood donors, found the median fluorescence for the A/A Vs A/G Vs G/G groups were 8.71±2.59, 12.39±3.94, and 15.89±4.51 respectively (all p-values between groups<0.001). A more detailed sub-study in 21 subjects (7/genotype group) failed to show any differences in spontaneous, thrombin or collagen induced aggregation. Huang et al.[188], in a study of 54 healthy Caucasian subjects, also demonstrated a relationship between the G873A and GPIa expression (G/G Vs. G/A Vs. A/A = 2.6±0.3, 3.4±0.2, 4.3±0.3, overall p < 0.002) but found no affect on platelet reactivity (measured by p-selectin expression), p = 0.20. Both these results are similar to the PRAMIS study in terms of magnitude of genotype effect on GPIaIIa expression and lack of correlation to agonist stimulation

Cadroy et al.[191] studied experimental ex-vivo thrombus formation in 40 normal subjects (mean age=25±4) under conditions of low ($650s^{-1}$) and high shear rates ($2600s^{-1}$), therefore mimicking normal and moderate stenosis arterial flow conditions. The normal flow conditions gave no statistical difference in the rate of platelet deposition on the

type I collagen-coated cover slip. However, under high shear stress conditions there was a significant higher rate seen in the A allele carriers (G/G Vs G/A Vs A/A = 3.47±1.36, 4.22±1.46, 5.88±2.61($x10^{-7}/cm^2$) compared to the G/G carriers, overall p-value=0.03.

More recently, Luzak et al.[226] described the effect of the G873A polymorphism in 290 healthy volunteers (age=30±12, 128 males and 162 females). In a subset of 17 G/G and 15 A/A individuals detailed platelet function tests were performed. No differences between the groups were seen for PFA-100 closure time (type I collagen coated membrane aperture under normal flow conditions) with ADP and epinephrine stimulation. Under static conditions no significant differences were observed for fibrillar type I collagen binding. However, with monomeric collagen, stimulation of platelets with thrombin, ADP and a thromboxane analogue all significantly increased collagen binding in the A/A group whereas in the G/G subjects a significant increase was only observed with thrombin and the thromboxane analogue ($p<0.001$). Also, Gi9, a mab GPIaIIa receptor antagonist was more effective in inhibiting spontaneous and thrombin induced binding to fibrillar collagen (both p-values <0.05) in the A/A group compared to the G/G subjects. Therefore, the C873A polymorphism influenced ADP induced monomeric collage binding and sensitivity to a GPIaIIa antagonist.

Due to the single visit strategy and the size of the cohort (450 subjects recruited) a direct assessment of platelet response to collagen could not be made in the PRAMIS study. Collagen studies by flow cytometry are technically very difficult and assessment using aggregometry or flow chambers in addition to flow cytometry were not feasible under the time constraints of this study. However, carrying out such a study to confirm the genotype, receptor expression and attachment or response to collagen would now be possible in a much smaller cohort as the genotypes are known. This type of study carried out by Cadroy et al.[191] and Luzak et al.[226] have confirmed that platelets with increased GPIaIIa expression, related to the G873A polymorphism, display increased responses to ADP and collagen stimulation and can alter sensitivities to receptor antagonism.

It is unknown whether the G873A polymorphism is itself involved in differential α_2 expression or is in linkage disequilibrium with a polymorphism responsible for

modulating α_2 level. The exact mechanism of the genotype-phenotype relationship remains unclear but there are a number of ways in which DNA sequence alterations could influence gene expression. Changes in specific promoter elements could influence promoter activity, resulting in increased or decreased mRNA levels. Such enhancer and silencer elements have been located within the α_2 gene and shown to affect α_2 expression specifically in megakaryocytic cells (the only other cell type to express the GPIaIIa molecule)[227] . Similarly, sequence variations within sites for transcription factors and other regulatory molecules could have a profound impact on expression levels of GPIaIIa. Other potential mechanisms of modulating protein expression include influences on mRNA stability by the 3' untranslated regions[228] or the polymorphism itself may have a direct allelic affect on GPIaIIa expression. Finally, differences in the intragenic sequences may play a regulatory role[229]. The clustering of the polymorphisms, identified to date, in one region of the α2 gene (between exon 7 and 8) suggests this region may harbour the critical control elements in the regulation of GPIaIIa expression.

In conclusion, considering the functional results, the GPIaIIa G873A polymorphism is significantly associated with receptor expression, and GPIaIIa receptor expression significantly influences platelet agonist fibrinogen binding. A plausible and reproducible biological mechanism therefore exists to consider the A allele as a potential risk factor for thrombotic disease and the GPIaIIa receptor for strategic targeting of anti-platelet therapy in a pharmaco-genetic manner.

5.5.1.2 Association studies

A number of association studies have investigated the G873A polymorphism as a thrombotic risk factor. Disease phenotypes tested include coronary disease, MI[154-159], venous thrombo-embolism[153], cerebrovascular disease[153,160] and thrombosis after stent insertion[230]. The results have been conflicting to date and no clear consensus exists as to the validity of the A allele as a thrombotic risk factor, despite the accepted effect on GPIaIIa expression and platelet function. The variety of disease phenotypes tested may explain some of this discrepancy. Although platelets play a crucial role in atherosclerosis, the functional studies on the G873A genotype to date would support an

enhanced platelet reactivity (related to increased GPIaIIa expression) predisposing to thrombotic disease rather than coronary artery disease per se. For the purposes of this discussion, only studies that have looked at MI as the disease phenotype will be considered. Some of the non-MI studies have been highlighted in Table 3, Chapter 1.

Moshfegh et al. carried out the first case control study in 177 MI survivors and 89 age and sex matched controls (median age 57, range32-72). They reported an association between the 873A allele and increased risk of MI (A/A Vs. G/A and G/G, odds ratio = 3.3 (95% CI 1.2-8.8, p = 0.022). The homozygous A genotype remained an independent risk factor when age, sex, smoking, hypertension, diabetes, BMI, HDL, LDL and fibrinogen were adjusted for by logistic regression (odds ratio = 6.2 (1.8-21.9), p = 0.005). Santoso et al.[155] genotyped 2250 consecutive male patients undergoing diagnostic angiography and divided them into those with (n=1050, age=62.2±9.5) and those without a history of MI (n=1187, age=61.4±9.9). 167 individuals with no significant angiographic coronary disease were excluded from the analysis. Although overall no detectable significant risk for MI was associated with the A allele (A/A and G/A genotype Vs. G/G, odds ratio = 1.13(0.91-1.40) p=0.25), when the analysis was restricted to those under the age of 62 (n=1057, odds ratio=1.57(1.14-2.13) p=0.004) or under the age of 49 (n=223, odds ratio=2.61(1.26-5.41) p=0.009), the results became significant. Further support for the A allele increasing MI risk came from a prospective study carried out by Roest et al.[156] in 12239 normal women followed up for 6 years as part of a breast screening programme. The nested case-control analysis of 480 cases that died of cardiovascular (MI and CVA) causes and 496 controls showed no significant association between the A/A homozygotes to cardiovascular mortality compared to G/G subjects (odds ratio = 1.2 (0.8-1.7) p>0.05). A similar analysis for the MI deaths alone (n = 217) also did not reveal a significant result (odds ratio A/A Vs. G/G = 1.3(0.8-2.1) p>0.05). However, a smoking history together with the A/A genotype did increase cardiovascular mortality (odds ratio = 2.2 (1.1-4.4) p<0.05), unfortunately an analysis of smoking history on MI death was not given. Further sub-analyses showed that the presence of two indicators of compromised endothelium (diabetes, microalbuminaemia, smoking history) significantly increased cardiovascular mortality in the A/A genotype compared to G/G genotype without such risks (odds ratio = 14.1 (5.0-39.9) p =0.007).

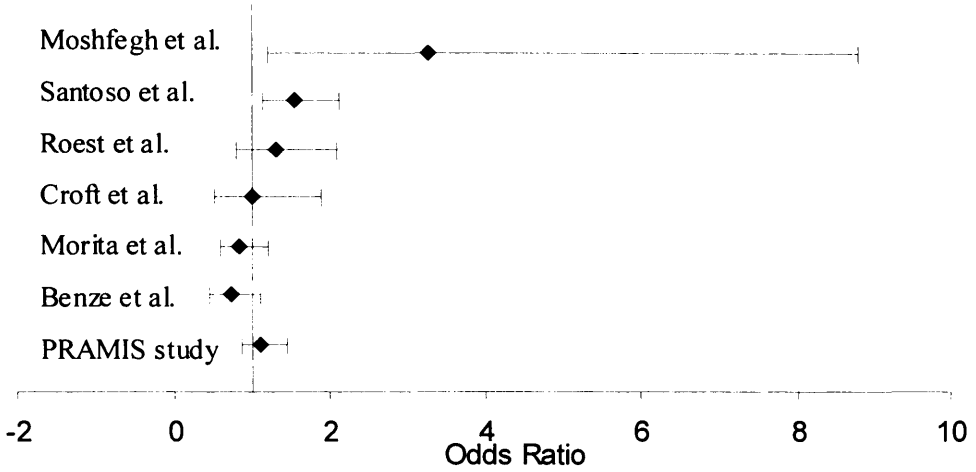
Croft et al.[157] published a study of a relatively elderly group of 546 acute MI subjects (mean age 61.9±9.2) and 507 controls (mean age 58.6±10.6) which showed no association with the A allele either in the whole cohort (odds ratio 0.88 (0.74-1.05) p=0.17) or a subgroup analysis of cases under 55 years old (odds ratio = 1.00(0.53-1.90) p= 0.99). Morita et al.[158] published a study of a similar aged cohort of 210 MI (mean age 60.9±8.5) and 420 age and sex matched controls (mean age 60.9±8.5). In this Japanese population based study, there was no increased risk of MI between A/A and G/A genotypes compared to G/G genotype (odds ratio = 0.84 (0.59-1.21) p= 0.35). Neither was there an increased risk demonstrated in the sub-set of less than 55 years old subjects (odds ratio = 0.94, (95%CI not given, p = 0.89). Benze et al.[159] reported a study on 287 particularly young men (age 40.5±3.4) who had suffered a MI and 138 healthy controls (age 40.5±3.4). They found no association with the A allele and risk of premature MI (0.73 (0.47-1.12) p> 0.05). Neither did this allele have an additive effect when analysed in conjunction with the C196T GPIIbIIIa polymorphism.

Finally, Von Beckerath et al.[230] looked at the relationship between the G873A polymorphism and 30-day adverse cardiac events (death, MI, urgent target vessel revascularisation) after coronary artery stenting. They studied 1797 consecutive patients and found no significant increase in the incidence of the composite endpoint in the A allele carriers (odds ratio = 1.23(0.81-1.86), p = 0.33). A similar study carried out by Meisel et al.[231] in 673 coronary intervention patients (PTCA, n=272; coronary atherectomy, n=104; stent insertion, n=276) found no increase in the same composite endpoint between the genotype groups (G/G Vs G/A Vs A/A = 7% Vs 5.9% Vs 7.1%, p = 0.84).

A major flaw in genetic association studies of complex multi-factorial disease is that it is unlikely a single gene polymorphism will have a major impact on risk stratification. More likely, complex gene-gene and gene-environment mechanisms will be involved to alter disease susceptibility. Many of the studies to date would therefore have been underpowered, especially if there is a failure to define an accurate phenotype, which allows unrelated disease modifying mechanisms to interfere with the biological or causal pathway of interest. The association studies listed above are typical of the difficulties in the ability to compare genetic based association studies due to differences

in phenotype, gender, race, age and inadequacy of size. Figure 29 is a comparison of the association studies for MI risk. For the data to be matched to the PRAMIS study cohort, where possible sub-analysis of younger age groups from the larger studies is included, rather than the entire cohort. It is clear from this figure that despite publication bias, the association studies to date do not support the hypothesis that the 873A allele is a robust independent risk factor for MI.

Figure 29 ***Risk of MI associated with the GPIaIIa G873A polymorphism A allele carrier status***



Data from 7 association studies with MI phenotype. Above graph represents odds ratio, with 95% confidence intervals, for MI for A allele carrier status compared to G/G genotype.

Only 3 positive studies have supported the increased risk of MI with A allele carrier status. Moshfegh et al.[154] analysed only 89 controls, where the A allele frequency was far lower (0.32) compared to the PRAMIS (0.39) and most other Caucasian population based studies (0.38-0.43, Table 3). The often-quoted positive data from the Roest et al.[156] is misleading. The overall cohort analysis was clearly negative as was the sub-analysis of MI deaths alone. Data for the A allele only became positive once smoking status or other risk factors for endothelial damage were considered. The study by Santoso et al.[155] is the most convincing. It involved a large cohort selected prospectively with well-defined angiographic coronary disease and MI history. The sub-analysis of the under 62 years populations still involved relatively large numbers and became more positive once restricted to under 49 years.

The PRAMIS findings are consistent with the general consensus from other study findings, that the G873A polymorphism significantly affects GPIaIIa receptor expression and therefore platelet function, yet this does not translate into independent increased risk of MI. This was despite the PRAMIS study being powerful enough to detect a 1.5 fold increase in MI risk (odds ratio of MI risk using a co-dominant model in association with the A allele = 1.12 (95%CI 0.86-1.46), p=0.413). The results suggest that moderately large variations in collagen binding to the GPIaIIa receptor (allowing anchoring of the platelet to exposed collagen on the endothelial surface) may not play a significant aetiological role in MI. In the high shear flow setting of the coronary circulation, the initial platelet attachment by the GPIb-IX-V complex to vWF and subsequent GPIIbIIIa activation leading to fibrinogen binding and platelet aggregation, as well as the coagulation mechanisms leading to thrombosis, may have a more prominent role in determining clinical outcome following endothelial damage.

In conclusion, despite the promise from the functional studies, the association studies to date have failed to convince that the 873A allele carrier status is an independent risk factor for MI. In the PRAMIS study, an extreme phenotype of 205 premature MI subjects was also unable to detect an increased risk of MI related to the polymorphism. Many of the studies to date have been carried out in non ideal populations in terms of age and disease phenotype and given the multifactorial nature of MI pathogenesis a small or modest increase in risk cannot be ruled out. Therefore, larger and ideally prospective population based studies on accurately defined young subjects are still required.

5.5.2 The GPIIbIIIa C196T polymorphism

5.5.2.1 Functional studies

Unlike the GPIaIIa G873A polymorphism, the GPIIbIIIa C196T (leu33pro, PL^{A1}/ PL^{A2}) was first associated with increased risk of MI[163] rather than an aspect of platelet function. The strategic role the GPIIbIIIa receptor plays in platelet aggregation and thrombus formation has generated a huge interest in the polymorphism and its possible functional effects as well as association with MI.

The functional studies have essentially been conducted in healthy subjects and analysed by genotype and have taken the form of either platelet function studies (aggregation, [182,186,187,232], flow cytometry[188,189], plasma markers[190,191]) and/or altered sensitivities to anti-platelet agents[184].

Feng et al. were the first to describe an association between the 196T allele (PL^{A2}) and increased platelet reactivity[182,232]. 1422 subjects from the Framingham Offspring Study were genotyped and platelet aggregometry by the Born method determined. The presence of one or two T alleles was associated with an incrementally lower threshold concentration for epinephrine-induced aggregation (C/C Vs. C/T Vs. T/T = 0.9(0.9-1.0), 0.7(0.7-0.9) and 0.6(0.4-1.0), p=0.007) therefore indicating increased platelet aggregability related to the T allele. This remained significant after adjustment for a number of cardiovascular risk factors and accounted for 0.7% of the total variance of epinephrine–induced aggregation (p=0.007). The ADP-induced thresholds did not differ between the groups. The same group reanalysed this data with the inclusion of plasma fibrinogen levels to look for interaction between the polymorphism, aggregation and plasma fibrinogen. They found that a higher fibrinogen level was associated with increased epinephrine induced platelet aggregability but that this affect was genotype specific, being significantly present in C/C individuals but not in T allele carriers (C/T and T/T), p = 0.002 and 0.90 respectively. In contrast to the above findings, Lasne et al.[186] described TRAP induced biphasic aggregation thresholds in 102 healthy volunteers and found that the T allele carriers (n=14) required higher TRAP concentrations to induce aggregation than for the whole population (8.2 ± 3.5 Vs. 5.9 ± 1.5 μmol, p = 0.0012). Similarly, the mean ADP concentration to induce biphasic aggregation was 1.6 ± 0.3 μmol in C/C subjects (n = 8) and 2.7 ± 1.1 μmol for C/T allele subjects (n = 8), p = 0.023. These results suggest that the T allele polymorphism is associated with platelet hypoaggregability. More recently an aggregometry based study by Frey et al.[187] assessed both the effects the β3 subunit G protein polymorphism, GNB3 C825T, and the IIIa C196T polymorphism. There were no significant differences between 196C/C genotype subjects and 196T allele carriers for aggregometry induced by ADP, TRAP, epinephrine or U46619 (a thromboxane

analogue), data not reported. The group did report a synergistic affect of the GNBT/T genotype and 196T allele to ADP, epinephrine and U46619.

Assessment by flow cytometry has also yielded conflicting results. Goodall et al.[181] described a significantly higher amount of bound fibrinogen, after ADP (but not thrombin) stimulation, to platelets from T allele carriers compared to non-carriers ($p<0.0001$). The result was independent of GPIIbIIIa receptor expression, plasma fibrinogen, platelet volume and count. In contrast, Meiklejohn et al.[189], in a study of healthy volunteers (35 C/C Vs. 35 C/T and T/T) found no significant differences between the groups for either baseline (0.98±0.62 Vs. 1.01±1.0%, $p=0.90$) or ADP stimulated (64.3±14.7 Vs. 62.2±15.3%, $p=0.60$) fibrinogen binding by flow cytometry. Huang et al.[188] studied 54 healthy Caucasians and also found no association between the C196T genotype and flow cytometric determined GPIIbIIIa expression or platelet activation quantified by p-selectin, ($p = 0.54$ and 0.89 respectively).

In a study of acute stroke patients (n=609) and age and sex matched controls (n=435), Carter et al.[233] found no association between the C196T polymorphism and plasma levels of platelet factor 4 or β thromboglobulin, both markers of platelet activation. Bennett et al.[190] genotyped 100 healthy subjects and then carried out detailed platelet function tests in a small subset by genotype (C/C, C/T, T/T, n= 10, 11, 5 respectively). Although there were some significant results between the individual groups for maximum fibrinogen binding and the fibrinogen binding dissociation constant, overall these were not significant. The authors also found no genotype affect on TRAP induced aggregation or p-selectin expression (data not given). Neither did they find differences in occlusive thrombus formation on the PFA100 analyser nor sensitivity to RWJ 53308, a GPIIbIIIa receptor antagonist, between the genotype groups. Although this study was comprehensive, the small number of subjects analysed would make it significantly underpowered, especially as most platelet function tests display large inter and intra individual variation. The same criticism applies to the small study by Cadroy et al.[191] comparing collagen induced thrombus formation at normal ($650s^{-1}$) and high ($2600s^{-1}$) shear rates. They found no statistical difference between the 21 C/C and 19 C/T genotype subjects (platelet deposition, normal shear = 0.97 ± 0.42 and 0.94 ± 0.58 x $10^{-7}/cm^2$, $p>0.05$; high shear = 4.08±1.77 and 4.23±1.82 x$10^{-7}/cm^2$, $p>0.05$ respectively).

The final group of functional studies have all assessed whether the C196T genotype influences platelet sensitivity to platelet antagonists, mainly aspirin. Cooke et al.[184] compared 15 C/C to 11C/T genotypes by platelet aggregation (epinephrine±ADP). They found that the mean aspirin concentration required to induce 50% inhibition of the aggregation response (IC_{50}) was significantly greater in the C/C subjects compared to the C/T heterozygotes (22.8±5.8 and 2.3±1.2 μmol/l respectively, p=0.005), indicating increased aspirin sensitivity in the T allele carriers. A more comprehensive study by the same group, Michelson et al.[183], was conducted in 56 healthy subjects (20 C/C, 20 C/T and 16 T/T) and found that the C/T heterozygotes were the most sensitive to aspirin and abciximab (a GPIIbIIIa antagonist) but that the A and T allele homozygotes were actually similar (IC_{50} for aspirin C/C Vs. C/T Vs. T/T = 13.1±3.7, 7.4±2.5 and 14.0±2.1 overall p=0.024; IC_{50} for abciximab C/C Vs. C/T Vs. T/T = 2.27±0.19, 1.90±0.21 and 2.13±0.14, overall p=0.099). In the same study there were significant T allele influences on resting p-selectin expression (p=0.01) and low dose ADP stimulation for GPIIbIIIa activation (p=0.005) and fibrinogen binding (p=0.01), indicating platelet hyper reactivity associated with the T allele. However, the aspirin and abciximab results are difficult to explain with genotype mediated molecular mechanisms. Undas et al.[185] quantified thrombin generation by plasma prothrombin fragment measurements after bleeding time wounds, before and after 7 days of aspirin, in 25 C/C and 15 T allele carriers. They found no significant differences in the reduction of thrombin generation after aspirin between the groups (C/C 14.6± 10.0 to 5.1± 3.1x10^{-7}, 68%; T allele carriers 12.6± 7.9 to 8.8± 7.61x10^{-7}, 28%). Finally, Andrioli et al.[192] found lower platelet responses in T allele carriers after arachidonic acid and thromboxane A_2 analogue (U46619) stimulation both for adhesion to fibrinogen (C/C Vs C/T and T/T = 18.5±1.4 Vs. 13.5±2.9, p<0.003 for arachidonic acid and 18.3±1.7 Vs. 16.0±1.2, p<0.005 for U46619) and aggregation (C/C Vs C/T and T/T = 65.2±12.7 Vs. 28.4±17.2, p<0.01 for arachidonic acid and 68.8±9.4 Vs. 37.9±23.3, p<0.01 for U46619). They went on to show that the amount of aspirin required to reduce arachidonic acid stimulated aggregation by 50% (IC_{50}) was significantly less in the T allele carriers (C/C Vs. C/T and T/T = 23.4±3.3 Vs. 2.7±0.6 μmol/l , p<0.005). These results found the T allele to be associated with decreased platelet response to arachidonic acid and thromboxane A_2 analogue as well as increased sensitivity to aspirin.

In this study of 200 healthy and 205 premature MI survivors, the GPIIbIIIa C196T genotype did not influence (i) resting platelet GPIIbIIIa receptor expression; (ii) ADP or TRAP stimulated receptor expression; (iii) platelet fibrinogen binding to the receptor at rest or (iv) platelet fibrinogen binding with stimulation with low, intermediate and high concentrations of ADP and TRAP. The PRAMIS study represents the largest analysis of the influence of the C196T polymorphism on platelet function in MI survivors. Only one larger functional study of this polymorphism exists, and this analysed platelet aggregation responses in healthy subjects only[182]. Previous functional studies have primarily been done in young healthy adults with low cardiovascular risk and in relatively small numbers of subjects[181,183-191]. While one may argue that a significant genotype effect should be evident in such 'clean' populations, in reality such individuals do not match the phenotype of the clinically relevant 'at risk' group (premature MI). Indeed, it may be interaction of the genotype affect with a number of intermediate cardiovascular risk factors (smoking, hypercholesteraemia, diabetes, hypertension) that may enhance genetic influences leading to clinically detectable events. The studies to date have analysed platelet function by a variety of methods (aggregometry, plasma activation markers, flow cytometry, thrombin formation, PFA 100 analyser and sensitivities to anti-platelet agents), which makes comparison of studies difficult as they each analyse different aspects of platelet function. Even in the previous flow cytometry based studies[181,187-189], only one of these[188] assessed the influence of the C196T polymorphism on receptor expression, but only in resting samples rather than resting and in response to ADP and TRAP as in the PRAMIS study. Overall, the results reported to date have been contradictory. A possible explanation for some of these studies may be that the negative results in such small cohorts simply indicate low power.

The precise mechanism by which a single amino acid substitution at position 33 of GPIIIa might influence receptor function remains unclear. However, the fact that the substitution is responsible for the formation of an alloantigen indicates that a significant conformational change occurs[234] and this may alter receptor affinity or inside-out and outside-in signalling. This is the most favoured hypothesis in trying to explain the increased platelet reactivity reported in various studies with T allele carrier status, either through enhanced affinity of the GPIIbIIIa receptor to fibrinogen and/or differences in

post agonist receptor or pre GPIIbIIIa receptor signalling pathways. This hypothesis was directly studied by Vijayan et al.[235] in Chinese hamster ovary and human kidney embryonal 293 cells overexpressing either the C or T allele polymorphic forms of IIbIIIa. They found that the cells overexpressing the T allele polymorphism exhibited greater adhesion to immobilised fibrinogen, greater spreading and actin cytoskeleton rearrangement, and greater clot retraction. After specific analysis of cell adhesion signalling pathways, they concluded that the influences appeared to depend on a signalling mechanism sensitive to receptor occupancy and that the polymorphism affect was through outside in signalling.

It cannot be ruled out that the C196T polymorphism is linked to another yet unidentified genetic marker, which is the true risk determinant, or to other platelet disorders. Also, other cellular sources apart from platelets may be affected, as the IIIa subunit is part of the vitronectin receptor expressed on endothelial cells and smooth muscle cells and therefore highly involved in angiogenesis and cardiovascular pathophysiology.

In conclusion, the C196T polymorphism and its functional consequences for the platelet have been studied using a wide variety of approaches that are difficult to compare and have given conflicting results. The majority of these studies have been small and in young healthy cohorts, an entirely different group to the pro-thrombotic high-risk subjects reported in the positive association studies that have proposed the polymorphism as an independent risk factor. The PRAMIS study has addressed a number of these issues and found no significant differences related to the polymorphism in either flow cytometry detected receptor expression or platelet fibrinogen binding at rest and after ADP and TRAP stimulation.

5.5.2.2 Association studies

The C196T (leu33pro, PlA1/PlA2) polymorphism has been one of the most studied genetic cardiovascular risk factors to date. Studies have used a wide variety of phenotype to assess the association of this polymorphism with cardiovascular risk. These include history of or extent of coronary artery disease[170,171,177,236-240], stroke[241], MI[159,163-173,175,242] and complications following percutaneous and surgical

intervention[176-179]. For more accurate comparison to the PRAMIS cohort, and to keep the discussion to a manageable length only studies with MI phenotype and populations greater than 100 cases (apart from the original paper by Weiss et al.) will be reviewed. Where possible, data for younger aged sub-populations are considered. An odds ratio graph encompassing the retrospective and prospective studies is shown in Figure 30.

Weiss et al.[163] published the first GPIIbIIIa polymorphism association study and found the T allele to be associated with MI, odds ratio = 2.8(1.2-6.4), $p < 0.05$. However, this was in a very small population size and there was an unusually low percentage of T allele carriers in the controls (T allele frequency in controls = 0.10, most studies give frequencies between 0.12-0.15). In a slightly larger study of 156 MI cases and 216 controls, Carter et al.[166] found a modest increase in risk of MI (odds ratio = 1.66 (1.15-2.39), $p = 0.007$) and this was further increased in the small subset (24 cases, 45 controls) of under 47 year olds (odds ratio = 2.30 (1.01-5.22), $p = 0.05$). In a study of 104 MI and 164 controls, Marian et al.[164] could not reproduce the association of MI with the T allele (odds ratio 1.5(0.9-2.6), $p = 0.13$). Little detail was given of the age of the subjects, and the analysis of those < 60years (77 cases and 90 controls) was also not significant (odds ratio = 1.7(0.8-3.4), $p = 0.17$). A much larger negative study (619 cases and 699 controls) was reported on behalf of the ECTIM group, by Herrmann et al.[165]. They found no increase in T allele frequency in the MI cases compared with controls (0.16 Vs. 0.15) and this was also the case for sub analyses of subjects under 55 (0.15 Vs. 0.15) and under 45 (0.17 Vs. 0.14). Zotz et al.[167] compared 298 men who had undergone angiography by dividing them into history of MI (124), CHD only (83) and controls (91). There was no increased T allele frequency between the MI and control group, either as a whole (0.9(0.7-1.4), $p = 0.75$) or the sub-group of less than 60 years (1.3(0.9-2.0), $p = 0.19$). Some positive associations were teased out in sub-set analyses of recent MI history and comparing MI to CHD subjects less than 60 years but the group numbers were very small.

The study by Scaglione et al.[168] is worth mentioning (98 cases and 98 controls) due to the very young cohort, all MIs (67% q wave and 33% non q wave MI) under 45 years, mean age 40±4. Despite this extreme phenotype, there was no increased risk of MI demonstrated, odds ratio = 0.8(0.4-1.4), $p > 0.05$. However, an equally young cohort,

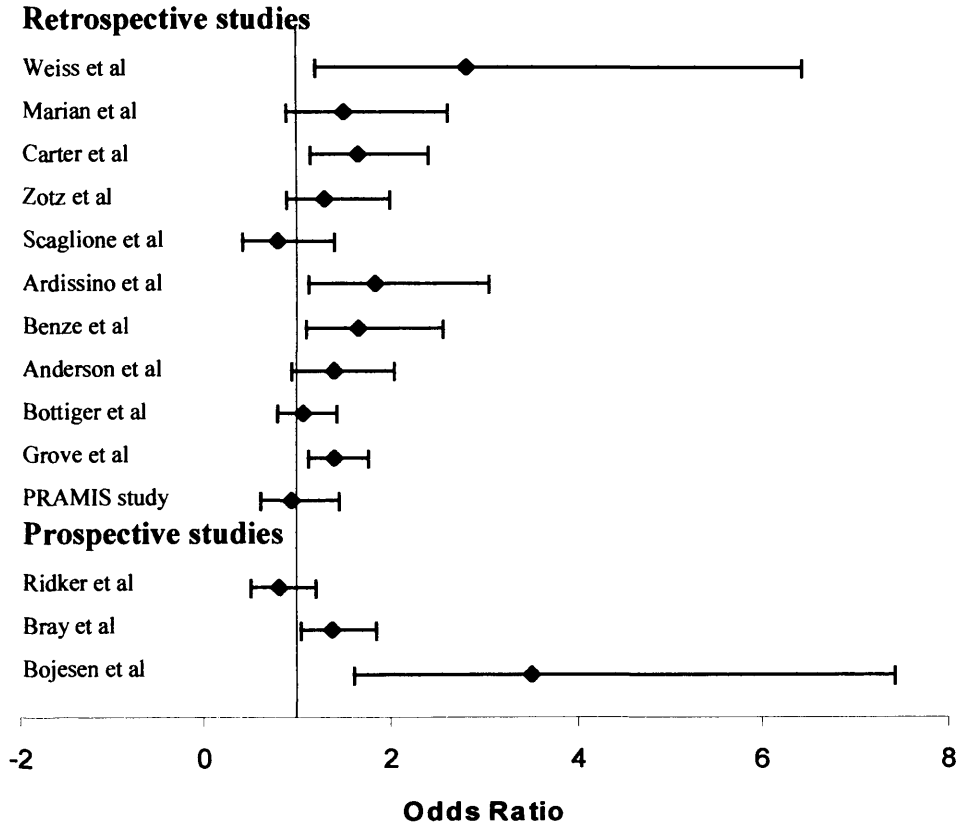
reported by Ardissino et al.[169] of 200 MI (mean age 40.7±4.1) and 200 matched controls did produce a significant positive association, odds ratio = 1.84(1.12-3.03), $p < 0.05$. When the effect of T allele was assessed for interaction with smoking the risk of MI increased further, odds ratio = 2.03(1.04-4.01), $p < 0.05$. A third cohort of very young premature MI subjects was described by Benze et al.[159], 287 men with MI under the age of 45 and 138 healthy controls. This study also showed a positive T allele association with MI (odds ratio = 1.65(1.09-2.54), $p = 0.01$), however, this did not remain significant once a logistic regression analysis was performed to include other cardiovascular risk factors.

Anderson et al.[170], in 225 MI subjects and 276 controls, found no association with the T allele and MI, odds ratio = 1.39(0.95-2.04), $p = 0.09$. Similarly, neither did Bottiger et al.[171] in a study of 793 MI and 340 controls, odds ratio = 1.06(0.79-1.42), $p > 0.05$. In the study described by Boekholdt et al.[242], in 455 angiographically proven CHD, the T allele did not correlate to MI risk, odds ratio = 0.81, $p>0.05$. However, there was a significant interaction with the plasma fibrinogen levels and the C196T polymorphism ($p=0.002$), with the T allele together with high plasma fibrinogen leading to increased event rate, relative risk = 2.7(1.1-7.1), $p = 0.03$. Finally, in a recent published study by Grove et al.[172], which included 1019 angiographic CHD (529 MI) and 1191 controls, the T allele was significantly associated with MI in comparisons with the control subjects (odds ratio = 1.40(1.13-1.75), $p = 0.002$) and CHD subjects without MI (odds ratio = 1.4(1.1-1.8), $p = 0.014$).

Ridker et al.[173] used the Physicians Health Study participants (14916 subjects with 8.6 years follow up) to compare the 374 first MI men to 704 matched controls. They did not find an increased T allele frequency in the cases and there was no significant correlation to MI (odds ratio = 0.93(0.7-1.2), $p = 0.40$). Analysing only subjects under the age of 60 did not affect the results (odds ratio = 0.81(0.5-1.2), $p = 0.30$). Bray et al.[174] adopted a nested case-control approach to prospectively assess the risk of recurrent event (cardiovascular death or MI) with the T allele in 385 cases and 382 controls and found an odds ratio of 1.38(1.04-1.83), $p = 0.028$. The subjects were all participants of the Cholesterol and Recurrent Events study (CARE study, 4159 participants randomised to pravastatin or placebo for 5 years), and the positive association only held for the

placebo group, as there was no significant increased risk in the active pravastatin arm. Bojesen et al.[175] published the largest study on the C196T polymorphism to date, using participants of the Copenhagen city heart study (n=9149). This was the first study powerful enough to assess the risk of T allele homozygotes (n=110 men, 134 women) with the risk of MI. Significant results were obtained in the men only, relative risk of MI for T/T genotype compared to C/C = 5.4(1.6-19), $p<0.05$ for under 40 age group, 3.5(1.6-7.5), $p<0.05$ for 40-50 group and 1.1(0.8-1.5), $p>0.05$ for above 50 age group. No significant results were obtained in the heterozygotes.

Figure 30 ***Odds ratio of risk of MI related to the T allele of the GPIIbIIIa C196T polymorphism***



For study details and discussion see text

Thrombosis is a major adverse complication of percutaneous intervention. Therefore, the setting provides the ideal opportunity to assess potential pro-thrombotic risk factors. Walter et al.[176] found a significantly increased 30 day risk of stent thrombosis associated with the T allele (odds ratio 5.26(1.55-17.85), in 318 consecutive patients undergoing percutaneous intervention. However, in a much larger study (653 cases-271 PTCA, 102 atherectomy, 280 stents) reported by Laule et al.[177] there was no increase in the composite endpoint (target vessel revascularisation, MI, death) associated with the T allele, odds ratio = 1.36(0.70-2.70), p = 0.37. Kastrati et al.[178] carried out a detailed study of 1150 subjects with coronary stent placement with 6 month angiogram follow up and demonstrated a significantly higher restenosis rate in the T allele carriers, odds ratio = 1.42(1.09-1.84), p = 0.009.

In the setting of coronary artery bypass grafting Zotz et al.[179] reported an increased risk of postoperative complications (graft occlusion, MI, death) at 1 year in 261 consecutive patients with the T allele, odds ratio = 4.73 (1.3-17.4), p = 0.011.

Two large meta-analyses have been published to date. Both try to compare the large number of association studies and perform statistical analyses on the published data to form a consensus opinion as to the role of the C196T polymorphism.

The first published meta-analysis, by Di Castelnuovo et al.[243] in 2001, compared data from 34 studies for coronary artery disease and 6 for restenosis after revascularisation. This culminated to a total of 9095 cases and 12508 controls. The overall odds ratio for 196T allele carriers for coronary artery disease was 1.10(1.03-1.18) and it was 1.21(1.05-1.38) in subjects younger than 60 (1695 cases and 2276 controls). Analysis of the MI phenotype studies (4384 cases and 5244 controls) gave an odds ratio of 1.09(0.97-1.22), which was not significant. Unfortunately, no data on MI phenotype in subjects less than 60 years of age was given. There were also odds ratio calculations for the T/T genotype (170 cases and 173 controls) in comparison with C/T (1874 and 1735) or C/C (5104 and 5220), giving odds ratio of 0.89(0.70-1.13) and 0.97(0.77-1.22) respectively for risk of coronary artery disease. For revascularisation procedures the overall odds ratio was 1.31(1.10-1.56). This meta-analysis concluded that a significant but weak association exists between the 196T allele and coronary artery disease but that the meta-analysis was affected by publication bias and inadequate co-factor adjustment.

A second meta-analysis carried out by Burr et al.[244] was published in 2003 and gave detailed descriptions of various statistical models employed to carry out such comparisons. This paper was written in early 2001 and only analysed 12 association studies (all included in the Di Castelnuovo paper) giving a total of 3400 cases and 3500 controls. Again, the authors commented on the heterogeneity of the studies for various parameters including phenotype (MI or CAD), ethnic distribution, age, sex and health of the control population. The overall conclusions stated the odds ratio for CAD with the 196T allele was 1.22(CI not given), $p = 0.049$ and for subjects under the age of 60 the odds ratio was 1.28 but this was non-significant, $p = 0.17$. The MI phenotype was not analysed separately.

The PRAMIS study did not find the 196T allele to be a significant risk factor for MI in a group of 205 young subjects all of whom had suffered an MI under the age of 50, despite the study being powerful enough to detect a 1.5 fold risk increase. There have been 3 previous studies that have also used such an extreme phenotype, Scaglione et al.[168] (OR = 0.8(0.4-1.4), $p > 0.05$), Ardissino et al.[169] (OR = 1.84(1.12-3.03), $p < 0.05$) and Benze et al.[159] (OR = 1.65(1.09-2.54), $p = 0.01$), however, this did not remain significant once a logistic regression analysis was performed to include other cardiovascular risk factors. These studies in themselves or in combination with the other 11 studies represented in Figure 30 highlight the variation of results from the association studies to date. The two meta-analyses were based more on the CAD phenotype and concluded significant but weak association with the 196T allele. Neither of these analyses looked at the MI phenotype in the under 60 age group.

A number of molecular mechanisms have been proposed, related to the effects of the C196T polymorphism on platelet function, and its association with increased MI or CAD or intervention procedural risk of thrombosis (Section 5.6.2.1). These results remain inconsistent and the PRAMIS study was unable to support the increased platelet reactivity hypothesis.

Our data does not suggest that the 196T allele is a significant risk factor for myocardial infarction. Two large previous meta-analyses have reported a significant but small increase in CAD risk of the magnitude of 10-20%. When considering the global disease

burden of CAD in terms of mortality and morbidity, this increased risk cannot be ignored, especially taken in conjunction with its multi-factorial aetiology. In particular, if the T allele does prove to be implicated as a risk factor it could be used in primary prevention strategies, by targeting increased anti-platelet medications, not just for CAD or MI but also percutaneous intervention and bypass grafting.

Chapter 6

Emerging Risk Factors

Not all the risk of coronary artery disease is explained by conventional risk factors such as diabetes, smoking, hypertension and hyperlipidaemia[245]. Recent advances in molecular techniques and a more in-depth understanding of the pathophysiological processes involved have led to the proposal of a number of emerging/additional risk factors which may influence coronary risk[246]. These include genetic mediated polymorphisms (Chapter 2, table 10), factors influencing thrombosis (fibrinogen, homocysteine, vitamin B12) and factors affecting atherosclerosis either through dyslipidaemia (Lp(a)) or inflammation (C-RP).

This chapter analyses the assessment of a number of these proposed risk factors in the 200 controls and 205 cases collected in PRAMIS. These include fibrinogen, homocysteine, lipoprotein(a) (Lp(a)) and C-reactive protein (C-RP).

6.1. Homocysteine

The results of plasma homocysteine (and fibrinogen, , C-RP and Lp(a)) levels are shown in Table 27. The Kolmogorov-Smirnov (K-S) test confirmed a non-normal distribution in all the tests and that transformation (the log value to base 10) was able to give normal distributions with the homocysteine and C-RP results. Subsequent analyses for these parameters were carried out using transformed data, which was back transformed appropriately to give meaningful numerical values. The raw fibrinogen data was not transformed as the K-S test was only weakly significant (p=0.019), however, to confirm validity all results were analysed using parametric and non-parametric tests. Non-parametric analysis was used for the Lp(a) data as transformation to a normal distribution was not possible.

Table 27 (a) ***Homocysteine, Fibrinogen, C-RP and Lp(a) levels in the controls***

	Raw data		Transformed Data	
	Mean ± SD (range)	**K-S test for normal distribution**	**Mean (95% CI)**	**K-S test for normal distribution**
Homocysteine (μmol/l)	11.9±4.0(5.1-36.3)	Z=1.88,p=0.002	11.4(11.0-11.9)	Z=0.96,p=0.317
Fibrinogen (g/l)	2.84±0.53(1.4-5.1)	Z=1.53,p=0.019	-	-
LP(a)	132(46-388)*	Z=3.53,p<0.001	-	-
C-RP (mg/dl)	2.1±2.2(0.1-16.3)	Z=2.85,p<0.001	1.35(1.19-1.53)	Z=0.60,p=0.859

* unable to transform LP(a) data to fit normal distribution, median (inter-quartile range) given

Table 27 (b) ***Homocysteine, Fibrinogen, C-RP and Lp(a) levels in the controls and cases***

	Controls	Cases	p-value
	Mean (95% CI)	**Mean (95% CI)**	
Homocysteine (μmol/l)	11.4 (11.0-11.9)	11.6 (11.2-12.2)	0.497
Fibrinogen (g/l)	2.84 (2.75-2.92)	3.15 (3.04-3.25)	<0.001
LP(a) (mg/l)	69(46-93)*	132(46-388)*	0.003
C-RP (mg/dl)	1.35 (1.19-1.53)	1.25 (1.09-1.44)	0.440

* unable to transform LP(a) data to fit normal distribution, median (inter-quartile range) given

High plasma homocysteine levels have previously been associated with increased risk of coronary artery disease and myocardial infarction[241,247-249]. An extreme example of this is the clinical manifestations of homocystinuria, an autosomal recessive metabolic condition, predisposing to both arterial and venous thrombosis[221]. Moreover, low levels of vitamin B_{12} and folate in the plasma (through low dietary intake) leads to higher homocysteine levels. This mechanism of increased MI risk is particularly attractive for primary and secondary risk prevention strategies as it is amenable to dietary supplementation. Finally, two polymorphisms have been proposed as risk factors for MI through decreased homocysteine metabolism associated with mutant alleles leading to increased plasma homocysteine levels. These are the methyltetrahydrofolate reductase (MTFHR) C677T polymorphism and the cystathione β synthase (CBS) ile278thr polymorphism.

In PRAMIS, there was no statistical difference in the plasma homocysteine levels between the controls and cases (Table 27(b)), or the plasma vitamin B_{12} (353±95 and 364±101 ng/l respectively, p = 0.258). The cases had a significantly lower plasma folate compared to the controls (9.4±4.7 Vs. 11.3±4.6μg/l, p = <0.001). There was a significant negative correlation between homocysteine and both folate and vitamin B_{12}, i.e. low folate and vitamin B_{12} levels were associated with high plasma homocysteine, in both the controls (Pearson correlation = –0.374 for folate and -0.282 for B_{12}, p<0.001) and cases (Pearson correlation = -0.343, p<0.001 for folate and -0.197, p = 0.005 for B_{12}). There was no significant correlation between plasma homocysteine level and any of the platelet function parameters analysed in either of the groups (data not shown).

Two polymorphisms thought to modulate plasma homocysteine levels were genotyped, the MTFHR C677T and CBS ile278thr. A summary of the findings is given in Table 28. There was no significant difference in the genotype distribution of the MTFHR (Chi squared = 0.739, p = 0.691) or CBS (Chi squared = 0.729, p = 0.393) polymorphisms between the controls and cases. The MTFHR C677T polymorphism did not affect plasma homocysteine levels in the controls or cases. The CBS polymorphism heterozygotes demonstrated a significantly lower plasma homocysteine compared to the wild type homozygotes in the cases but not the controls (Table 28).

Table 28 ***The MTFHR and CBS polymorphisms and effect on plasma homocysteine***

MTFHR genotype	Controls (μmol/l)	Cases (μmol/l)
C/C	11.17 (10.57-11.78) n=94	11.64 (10.86-12.47) n=88
C/T	11.56 (10.76-12.30) n=81	11.35 (10.64-12.11) n=90
T/T	11.91 (10.20-13.90) n=23	12.74 (11.17-14.45) n=27
Overall p-value	0.571	0.262
CBS genotype	**Controls**	**Cases**
ile/ile	11.56 (11.03-12.11) n=153	11.94 (11.37-12.58) n=164
ile/thr	10.94 (9.97-11.99) n=45	10.54 (9.61-11.58) n=41
thr/thr	- n=0	- n=0
Overall p-value	0.380	0.025

Discussion

Homocysteine, a thiol containing amino acid, is formed during the metabolism of methionine, an essential amino acid. Higher plasma homocysteine levels have been associated with aging, menopause, chronic renal insufficiency, low plasma levels of vitamin cofactors (B_6 B_{12} and folate) and cardiac transplantation[250]. A meta-analysis of 27 studies indicated that increased homocysteine levels (>15 μmol/L) are associated with an increased risk of CAD, peripheral arterial disease, stroke and venous thromboembolism[251]. The mechanism of this increased risk is thought to be through increased vascular damage mediated by high homocysteine levels including endothelial damage, mitogenic effect on smooth muscle cells, enhancement of coagulation factors and platelet activation[250]. Although the PRAMIS study did not find an increased level of homocysteine in the cases compared to the controls or a correlation between levels and platelet function, a number of important regulatory factors affecting plasma homocysteine levels were highlighted by the results. Both low plasma vitamin B_{12} and folate correlated with higher homocysteine levels in the controls and in the cases.

Individuals heterozygous for the CBS polymorphism had lower homocysteine levels, a finding that is in contrast to previous associations with this polymorphism, which have

found increased plasma homocysteine in homozygotes of the mutant allele (no thr/thr homozygotes were genotyped in the PRAMIS cohort)[252].

Discrepancy of these results from previous studies may have a number of explanations. The sample size of the PRAMIS study was relatively small and therefore may have been inadequately powered. In the UK dietary fortification of cereals and flour already exists and may have masked any genetic influences. In this study plasma homocysteine levels were determined by HPLC using a commercially available kit (Chapter 2) on fasting morning samples. This is a single measurement of a fluctuating biochemical protein and alternative methods include measurement after loading doses of methionine, which may more accurately assess the metabolic capacity of an individual and possibly be a more appropriate phenotype to assess the influence of the polymorphisms considered.

6.2 Fibrinogen

Fibrinogen has an essential role in thrombosis, as it is the main substrate for fibrin laden clot formation. A raised plasma level of fibrinogen has been established as an independent risk factor for acute coronary events[141,144,218]. More recently the association between plasma fibrinogen level and inflammation, as it is an acute phase protein, has highlighted another potential causal mechanism for promoting atherosclerosis and thrombosis[253,254].

The fibrinogen gene polymorphism G455A has been proposed as a risk factor for MI by increasing plasma fibrinogen levels in association with the A allele.

There was a significantly higher plasma fibrinogen level in the cases compared to the controls (3.15 vs. 2.84 g/l, $p<0.001$). In total there were 6 significant predictors of plasma fibrinogen level when the entire cohort was analysed by stepwise regression analysis. These were C-RP, gender, case control status, homocysteine, platelet count and BMI (standardized β coefficients = 0.370, 0.199, 0.158, 0.139, 0.131, 0.099 respectively, p values = 0.001, 0.001, 0.001, 0.003, 0.007 and 0.048 respectively). Therefore, in the analysis of the entire cohort females had higher fibrinogen levels compared to the males (3.32 vs. 2.94 g/l, $p<0.001$) and current smokers did not have significantly higher fibrinogen levels compared to non-current smokers (3.13 vs. 2.96, p

= 0.059). Plasma fibrinogen levels demonstrated a weak but significant negative correlation with platelet fibrinogen binding with ADP and TRAP (see chapter 4 for details and discussion of results).

In the PRAMIS study, there was no difference of plasma fibrinogen levels when analysed by the fibrinogen G455A polymorphism genotype status in the entire cohort or in the cases (Table 29). In the control group there was a significantly higher fibrinogen level in G/A heterozygotes compared to G/G homozygotes (p = 0.011), suggesting a dominant effect. The A/A homozygotes displayed a similar fibrinogen level to the G/G group, but the small size of this group (n = 7) may have affected this result. Indeed, when the G/G homozygotes were compared to the combined G/A and A/A groups the result became more significant (fibrinogen level = 2.75 vs. 3.00 g/l respectively, p = 0.006). There were no differences between the genotype groups in the control, case or combined analyses for C-RP, gender, homocysteine, platelet count and BMI (data not shown). There was no difference in the genotype distribution of the G455A polymorphism between the cases and controls, Chi squared = 0.033, p = 0.983.

Table 29 ***The fibrinogen G455A polymorphism and effect on plasma fibrinogen***

G455A genotype	Controls	Cases	Combined
G/G	2.75 ± 0.56 g/l	3.17 ± 0.79 g/l	2.96 ± 0.71 g/l
	n = 124	n = 127	n = 251
G/A	3.02 ± 0.67 g/l	3.10 ± 0.66 g/l	3.06 ± 0.66 g/l
	n = 63	n = 63	n = 126
A/A	2.77 ± 0.60 g/l	3.16 ± 0.71 g/l	2.93 ± 0.65 g/l
	n = 7	n = 5	n = 12
Overall p-value	0.014	0.798	0.436

Discussion

The fibrinogen molecule plays a central role in thrombosis and plasma levels have been shown to correlate with thrombotic and CAD risk[144,218]. The PRAMIS study results support these previous observations, as the cases had a significantly higher fibrinogen

level compared to the controls. The plasma fibrinogen levels in the cases may also have a prognostic value as previous studies have correlated them to future cardiovascular events[141,142].

The mechanism of increased risk is thought to be through increased plasma viscosity, enhanced platelet aggregation and stimulation of smooth muscle proliferation[250]. Our study does not support increased plasma fibrinogen being associated with increased platelet reactivity. However, there was a strong correlation between plasma fibrinogen and C-RP levels in the combined and separate analyses, indicating the influence of inflammation on these molecules, as both are known to be hepatic acute phase reactants[141]. This correlation was particularly striking in the control population, standardized β coefficients = 0.546, none of who were on treatments which may effect inflammation (aspirin or statins), and therefore dampen the association.

It has been estimated that 30-50% of the variability of fibrinogen level is genetically mediated[255]. The 455A allele of the fibrinogen β chain G455A polymorphism is thought to increase basal rates of gene transcription and has been associated with increased fibrinogen plasma concentrations[256] in the order of 10%. The PRAMIS data supported this influence in the control population, however the cases did not demonstrate this effect nor was there a difference in the genotype distribution between the groups. Again various medications in the cases may have effected plasma fibrinogen levels or contributed to the lack of genotype influence. Previous case-control studies have mainly been negative and the role of the 455A allele as a MI risk factor remains controversial[257].

6.3 Lipoprotein (a)

Lipoprotein(a) (Lp(a)) refers to a family of lipoprotein particles similar to LDL in core lipid composition and in having apo B-100 as a surface apolipoprotein and a unique glycoprotein, apo(a), which is bound to apo B-100. Lp(a) has been proposed as a risk factor for atherosclerosis and thrombosis mediated through its LDL-like properties and its structural homology to plasminogen interfering with fibrinolysis[258] . Plasma levels vary widely among persons but are stable among individuals, suggesting a strong

heritable component[259]. Two polymorphisms within the apolipoprotein gene have been described (C93T and G121A) which may alter Lp(a) levels and therefore modify MI risk[260]. The methods used to quantify Lp(a) levels are described in Chapter 2.

The distribution of Lp(a) in the controls and cases was not normal (Kolmogorov-Smirnov test) and skewed to the right. As transformation of the data to a normal distribution was not possible, non-parametric tests (Chi square and Man whitney U test) were used for all the analyses. Lp(a) levels were significantly higher in the cases compared to the controls (median(inter-quartile range) = 132(46-388)mg/l vs. 69(46-93)mg/l respectively; p=0.003). Stepwise regression analysis of the entire cohort found 4 significant influencing factors of Lp(a); case-control status, cholesterol, triglyceride and fibrinogen (standardized β coefficients = 0.162, -0.188, 0.120 and 0.107; p-values = 0.004, 0.002, 0.038 and 0.049 respectively). However, on direct analysis, Lp(a) did not significantly correlate with cholesterol, LDL, HDL or triglyceride levels (data not shown) and there was a weak correlation with fibrinogen (Pearson correlation = 0.110, p = 0.038). In the cases there was no significant difference in the Lp(a) levels of subjects taking or not taking statin treatment, 130(46-375) mg/l vs. 140(46-429)mg/l respectively(p=0.634).

The polymorphism effect on Lp(a) levels are represented in Table 30. In the group as a whole, the T allele of the C93T polymorphism was associated with a significantly lower Lp(a) level acting in a dominant fashion (C/C vs. C/T and T/T = 108(46-390)mg/l vs. 79(46-167)mg/l, p=0.044). There was no significant difference between the genotype groups for fibrinogen (C/C vs. C/T and T/T = 3.00 vs. 2.97 g/l, p = 0.68), cholesterol (C/C vs. C/T and T/T = 5.12 vs. 4.94 mmol/l, p = 0.16) or triglyceride (C/C vs. C/T and T/T = 1.75 vs. 1.64 mmol/l, p = 0.30). A similar trend was observed in controls and cases but did not reach statistical significance (C/C vs. C/T and T/T = 84(46-236) mg/l vs. 63(46-126) mg/l, p=0.115 and 149(46-465) mg/l vs. 105(52-214) mg/l, p=0.100 respectively). There was no significant difference in the genotype distribution between the groups (C/C, C/T, T/T = 145,51,5 in cases and 123, 45, 2 in controls, Chi squared = 1.46, p=0.481). The G121A polymorphism did not influence Lp(a) levels and there was no difference in the genotype distribution between the controls and the cases (G/G,

G/A, A/A = 113, 50, 7 in the controls and 140, 54, 7 in the cases, Chi squared = 0.096, p = 0.953).

Table 30 ***Influence of the apolipoprotein A C93T and G121A polymorphisms on LP(a) levels***

	Apolipoprotein A C93T polymorphism			
	C/C	C/T	T/T	p-value
Controls	84(46-236) mg/l (n=123)	63(46-126) mg/l (n=45)	(n=2)	0.115
Cases	149(46-465) mg/l (n=145)	105(52-214) mg/l (n=51)	(n=5)	0.100
Overall	108(46-390) mg/l (n=268)	79(46-167) mg/l (n=96)	(n=7)	0.044

	Apolipoprotein A G121A polymorphism			
	G/G	G/A	A/A	p-value
Controls	68(46-250) mg/l (n=113)	81(46-145) mg/l (n=50)	(n=7)	0.601
Cases	126(46-364) mg/l (n=140)	140(46-463) mg/l (n=54)	(n=7)	0.745
Overall	95(46-338) mg/l (n=253)	107(46-263) mg/l (n=104)	(n=14)	0.726

Median values (inter-quartile range) given

Discussion

The above results support the hypothesis of Lp(a) predisposing to MI as the cases demonstrated a significantly higher level compared to the controls. Previous studies have demonstrated Lp(a) to be an independent risk factor for MI in both retrospective[261] and prospective studies[262,263]. A meta-analysis of almost 5500 CAD patients followed up for 10 years reported that individuals with Lp(a) levels in the top tertile had an approximately 70% increased risk of CAD compared to those in the bottom tertile (RR 1.76; 95% CI, 1.4-1.9)[264]. Of particular interest is the fact that none of the conventional lipid components (LDL, HDL and triglyceride) correlate with Lp(a) levels and that statin treatment in the cases has no effect on Lp(a) level either. Levels of Lp(a) therefore are independent of other lipid constituents and are not influenced by traditional anti-lipid treatment.

There also appears to be a significant genetic influence on Lp(a) levels. Three main types of genetic variation have been described to affect the apolipoprotein(a) gene: a

size polymorphism in the coding region for variable number of kringle IV repeats; a pentanucleotide TTTTA repeat polymorphism at position-1270 of the promoter; and several single base pair variations in the coding and non-coding regions of the gene[257]. The T allele from the C93T polymorphism associates with lower Lp(a) levels in the group as a whole, in a dominant manner. The separate analyses of the controls and cases gave similar trends although this did not reach significance.

The mechanisms involved by which Lp(a) may exert its increased MI risk include atherogenicity (LDL-like properties), thrombogenic properties (by impairing fibrinolysis), stimulation of vascular smooth muscle cells, enhanced expression of ICAM-1 in endothelial cells and inhibiting the activation of plasminogen[250]. Previous and above findings suggest that particularly in premature or familial coronary artery disease, LP(a) levels should be determined to allow testing and counseling of family members. Lp(a) is a particularly attractive genetic risk factor to target because although levels vary significantly between subjects there is little variation within individuals. Pharmacological therapies are currently limited to apheresis but higher levels should lead to aggressive lowering of LDL with which high levels of Lp(a) have a synergistic atherogenic effect.

6.4 C-reactive protein

C-reactive protein, an acute phase reactant, is a marker and regulator of inflammation[265,266]. The development of high sensitivity assays has allowed more accurate quantification of low-grade inflammation and increased levels have been associated with CAD as well as a predictor for cardiovascular events[267].

There was no significant difference between the C-RP levels in the controls and cases (1.35 vs. 1.25 mg/dl respectively, $p = 0.440$). However, there were significant correlations between C-RP and other markers of inflammation in the controls and cases, fibrinogen (described earlier) and the white cell count (Pearson correlation = 0.336 and 0.196, $p<0.001$ and 0.006 in controls and cases respectively). There were significantly lower C-RP levels in the females compared to the males (1.13 mg/dl vs. 1.44 mg/dl, $p = 0.012$) in the controls but not the cases (1.55 mg/dl vs. 1.21 mg/dl, $p = 0.217$). Current smoking status, a history of hypertension, diabetes and family history did not affect C-

RP levels in either the controls or cases. In the cases the statin treatment group (n = 158) had lower C-RP levels (1.18mg/dl) compared to the no statin treatment group (1.55mg/dl), however, this difference was not significant (p = 0.105). There were no significant strong correlations between C-RP and the platelet function parameters analysed (see chapter 4).

Discussion

Multiple prospective studies have demonstrated that high-sensitivity C-RP is a potent predictor of future cardiovascular events[268]. The American Heart Association has recently endorsed C-RP to be used in conjunction with lipid evaluation as part of global risk prediction[269]. C-RP is now thought to play a number of direct roles in atherothrombosis (enhance complement activation, oxidation and uptake of LDL by macrophages, tissue factor production) and is not merely a marker of inflammation[268].

In the PRAMIS study no significant difference was found between the controls and cases. The study may have been underpowered to detect such differences. Also, although study visits were only made if subjects denied acute illnesses in the previous 2 weeks, the range of C-RP levels (0.1-52.0 mg/dl) suggest a few subjects would have been suffering from acute illnesses and this may have influenced the results despite only 8 subjects in the entire cohort having C-RP levels greater than 10. However, even after eliminating such individuals from the analysis they remained non significant. Also, it must be appreciated that the cases were universally on aspirin and the majority on statins (80% of cases), both of which may affect C-RP levels by their anti-inflammatory properties[201 219 220] and help explain this discrepancy. The range of C-RP in the cases (0.2-52.0 mg/dl) could be used to assess further cardiovascular events prospectively.

Chapter 7

General discussion and future perspectives

7.1 General Discussion

Understanding the genetic aetiology of complex common diseases remains a challenge. A myocardial infarction is caused by the interaction of many acquired and genetic factors. A number of the risk factors are well established and include smoking, diet, abnormalities of lipid metabolism, elevated blood pressure and abnormalities of sugar metabolism. Some of these risk factors may themselves have genetic components. There is an increasing body of work addressing the role of haemostatic disorders in the development of arterial thrombosis. An acute myocardial infarction arises from two processes, atherosclerosis and thrombosis. Atherosclerosis is a disease of the vessel wall resulting in chronic changes in vessel wall cellular phenotypes occurring over many years. The thrombotic event is an acute process triggered by a plaque rupture and influenced by haemostatic factors, fibrinolytic factors and the reactivity of the platelet.

There is great interest in defining the genetic influences in arterial thrombotic disease. Knowledge of the genetic factors will help further define the mechanisms of disease and could ultimately assist in the rational design of selective prophylaxis and therapy. The PRAMIS study addressed potentially important genetic mediated haemostatic risk factors in the form of the platelet glycoprotein receptor polymorphisms.

The PRAMIS study recruited an extreme phenotype of premature MI subjects with the mean age of MI of only 42.3 years. The group demonstrated relatively less coronary burden (assessed by angiography results) compared to more elderly populations and showed a strong genetic predisposition, highlighted by a high proportion of positive family histories (Chapter 3).

The detailed platelet function analysis by flow cytometry (Chapter 4) demonstrated large inter-individual variations in both the controls and cases, and a high degree of correlation between the various platelet functional parameters assessed. Factors significantly influencing platelet function in the controls were MPV, gender, age, GPIaIIa expression, GPIaIIa G873A polymorphism, smoking status and fibrinogen whereas in the cases they were MPV, GPIaIIa expression, fibrinogen levels and the G873A polymorphism. Generally, the cases demonstrated lower platelet reactivity

compared to the controls and this may have been secondary to medications (particularly aspirin) or the disease status.

The GPIaIIa G873A genotype demonstrated a significant genotype effect on the GPIaIIa receptor expression in the controls and cases and accounted for 28 and 16% of the total receptor expression variation respectively (Chapter 5). This did not correlate with either an increase in the other platelet function parameters assessed or increased predisposition to MI. The GPIIbIIIa C196T polymorphism did not effect receptor expression, platelet reactivity or MI risk.

A number of emerging biochemical risk factors such as fibrinogen and lipoprotein(a) were significantly higher in the case group compared to the controls (Chapter 6). Genotyping of a number of polymorphisms thought to modulate MI risk revealed significant genotype phenotype effects on homocysteine, fibrinogen and lipoprotein(a).

The PRAMIS study results reinforce the fact that genetic mediated biological variation exists in many of the conventional as well as novel risk factors for MI. These results demonstrate some of the complex gene-gene and gene-environmental interactions involved in MI pathophysiology.

7.2 Additional studies

The PRAMIS resource has also been used to conduct three additional studies related to the initial investigations:

(i) The PRAMIS offspring study[270].

The case subjects recruited in the Pramis study represented an extreme pro-thrombotic phenotype, and given their young age at time of MI they also had an increased genetic predisposition. This was highlighted by 29.8% of the cases having a positive family history in a first-degree relative of an MI under the age of 50. By recruiting offspring of the PRAMIS cohort, this study was able to select young individuals (mean age 26.1±6.1 years) with a two generational family history of MI under the age of 60 (n = 20) for

comparison with controls (mean age 27.1±6.4 years) with no such history (n = 22). Despite neither group being on any medication nor having any diagnosis of CHD or MI, the cases demonstrated greater platelet reactivity in response to ADP, TRAP and collagen related peptide and had a greater capacity to generate thrombin compared to controls (Table 31). The results highlight inherent predisposition to thrombosis in such individuals at a young age and give insight into some of the mechanisms involved. These could potentially be targeted in future primary prevention strategies.

Table 31 ***Comparison of platelet function between healthy individuals with a two-generation history of myocardial infarction to healthy individuals with no history***

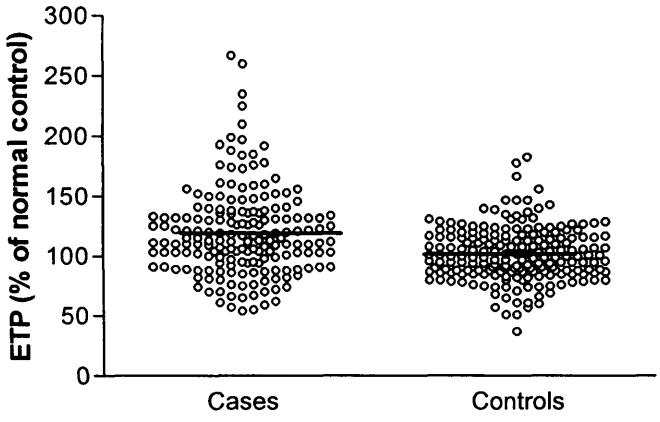
Platelet function	Controls	Cases	p-value
Fibrinogen binding % positive ADP 1 x 10^{-6}	57.2±2.8	67.6±2.5	0.008
Fibrinogen binding % positive TRAP 1 x 10^{-6}	66.6±3.4	78.4±2.2	0.007
Fibrinogen binding % positive collagen related peptide 1 x 10^{-6}	80.4±2.7	87.3±1.7	0.04
Fibrinogen binding % positive collagen related peptide 1 x 10^{-7}	38.7±5.1	52.5±4.1	0.04
P-selectin expression % positive ADP 1 x 10^{-6}	28.0±1.7	34.2±2.0	0.021
P-selectin expression % positive TRAP 1 x 10^{-6}	77.1±3.3	84.7±1.4	0.040
Thrombin generation assay (units of thrombin/minute)	29.8±1.7	39.5±2.4	0.002

(ii) The thrombin generation study[271].

Thrombosis results from a complex series of reactions involving platelets, the coagulation system and the fibrinolytic system. The coagulation system already has a number of factors proposed as independent risk factors for MI (factor V, factor VII, fibrinogen), however it is the overall thrombotic potential that is important. The thrombotic potential in an individual can be related to the overall ability of his/her plasma to generate thrombin (the endogenous thrombin potential or ETP). This study compared the overall ability to generate thrombin between the PRAMIS cases (n = 162) and controls (n = 186), and between the PRAMIS offspring cases (n =20) and controls (n = 22).

This study demonstrated significantly enhanced ETP in the PRAMIS cases compared to the controls (119.4±37.0 vs. 101.8±22.8%; p<0.001) (Figure 31), as well as the PRAMIS offspring study cases compared to the offspring controls (116.7±20.7 vs. 98.0±19.1% p=0.0042). Therefore, increased thrombotic potential may contribute to the primary and recurrent risk of premature MI.

Figure 31 ***Endogenous thrombin potential in cases and controls***



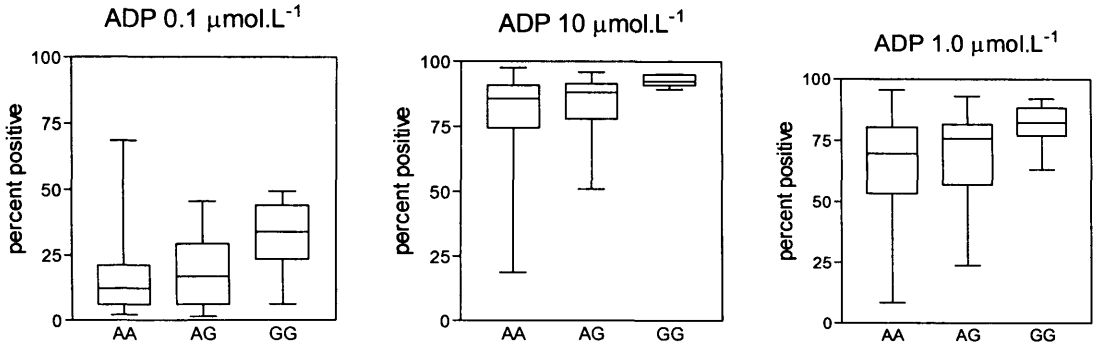
ETP is expressed as a percentage of the value for a standard, prepared using a 20-donor normal human pooled plasma, and arbitrarily assigned an ETP value of 100%.

(iii) The ADP receptor polymorphisms study[272].

The PRAMIS study has demonstrated there is marked inter-individual variation in platelet response to ADP and this was not explained by the GPIaIIa C873T or GPIIbIIIa C196T polymorphisms. The 200 PRAMIS control subjects and their detailed flow cytometric platelet function analysis was used to investigated whether genetic variation at the $P2RY_1$ and $P2RY_{12}$ ADP receptor loci affect platelet response to ADP. The study found a silent $P2RY_1$ gene polymorphism, A1622G, was associated with a significant effect on platelet ADP response. The G allele was associated with increased levels of platelet fibrinogen binding in response to all three concentrations of ADP stimulation

(Figure 32). Identification of this genotype effect partly explains the inter-individual variation in platelet response to ADP, and may have important clinical implications.

Figure 32 ***Fibrinogen binding to platelets following stimulation with 0.1, 1.0 and 10 μmol.L^{-1} ADP, in relation to the P2RY$_1$ 1622 genotypes (AA, n=147; AG, n=46; GG, n=7).***



p = 0.005 p = 0.020 p = 0.005

Data shown as box plots demonstrating medians, 25th and 75th percentiles, and range for each genotype

Gene Polymorphism	Genotype	Frequency n (%)	Mean platelet response (95% C.I.) to ADP (% positive for bound fibrinogen)		
			0.1 μmol.L^{-1}	1.0 μmol.L^{-1}	10 μmol.L^{-1}
P2RY1	AA	147 (73.5)	12.5 (10.8-14.5)	68.4 (65.0-71.7)	84.5 (82.6-86.3)
1622	AG	46 (23.0)	15.0 (11.5-19.4)	71.9 (66.3-76.9)	86.5 (83.5-89.1)
	GG	7 (3.5)	28.8 (16.7-45.0)	82.8 (74.8-88.8)	93.0 (90.4-94.6)
			p = 0.005	p = 0.020	p = 0.005
			Combined p value = 0.007		

7.3 Future perspectives

7.3.1 Platelet function studies

Platelets play a central role in MI pathophysiology and evidence already exists that increased platelet response or reactivity in previously healthy subjects is a risk for future cardiovascular events[273] (Chapter 1). Similarly, heightened reactivity following a cardiovascular event predisposes to an increased risk of future events[117,119,125]. However, the results are inconsistent and the two largest prospective studies (Meade et al.[126] 740 subjects with 16 year follow up, Elwood et al.[127] 1812 subjects with 5 year follow up) failed to show a significant correlation between platelet aggregation and subsequent cardiovascular events.

There is little doubt to the importance of the platelet in MI and it still remains the key target of primary and secondary prevention strategies. The PRAMIS study has highlighted that there is tremendous biological variation in all aspects of platelet functions assessed (Chapter 4) in both the controls and cases and that this remains despite the cases all being on aspirin. Targeting anti-platelet treatments by platelet function assessment will become even more desirable as newer anti-platelet options become available, especially in an era where percutaneous intervention with coronary stent placement is becoming more widespread and the demand for more potent anti platelet agents (to prevent stent and coronary artery thrombosis) rises.

Currently, the main factors that have prevented platelet function assessments from having widespread clinical use, and remain a continuing challenge, are:

(i) Reproducibility of the assays.

There are a variety of techniques available to assess platelet function (Chapter 1) which are not directly comparable to one another and this may account for some of the inconsistencies seen in previous study findings. Platelet function is a very dynamic process and a number of external factors can influence the result. For the purposes of the PRAMIS study, all subjects were fasting, seen in the morning, refrained from smoking, remained supine for 15 minutes before blood samples were taken by trained

staff to minimize ex vivo platelet activation. This resulted in acceptable low levels of inter and intra assay variability. Such measures would not be practical in a clinical setting.

(ii) Cost of assays

Platelet aggregation, flow cytometry and flow chamber studies all require specialized equipment, trained staff and are time consuming. The level of throughput required for primary and secondary risk assessments (1227 MIs/year in Leicester coronary care units alone) would have major implications on cost. Plasma and urinary markers of platelet activation offer a more practical and cost-effective alternative but only provide insight into the resting status of platelets.

(iii) Clinical relevance

Until recently, aspirin was the only anti-platelet medication in widespread use. Over-riding clinical evidence[107] and its relative insignificant cost has dictated almost universal use in all atherothrombotic diseases. However, with the advent of new anti-platelet treatments such as the ADP antagonists and GPIIbIIIa receptor inhibitors, there are now options to the type and number of anti-platelet agents prescribed and this is likely to increase in the near future. There has also been an increase in the use of intra-coronary stents which often exposes patients to a planned but high thrombotic risk scenario. The clinical arena therefore demands large prospective studies in primary and secondary prevention populations to assess not only platelet function to future thrombotic events but also treatment strategies based on platelet function, incorporating the newer anti-platelet agents. The need for a reproducible, cost effective and clinically relevant platelet function assessment test has never been more pressing.

7.3.2 The difficulties and potential with genetic studies

Genetic studies may have great potential and the ease with which DNA samples can be collected and analysed continue to fuel a plethora of research into the genetics of CAD. Most genetic studies to date have involved variants in a particular gene (a polymorphism) and whether it is present more (or less) in affected subjects compared to non-affected controls (a case-control study). The main dilemma currently facing the field of CAD genetics is the lack of consistency of findings among studies despite the

early promise. For virtually all the polymorphisms studied to date, a consistent association with MI has not been found. In a pathophysiologicaly complex, and genetic heterogeneous, disorder such as MI, one would not expect all results to agree, but the discordance of findings has hampered progress and application of the results. Some of the factors accounting for this marked variability are discussed below.

(i) Lack of a clearly defined phenotype

Gene polymorphism association studies are easy to conduct. However, when hypothesising new risk factors or mechanisms, a clearly defined phenotype is essential[274]. The phenotype of MI has several positive attributes, not only is it clinically important but has well defined criteria for diagnosis. Furthermore, in most cases, the time of onset can be determined very precisely allowing any age-dependency of genetic effect to be examined. Likewise, controls who have not suffered an event by a particular age can be determined accurately. However, there is an important downside, namely the unavoidable loss of subjects through fatality when recruitment is based on an incident event. This is particularly relevant in MI where 50% of fatalities occur before subjects reach hospital.

At a molecular level, the GPIIbIIIa C196T polymorphism involves the major surface receptor responsible for platelet fibrinogen binding and therefore may alter platelet function. Therefore, phenotypes which involve thrombosis directly (MI, stroke and stent thrombosis) are more appropriate for a platelet receptor gene than less clearly defined associations (angina and coronary artery disease).

(ii) False positive results

Many of the published studies were conducted in relatively small cohorts and are particularly vulnerable to selection bias, which can easily lead to a false positive result. Publication bias towards the reporting of positive data further amplifies this problem and more stringent criteria for reporting association studies have been recommended[275]. The initial positive C196T polymorphism report by Weiss using only 71 cases and 68 controls may be an example of this.

(iii) Gene-environment and gene-gene interaction

Complex diseases have multiple pathophysiological interactions. Many of the risk factors for MI including hypertensions, lipid metabolism, inflammation and thrombosis may themselves have multiple gene-mediated effects. Therefore, study designs must incorporate such interactions to appreciate the true gene mediated effects. Gene-environmental and gene-gene interactions may amplify or suppress gene effects. For example gene variations involved in lipid metabolism may only become clinically relevant with the intake of diets rich in cholesterol (Western diet) as opposed to low cholesterol diets (Japanese diet).

(iv) Small effect

It is unlikely a single gene polymorphism will have a major impact on risk stratification as many of these genes may have a low individual polymorphism risk, but together may have an additive disease influencing effect. Therefore, in MI complex gene-gene and gene-environment mechanisms will be involved to alter disease susceptibility, leading to larger population size requirements. Studies in extreme phenotypes (premature MI) may enhance genetic susceptibility and minimize the environmental interaction (e.g. atherosclerosis) to try and reduce population size requirements.

(v) Lack of intermediate phenotype

A fundamental requirement for a gene change (polymorphism) to have an effect on disease is that it must be mediated through a plausible pathophysiological mechanism. Therefore, in studies that report consistent relationships linking polymorphisms, pathophysiological mechanism and clinical effect, there can be confidence that the genetic variation is influencing disease. A criticism of many of the initial case-control association studies was the reporting of only the associations between polymorphisms and disease. Such statistically significant associations could well have arisen for reasons unrelated to the effect of its phenotype (play of chance, linkage with another gene locus, and poor study design resulting in bias). The GPIIbIIIa C196T polymorphism is a good example of various studies finding positive associations with various phenotypes (MI, CHD, stroke) with little data supporting plausible genetic mediated mechanisms (Chapter 5). In the PRAMIS study flow cytometry determined platelet function tests were the intermediate phenotype assessed to try and explain any association between the G873A and C196T polymorphisms and MI.

The development of robotic and micro-array technology is now allowing large-scale genotyping to be completed with increasing efficiency. The recent sequencing of the entire human genome and The International HapMap Project which is constructing a map of the genome using 600,000 single nucleotide polymorphisms (SNPs) to define haplotype blocks (regions of DNA around 10,000 bases long that have been inherited without being broken up) will allow geneticists with the means to scan the whole genome rapidly for disease genes. Large-scale population-based genetic databases, or 'genebanks', have been proposed in eight international locations[276] and will soon be able to utilize such resources. The recruitment of large high genetic risk populations for the common diseases will also be an invaluable tool in identifying disease susceptibility genes. Examples of these large-scale approaches include the recruitment of 270,000 subjects in the Icelandic DeCode Study[276] and The British Heart Foundation Family Heart Study[277], which has recently completed recruitment of 2000 sets of two or more siblings that have suffered from coronary heart disease under the age of 65 years.

Once disease-associated gene-polymorphisms have been identified they may have a number of clinical applications:

(i) Tailoring of treatment

Polymorphisms may be used for the prescribing of treatments based on genetic profiling (pharmacogenetics). The clinical potential of such profiling is underlined by the recent approval by the FDA in the USA to relabel an approved drug to exclude poor responders by SNP analysis alone. Mercaptopurine is an intrinsically toxic agent used in the treatment of childhood leukaemias, and there is a narrow dose range within which the therapeutic benefits outweigh its toxicity (life threatening bone marrow suppression). 70% of cases of toxicity carry one or more mutations of three polymorphisms identified in the gene for the enzyme thiopurine methyltransferase, which metabolizes the drug. Therefore, SNP testing gives a simple method of risk assessment in the use of a potentially lethal medication.

(ii) Identification of new pathways

There is great anticipation that genetic research will unlock new pathophysiological mechanisms not previously studied in the development of common disease, leading to novel therapeutic targets and treatment. An example of this is the recent mapping of the gene (ALOX5AP) encoding 5-lipooxygenase activating protein (FLAP). Genetic variation within this gene (in the form of a SNP haplotype) was associated with two times greater risk of MI. The novel mechanism by which this risk is mediated involves increased neutrophil production of proinflammatory leukotreine B4, a key product in the 5-lipooxygenase pathway, by subjects who carry the at-risk haplotype[278]. Clinical studies are under way to assess whether such individuals would benefit from therapy with leukotreine inhibitors to negate the genetic mediated increased risk.

(iii) Risk stratification

Gene-polymorphisms represent an ideal resource for targeting primary and secondary prevention strategies due to the ease of analysis. The $P2RY_1$ A1622G polymorphism may help identify increased thrombotic risk individuals, through a novel pathway, whom may benefit from ADP receptor antagonists. The PRAMIS Offspring study underlines the fact that inherent pathological mechanisms exist prior to any clinical consequences and that identification of the genes mediating these influences may allow primary prevention strategies from a young age.

Fresh approaches especially in the scale and design of future genetic studies are required to clarify the possible importance of genetic mediated risk particularly in the clinical setting. These studies must be conducted in adequately powered and accurately phenotyped populations and include appreciation of important gene-environment and gene-gene interactions. Advances in genomic technology are already allowing progressively higher and more complex throughputs with relative ease. Such investigations may identify key individual as well as combination of polymorphisms that influence disease. Such polymorphisms will assist with risk stratification, rationalizing treatment and highlight novel pathophysiological mechanisms.

Presentations, publications and awards arising from the thesis

Presentations

Singh RK, Patel H, Warner E, Stribling J, Desouza P, Goodall A, Samani NJ. Flow cytometric assessment of platelet reactivity in normal population: a determinant of inter-individual risk of coronary thrombosis? Heart 2001; 85: (supp 1): 44.
Presented at the British Cardiac Society, Manchester, 2001.

Singh RK, Patel H, Braund P, Stribling J, Desouza P, Warner E, Samani NJ, Goodall A. Platelet GP1a expression in controls and young MI patients. Thrombosis and Haemostasis 2001; 86 (1): (supp): P1175.
Presented at Thrombosis and Hemostasis Symposium, Paris, 2001.

Singh RK, Patel H, Braund P, Stribling JM, Desouza P, Warner EL, Goodall AH, Samani NJ. Platelet GP1a polymorphism and expression in young MI patients and controls. European Heart Journal 2001; 22 (supp): 121.
Presented at the European Society Of Cardiology Symposium, Stockholm, 2001.

Singh RK, Patel HR, Warner EL, Stribling JM, Desouza PA, Samani NJ, Goodall AH. Platelet polymorphisms in premature myocardial infarction patients. Platelets 2002; 13: 58.
Presented at the European Platelet Meeting, Maastrict, 2001.

Singh RK, Patel H, Braund P, Stribling JM, Desouza P, Cheng S, Goodall AH, Samani NJ. Platelet GPIa (G873/873A) and GPIIbIIIa (leu33/33pro, PLA1/PLA2) glycoprotein receptor polymorphisms: An analysis of functional effects and association with risk of premature myocardial infarction. Journal of the American College of Cardiology 2002; 39 (supp): 229A
Presented at the American College of Cardiology congress, Atlanta, 2002.

Singh RK, Patel H, Braund P, Stribling JM, Desouza P, Warner EL, Goodall AH, Samani NJ. Persistent risk factors in premature myocardial infarction. Heart 2002; 87 (supp): P65

Presented at the British Cardiac Society meeting, Harrogate, 2002

Papers in preperation

Singh RK, Patel H, Braund P, Stribling JM, Desouza P, Warner EL, Goodall AH, Samani NJ. Flow cytometric assessment of platelet reactivity in premature MI and normal population: Highly significant correlation between ADP and thrombin response.

Singh RK, Patel H, Braund P, Stribling JM, Desouza P, Cheng S, Goodall AH, Samani NJ Platelet GPIa (G873/873A) and GPIIbIIIa (leu33/33pro, PLA1/PLA2) glycoprotein receptor polymorphisms: An analysis of functional effects and association with risk of premature myocardial infarction.

Singh RK, Patel H, Braund P, Stribling JM, Desouza P, Goodall AH, Samani NJ. Platelet function in normal and premature myocardial infarction subjects: Analysis and comparison of key determinants of response.

Awards

2000 British Cardiac Society, David de Bono Research Fellowship
2000 British Medical Association Edith Walsh Cardiovascular Research Award
2000 British Medical Association Geoffrey Holt and Ivy Powell Cardiovascular Research Award
2001 European Society of Cardiology, Young Investigator Award, 2nd place
2001 Pfizer Academic Travel Award
2001 The Ernest Frizelle Prize for Clinical Research, University Hospitals of Leicester, 1st place
2001 Pfizer Cardiovascular Research Awards, Trent and West Midlands, 3rd place

All the above awards were competitive, the first three were grants used to fund my fellowship and costs of the study. The remaining awards were all in relation to the PRAMIS study findings and involved abstract submissions and oral presentations. The travel award allowed me to travel to the ESC in 2001 for the award presentation.

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