Sequence Variation within the Platelet Adenosine Diphosphate Receptor Genes, Platelet Function and Premature Myocardial Infarction

Thesis submitted for the degree of
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by

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Author's declaration

I declare that the work described in this thesis has been done by myself, except where indicated.

I contributed to writing the grant proposal to secure the British Heart Foundation Junior Research Fellowship, and also the application for gaining ethical approval to re-approach subjects from the GRAPHIC study to allow their recruitment to the platelet function studies performed in this thesis.

The recruitment of subjects, measurement of platelet function and DNA extractions performed within the PRAMIS study were undertaken by Ravi Singh as part of his MD Thesis.

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Prizes

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Publications

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Abstracts

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Hetherington SL, Lodwick D, Goodall AH, Samani NJ. Molecular basis for platelet hyper-reactivity to ADP associated with the platelet P2Y1 receptor 1622 G allele. *Heart* May 2005; 91: (Suppl I): A60.
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Hetherington SL, Singh RK, Lodwick D, Thompson JR, Goodall AH, Samani NJ. Genetic regulation of the platelet ADP response. *Heart* May 2004; 90: (supp II): A54. Presented at the British Cardiac Society Annual Conference, Manchester, UK, May 2004

Hetherington SL, Singh RK, Lodwick D, Thompson JR, Samani NJ, Goodall AH. ADP-induced platelet activation is influenced by a P2RY1 gene dimorphism. *Platelets* 2003; 15(3): 190-1.
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Papers in preparation

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Chapter 1

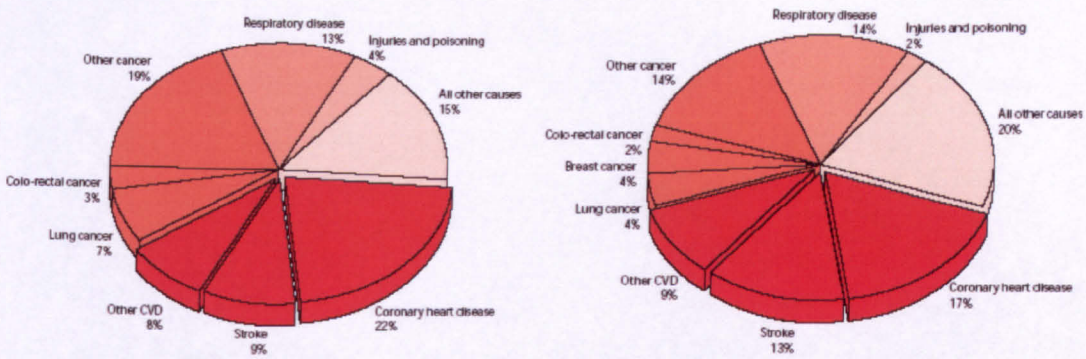
Introduction

1.1 Myocardial infarction

1.1.1 Definition, Burden and Significance

Myocardial infarction (MI) describes the necrosis of cardiac myocytes following severe ischaemic injury. This is usually as a result of total thrombotic occlusion of an epicardial coronary artery at the site of a disrupted atherosclerotic plaque.[Davies *et al*, 1985] Myocardial infarction is a significant cause of morbidity and mortality in England and Wales with over 40,000 deaths directly attributable to acute MI per year, and more than 50,000 deaths recorded as secondary to coronary artery disease (CAD).[Office for National Statistics, 2004] (Figure 1) This second group would consist of a significant number of individuals with a previous history of MI.

Figure 1 ***Deaths by cause in 2002 for the United Kingdom (adapted from Petersen et al, 2004)***



The latest statistics on the incidence and prevalence of fatal and non-fatal MI from the British Heart Foundation estimate that 268,000 individuals suffer an MI each year in the United Kingdom, and that the overall prevalence of individuals with MI is approximately 1.2 million.[Petersen *et al*, 2004] In addition to the risk of death associated with MI, survivors may continue to suffer with significant ill-health as a direct consequence of their previous MI. This can be as a result of left ventricular remodelling causing progressive heart failure, or from scar related ventricular arrhythmias. Survivors may also go on to develop further MIs in the future, or develop angina as a result of progressive coronary atherosclerosis. They are also at increased

risk of developing occlusive vascular disease in other peripheral arteries, which may for example lead to ischaemic stroke, or ischaemic gangrene of the lower limbs.

1.1.2 Pathogenesis

Although the presence of coronary atherosclerosis is often the underlying substrate for the development of an acute MI, other pathological processes, such as coronary artery spasm or linear dissection, may also form part of its aetiology. However, as atherosclerosis remains the commonest cause, this will be discussed below.

In the following sections I am going to briefly describe the formation of atherosclerotic lesions, and then go on to describe the acute events that culminate in thrombotic occlusion of a coronary artery, resulting in an acute MI.

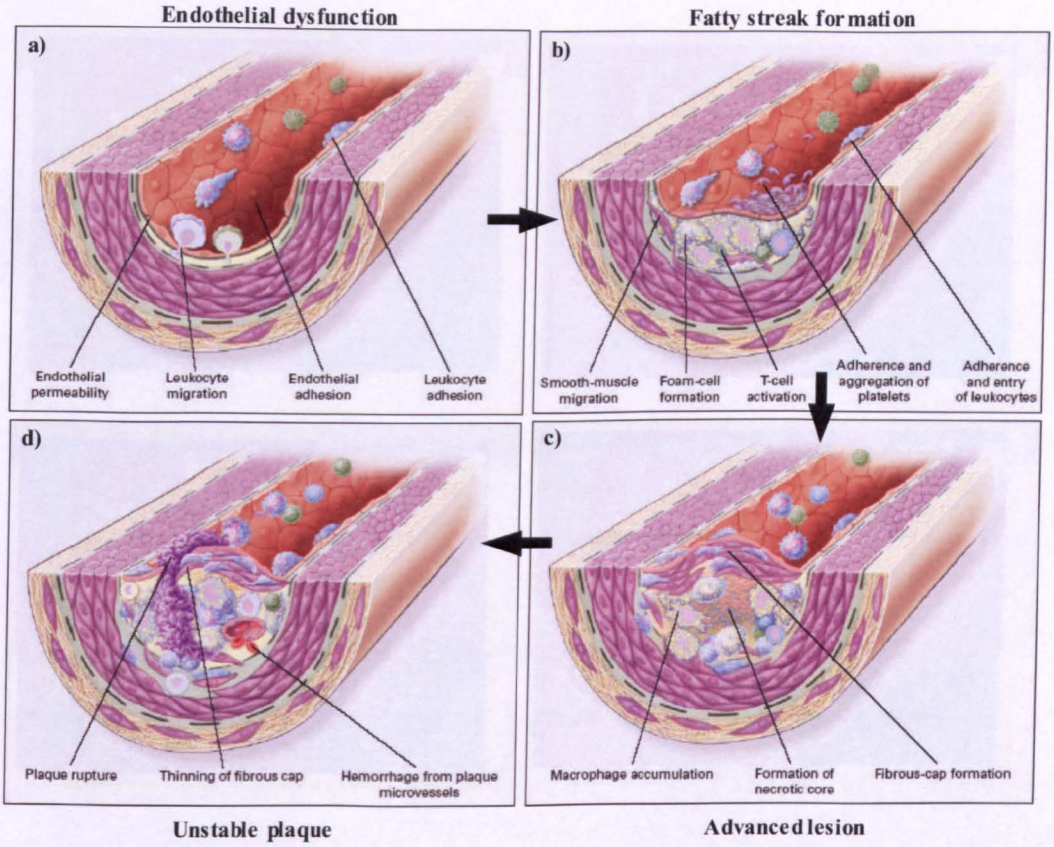
1.1.2.1 Atherosclerosis

Atherosclerosis is an inflammatory disease that develops as a response to injury to the arterial endothelium [Ross *et al*, 1973] and is ubiquitous in most adults of the developed world. The lesions of atherosclerosis occur mainly in the large and medium sized arteries of the circulation.[Ross, 1999] The principle triggering event is believed to be endothelial cell dysfunction or denudation and the sub-endothelial accumulation of lipids (for example, LDL and modified LDL). The various causes of endothelial dysfunction include the effects of smoking, hypertension, diabetes mellitus and the combination of these and other factors.[Ross, 1999]

Endothelium dysfunction leads to the reduced ability of the injured endothelium to produce nitric oxide (NO).[Ross, 1993; Weissberg, 2000] Nitric oxide plays a number of important roles in the maintenance of normal vascular homeostasis including modulation of vascular smooth muscle cell (VSMC) tone and the inhibition of platelet activation/aggregation, inhibition of VSMC proliferation and the inhibition of the formation of reactive oxidative free radical species. Dysfunctional endothelium also expresses a number of surface bound adhesion molecules, such as intracellular adhesion molecule 1 (ICAM 1) and vascular cell adhesion molecule 1 (VCAM 1).[Nakashima *et al*, 1998] The surface expression of these molecules allows the recruitment of monocytes and T lymphocytes to the sub-endothelial space (Figure 2a), where the monocytes mature into activated macrophages and ingest the extracellular lipid. Theses activated macrophages with intracellular accumulation of lipid are called foam cells.[Gerrity, 1981] At this stage the lesion formed by the inflammatory cells and sub-

endothelial lipid in the arterial wall is termed a fatty streak (Figure 2b).[Stary *et al*, 1994]

Figure 2 ***The Development and progression of atherosclerosis (adapted from Ross, 1999)***



The presence of sub-endothelial lipid, particularly in its modified, oxidised form perpetuates the chronic, local inflammatory process and maintains activation of the overlying endothelium.[Stemme *et al*, 1995; Weissberg, 2000] In addition, macrophages and T lymphocytes within the fatty streak express a number of pro-inflammatory cytokines and growth factors which act to recruit and activate further inflammatory cells from the blood,[Rajavashisth *et al*, 1990] and provide a stimulus to the VSMCs in the media of the artery to change to a synthetic/repair phenotype. In response to cytokine stimulation, the VSMCs express various proteinases, which are required to breakdown the surrounding basement membranes and allow the cells to migrate to the site of injury.[Pauly *et al*, 1994] Here, they continue to produce growth

factors, which facilitates their proliferation, and secrete matrix proteins, such as collagens and elastin, that are necessary to repair the vessel.[Libby, 1995] This process allows the formation of a fibrous cap over the highly thrombogenic lipid core of the atherosclerotic plaque thereby stabilising the lesion (Figure 2c).

The self-perpetuating process of endothelial dysfunction, lipid accumulation, inflammatory cell recruitment, VSMC proliferation and matrix synthesis leads to the growth of the atherosclerotic lesion into a fibrous plaque. The majority of lesions at this stage in the development of atherosclerosis are asymptomatic, as affected arteries undergo remodelling to accommodate the expanding plaque whilst maintaining a normal lumen diameter.[Glagov *et al*, 1987] Thus modest sized fibrous plaques are often silent, although larger lesions that do encroach into the lumen causing stenosis, may produce symptoms of stable angina pectoris. Lesions characterised by a relatively small lipid core with a thick fibrous cap are termed stable atherosclerotic plaques, as the likelihood of plaque rupture and subsequent thrombosis is low. However, as plaques continue to enlarge and evolve into advanced lesions, a number of morphological changes may become apparent which are associated with a higher risk of plaque rupture and thrombosis (Figure 2d).[Stary *et al*, 1995]

1.1.2.2 Plaque disruption and thrombotic occlusion

In the majority of cases, disruption of an atherosclerotic plaque is necessary for the development of acute MI. Post mortem studies have shown that this may take the form of either rupture or erosion of the fibrous cap.[Farb *et al*, 1996; van der Wal *et al*, 1994] Inflammation, however, remains a critical factor irrespective of the underlying plaque morphology.[van der Wal *et al*, 1994]

Continued recruitment of inflammatory cells, and in particular macrophages, to the shoulders of atherosclerotic lesions can lead to a more pronounced inflammatory reaction at these points.[Falk, 1989; Richardson *et al*, 1989] Expression of matrix metalloproteinases by these macrophages can lead to a localised reduction in the collagen content of the fibrous cap.[Shah *et al*, 1995] Inflammatory cytokines secreted by these cells can also inhibit proliferation and initiate apoptosis of the VSMC's found in the cap.[Geng *et al*, 1997] The combination of these two processes can lead to thinning and erosion of the fibrous cap, which increases the chance of plaque fissuring and rupture (Figure 2d). Finally, expansion of the lipid core with plaque enlargement

can also increase the risk of plaque rupture even despite a relatively thick fibrous cap, as a result of increased circumferential stress on the cap.[Richardson *et al*, 1989]

Disruption of an atherosclerotic plaque permits the exposure of a number of highly thrombogenic substrates within the lesion, to the circulating blood. This includes the exposure of collagen and von Willebrand Factor (vWF), which facilitates platelet adhesion and activation.[Barnes *et al*, 1998] Once activated, platelets bind fibrinogen via the glycoprotein IIbIIIa (GPIIb-IIIa) receptor and release adenosine diphosphate (ADP) from their dense granules.[Shattil *et al*, 1998] ADP, acting via specific agonist receptors, leads to amplification of the platelet activation response, and recruitment of further platelets to the growing thrombus. At the same time, exposure of the thrombogenic lipid core, which is rich in macrophage-derived tissue factor,[Toschi *et al*, 1997; Wilcox *et al*, 1989] enables activation of the clotting cascade via the intrinsic pathway. This results in the production of thrombin, another potent platelet agonist, and other activated clotting factors that facilitate the formation of a more stable fibrin thrombus. Ultimately, the consequence of these processes can lead to total thrombotic occlusion of the vessel, resulting in an acute MI. Thus, the thrombotic potential of the blood plays an important role in the progression to acute MI following plaque disruption, in addition to the underlying thrombogenicity of the lipid core of the atherosclerotic lesion itself.

The importance of platelets in this thrombotic process is further underscored by the evidence provided from various randomised controlled trials which have shown a number of anti-platelet agents, each blocking different aspects of platelet activation, to be of benefit in reducing the morbidity and mortality of acute coronary syndromes, including acute MI.[Antithrombotics Trialists' Collaboration, 2002; Boersma *et al*, 1999; Montalescot *et al*, 2004; Yusuf *et al*, 2001b]

1.1.3 Risk factors for acute myocardial infarction

The established risk factors for acute MI are similar to those for the development of any stable coronary artery disease. They are made up of a number of modifiable factors and a number of non-modifiable factors.

The most important of the modifiable risk factors are smoking, diabetes mellitus, hypertension and dyslipidaemia. Smoking is implicated in the pathogenesis of atherosclerosis due to its effects on endothelial function, and in the production of

reactive free radical species that may modify LDL, thereby increasing its atherogenic properties.[Heitzer *et al*, 1996] Age-standardised mortality rates for smokers are up to twice that of lifelong non-smokers with respect to ischaemic heart disease.[Doll *et al*, 2004] With respect to diabetes mellitus, data from the Framingham Study [Kannel *et al*, 1979] shows that the presence of diabetes is associated with a three to five-fold increase in the risk of suffering a future cardiovascular event. In addition to the independent effect of diabetes, there is often clustering of other cardiovascular risk factors in diabetic subjects.[Kannel *et al*, 1990] Increasing systolic and diastolic blood pressure predicts increasing mortality from IHD at all age groups.[Lewington *et al*, 2002] An increase in systolic blood pressure of 20mmHg at any age group leads to an approximate two-fold increase in mortality from IHD. A 10mmHg rise in diastolic blood pressure gives a similar increase in death rate. The importance of blood pressure as a risk factor for IHD is confirmed by numerous studies showing that lowering blood pressure leads to reductions in cardiovascular events.[Neal *et al*, 2000] There is also a graded relationship between total serum cholesterol and risk of suffering a cardiovascular event or death.[Stamler *et al*, 1986] Patients with total cholesterol in the highest quintile of the range compared with those in the lowest quintile have a greater than three-fold increase in IHD mortality. In addition, there is significant interaction between these modifiable risk factors in terms of risk of IHD death that is more than additive.[Neaton *et al*, 1992]

The major non modifiable risk factors for MI include age, sex and family history. Compared to subjects in their fifth decade, the risk of dying from IHD increases three, ten and thirty fold for subjects in their sixth, seventh and eighth decades respectively. This effect is irrespective of blood pressure, which also increases with age.[Lewington *et al*, 2002] Over a lifetime, men have approximately twice the risk of coronary heart disease (CHD) related morbidity and mortality compared to women. However, this difference in risk narrows somewhat after the age of 45 years due to catch-up from women.[Lerner *et al*, 1986] Family history is now accepted as an independent risk factor for CHD.[Perkins, 1986] In particular, in first-degree relatives of an affected individual who is under the age of 55 years, the risk of developing IHD is two to four fold greater than in those without such a history. The underlying cause of this increase in risk remains the focus of intense research, specifically with regard to the genetic basis of such an association. Recently, a number of genome wide scans and candidate

gene studies have revealed potential genetic loci that may confer some of this risk in large cohorts of subjects.[Helgadottir *et al*, 2004; Ozaki *et al*, 2004; Samani *et al*, 2005]

1.2 The Platelet

1.2.1 Morphology and thrombocytopoiesis

Platelets circulate in the blood as discoid pieces of membrane-bound cytoplasm that do not contain nuclei. The normal platelet count in whole blood is 150-400 x 10^3 μL^{-1}. They measure 2-3 μm in diameter and have a mean platelet volume (MPV) of 6-8 fL.[Zucker-Franklin, 2000] Platelets are generated by polyploid cells called megakaryocytes (MKs), which are predominantly found in the bone marrow.

The discoid shape of unstimulated platelets is derived from the presence of a microtubule coil and rigid cytoskeleton within the platelet. The microtubule coil is formed from a single filament, which in turn is composed of approximately 1.5 x 10^5 tubulin subunits.[Hartwig *et al*, 2003; Kenney *et al*, 1985] Actin makes up the rigid cytoskeleton in both unstimulated and activated platelets. It is present within platelets in large quantities both as polymers (typically 2000-5000 filaments), in addition to a large non-polymerised pool, maintained in a complex with β4-thymosin.[Safer *et al*, 1991] In unstimulated platelets, other proteins, such as α-adducin, cap the preferred assembly ends of the actin filaments, thereby preventing further actin polymerisation.[Barkalow *et al*, 2003] Following stimulation, these capping proteins dissociate from the actin to allow further assembly of filaments. Actin filaments connect to both the platelet-membrane skeleton (which is composed of spectrin strands) and to one another throughout the platelet cytoplasm, thus forming an integrated cytoskeletal network.[Hartwig *et al*, 2003] Cross-linking of actin polymers is facilitated by filamin and α-actinin, whereas interaction with the spectrin membrane skeleton is primarily via molecules of adducin.[Barkalow *et al*, 2003] A second important interaction between actin and the platelet-membrane skeleton is mediated via filamins.[Stossel *et al*, 2001] These connect the actin filaments to the cytoplasmic domain of the glycoprotein (GP) Ibα subunit of the von Willebrand factor (vWF) receptor (GPIb-V-IX).[Fox, 1985] Each actin filament in unstimulated platelets is linked to GPIb-V-IX via this interaction approximately six times.[Hartwig *et al*, 2003]

The majority of platelet organelles are preformed in MKs. Mature platelets contain α-granules, dense granules, mitochondria, the dense tubular system and the open canalicular system (Figure 3).[Zucker-Franklin, 2000] Platelets prematurely released during accelerated thrombocytopoiesis may also contain ribosomes and rough endoplasmic reticulum. α-granules contain lineage-specific membrane proteins (such as p-selectin and GPIIb-IIIa) in addition to proteins and substances that are endocytosed (e.g. fibrinogen). The contents of α-granules are shown in detail in Table 1.[Plow *et al*, 2000b] Dense granules predominantly contain low molecular weight platelet agonists, such as adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), epinephrine and 5-hydroxytryptamine (5-HT).[Ugurbil *et al*, 1984] Mitochondria provide energy, in the form of ATP, to the platelet by utilising glucose stored in glycogen particles within the platelet cytoplasm. The dense tubular system is a membrane bound sac that typically lies close to the plasma membrane. Its main function is as an intracellular store for calcium ions, as well as being a major site of prostaglandin synthesis.[Gerrard *et al*, 1978] The open canalicular system is probably derived from the demarcating membrane system (DMS) of the MK.[Italiano, Jr. *et al*, 2003] The main function of the canalicular system is to act as a channel through which granule contents can be secreted following stimulation of the platelet.[Zucker-Franklin, 2000] In addition, it is likely that membrane proteins residing in the α-granule membranes, such as p-selectin [Stenberg *et al*, 1985] and GPIIb-IIIa, are translocated to the plasma membrane surface, through the canalicular system, upon platelet activation.

Figure 3 ***Electron micrograph (x20,000) of a resting platelet demonstrating various intracellular organelles (adapted from George JN, 2000)***

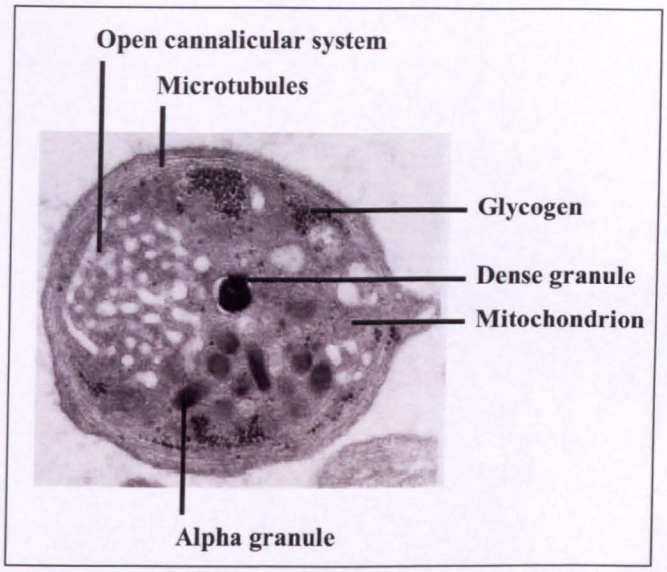


Table 1 ***Specific contents of platelet α-granules (adapted from Plow et al, 2000b)***

Adhesive proteins	Fibrinogen Fibronectin vWF Thrombospondin Vitronectin
Membrane proteins	GPIIb-IIIa P-selectin
Growth modulators	Platelet-derived growth factor Connective tissue activating peptide Transforming growth factor β Platelet factor 4 Thrombospondin
Coagulation factors	Factor V High-molecular-weight kininogen C1 inhibitor Fibrinogen Factor XI Protein S Plasminogen activator inhibitor 1

Thrombocytopoiesis is a complex process that is yet to be fully understood. On average approximately 2 x 10^{11} platelets are produced per day in the bone marrow [Harker *et al*,

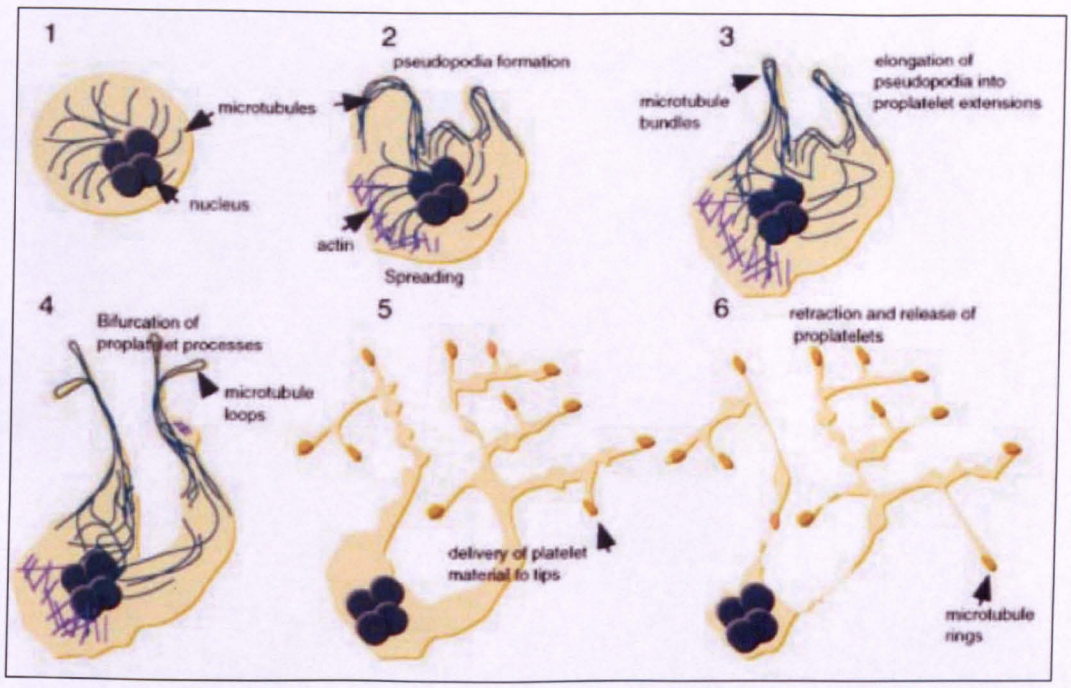
1969], with a single mature MK capable of producing approximately 1000 platelets.[Hartwig *et al*, 2003] Once released into the circulation, platelets survive for an average of seven days. Megakaryocytes are polyploid cells, with mature cells typically attaining a deoxynucleic acid (DNA) content of at least 16N.[Odell, Jr. *et al*, 1970] The increase in MK ploidy, as a result of endomitosis (prematurely terminated mitosis), is largely driven by thrombopoietin, acting on its receptor c-Mpl. The increase in DNA content is thought to facilitate the increase in cell mass required to generate the large number of platelets each MK produces.[Italiano, Jr. *et al*, 2003]

Megakaryocyte development can be divided into three stages: (i) proliferating stage (progenitor cells, consisting of burst-forming units and colony-forming units), (ii) transitional stage (promegakaryoblasts, which are transitional between the progenitor and mature cells and have an intermediate DNA content), (iii) maturation stage (megakaryoblasts, promegakaryocytes and granular megakaryocytes).[Long *et al*, 2000] Only the progenitor cells have the capability to proliferate, with this proliferation process driven by a number of growth factors, such as thrombopoietin, interleukins (IL-3, IL-6, IL-9 and IL-11), c-kit ligand, granulocyte-macrophage colony stimulating factor and possibly erythropoietin.[Kaushansky *et al*, 1995; Kaushansky, 1995] Endomitosis occurs predominantly in the transitional and maturation stages, and it is during the maturation stage that the cytoplasmic volume and granule content of the MKs increase.[Long *et al*, 2000] In addition proliferation and invagination of the plasma membrane occurs to form the DMS. The function of the DMS is to serve as a membrane reserve for proplatelet formation, and it is likely that the open canalicular system represents a remnant of this in the mature platelet.[Italiano, Jr. *et al*, 2003; Radley *et al*, 1982]

The final stages of MK maturation involve the development of pseudopodial proplatelet extensions, which are the intermediate structures essential for platelet release.[Italiano, Jr. *et al*, 1999] This process is driven by microtubule bundle formation and extension in loops, out from the main body of the MK. The initially thick pseudopodia elongate into thin tubules, which undergo repeated cycles of bending and branching. This bending and branching process is dependent on actin-based forces.[Hartwig *et al*, 2003] Platelet buds form at the ends of the proplatelet processes, and contain the characteristic microtubule coils found in mature platelets.[Italiano, Jr. *et al*, 1999] Following this, organelles and granules are delivered to the maturing platelet buds sequentially via

translocation down the microtubule bundle. Finally, rapid retraction separates the mature platelet buds from the main cell body either individually, or in short chains, which are then released into the circulation. At present it is not clear whether MKs release fully formed, mature platelets or whether platelet maturation terminates within the circulation. (Figure 4)

Figure 4 ***Platelet formation (adapted from Italiano, Jr. et al, 1999)***



1.2.2 Mechanisms of platelet activation

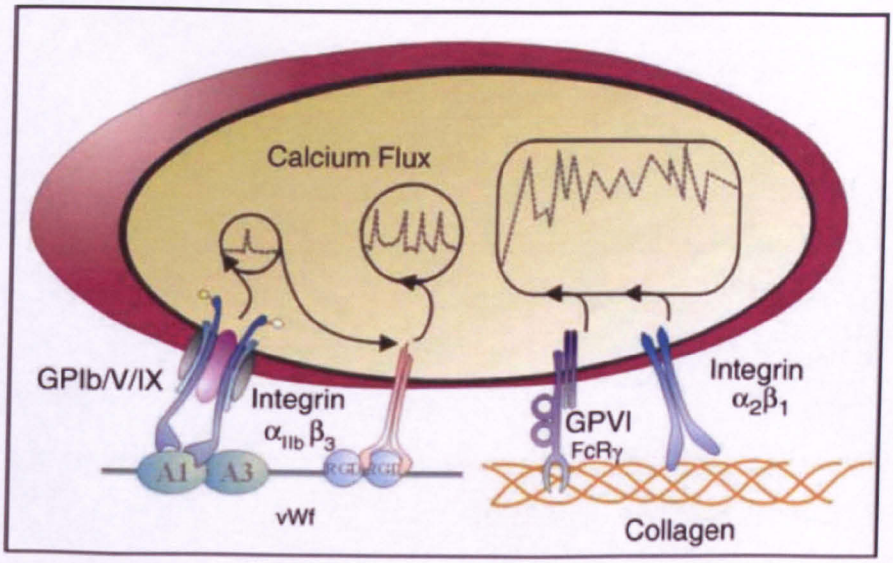
The main role of platelets within the blood is to provide haemostasis. In the normal circulation platelets are maintained in a resting state via the action of inhibitory molecules, such as prostacyclin and nitric oxide (NO), on specific platelet receptors. In order for platelets to fulfil their haemostatic function, they must first become activated. Platelet activation is characterised by the activated platelets undergoing shape-change, aggregating at the site of injury and secreting their granule contents, thereby propagating the wave of activation and recruiting further platelets to the thrombus. Platelet activation is mediated via the interaction between various ligands and agonists and their cell surface receptors, which, in turn, initiate a variety of signalling cascades.

1.2.2.1 Adhesion receptors

In vivo the initiating event in platelet thrombus formation, whether for normal haemostasis or as part of a pathological process such as MI, is the adherence of platelets to exposed subendothelial matrix.[Barnes *et al*, 1998] This matrix consists of a variety of substances such as fibrillar collagens and vWF. It is these molecules, through their interaction with specific, platelet cell-surface adhesion receptors that facilitates this adhesion process.

The four major receptors involved with initial platelet tethering are GPIb-V-IX, GPIIb-IIIa, GPIa-IIa and GPVI.[Jackson *et al*, 2003] (Figure 5) The most important ligands that bind to these receptors are vWF, fibrinogen and collagen respectively, although the receptors are not 100% specific for these ligands (for example GPIIb-IIIa will also bind vWF and collagen).[Plow *et al*, 2000a] In vivo, the first interaction to occur at the site of endothelial injury is commonly between vWF, which is bound to exposed collagen, and the GPIbα subunit of the GPIb-V-IX complex. This interaction is relatively weak and only occurs under conditions of high shear flow. The binding of vWF to GPIbα triggers a small rise in cytosolic calcium through tyrosine kinase-mediated signalling, which in turn induces low level activation of the GPIIb-IIIa receptor, resulting in affinity modulation of its ligand binding site.[Jackson *et al*, 2003] This process facilitates the more secure adhesion of the platelet to the exposed matrix through interaction between GPIIb-IIIa and vWF. Engagement of activated GPIIb-IIIa establishes a feedback loop termed "outside-in" signalling whereby binding of ligand to this receptor propagates further calcium influx in a phosphoinositide 3-kinase (PI3K)/phospholipase Cγ2 (PLCγ2) dependant manner.[Nesbitt *et al*, 2002; Yap *et al*, 2002] This process has been shown to depend on tyrosine kinase interaction (such as Src, Syk, focal adhesion kinase) with the cytoplasmic tail of GPIIb-IIIa.[Phillips *et al*, 2001] This mechanism amplifies GPIIb-IIIa activation and results in firmer adhesion of the platelets to the subendothelial matrix.

Figure 5 ***Receptor-ligand interactions in initial platelet tethering to vWF and collagen, resulting in a robust calcium signal (adapted from Jackson et al, 2003)***



The initial tethering process described above enables collagen to interact with its receptors GPVI and GPIa-IIa. Collagen binding to GPVI promotes the clustering of associated FcR γ-chains. This clustering process facilitates the phosphorylation of the cytoplasmic tail by various Src tyrosine kinases, which in turn allows the binding of Syk, and the initiation of a tyrosine kinase-based signalling cascade that results in further activation of PI3K and PLCγ2.[Jackson *et al*, 2003; Watson, 1999] The end effect of this is the generation of a robust and sustained calcium signal that results in efficient platelet activation. The contribution of the GPIa-IIa receptor to the platelet-collagen interaction has also been shown to depend on tyrosine kinase-mediated signalling cascades.[Suzuki-Inoue *et al*, 2001]

The receptor-ligand interactions described above are responsible for the initial phase of platelet adherence to the damaged blood vessel wall. However, other soluble agonists, acting on receptors that are largely dependant on guanine nucleotide binding-protein signalling mechanisms (G-protein-coupled receptors, GPCRs), are responsible for the recruitment of further platelets, thereby promoting platelet thrombus growth. It should also be recognised that there is substantial "cross-talk" between all of these receptors and signalling pathways, and significant overlap in their cellular effects (such as causing calcium release from the DTS).[Hardy *et al*, 2004; Nieswandt *et al*, 2001]

1.2.2.2 Soluble agonist receptors

The soluble agonists heavily involved in promoting thrombus growth consist of thrombin, thromboxane A2 (TxA2) and ADP.[Jackson *et al*, 2003] All of these soluble agonists interact with specific GPCRs, through which they exert their cellular effects.

G-protein-coupled receptors

G-proteins are αβγ heterotrimers.[Brass, 2000] In their inactive state, guanosine diphosphate (GDP) is bound to the Gα subunit. On receptor activation, GDP is released from the complex and replaced with guanosine triphosphate (GTP). This induces a conformational change in the Gα subunit, which allows it to dissociate from the Gβγ subunit. Both the Gα and Gβγ subunits are then free to mediate signalling via G-protein-coupled pathways. After an interval of time, the intrinsic GTPase activity of the Gα subunit hydrolyses the GTP back to GDP, thereby inactivating the complex.

There are four distinct families of Gα subunits found in platelets (Gαs, Gαi, Gαq, Gα12), each with their own specific signalling pathways and effects.[Brass *et al*, 1993] Gαs stimulates adenylyl cyclase, leading to an increase in adenosine 3',5'-cyclic monophosphate (cAMP) and activation of cAMP-dependant kinases such as protein kinase A. Gαi has the opposite effect to Gαs, causing inhibition of adenylyl cyclase. In addition, the Gβγ subunit of Gαi can activate PI3K with resulting activation of the small Ras family GTPase, Rap1B,[Woulfe *et al*, 2002] the serine/threonine kinase, Akt,[Woulfe *et al*, 2004] and has also been found to activate G-protein-gated, inwardly rectifying potassium channels.[Shankar *et al*, 2004] These have recently been shown to play an important role in platelet activation. Gαq signalling is mediated via activation of phospholipase Cβ (PLCβ), which hydrolyses membrane phosphotidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).[Brass *et al*, 1985; Jin *et al*, 1998a] IP3 acts directly on the DTS within the platelet to open calcium channels, thereby resulting in an increase in cytosolic free calcium concentration. DAG, on the other hand, activates protein kinase C (PKC, a family of serine/threonine kinases) [Brass, 2000; Quinton *et al*, 2002] which goes on to phosphorylate a number of platelet proteins involved in platelet activation, such as mitogen-activated protein kinase (MAPK), myosin light chain and pleckstrin. Finally, activation of Gα12 family members in platelets leads to activation of tyrosine kinases such as Syk and Src, in addition to activation of the small GTPase Rho.[Klages *et al*,

1999] Rho activates Rho-kinase and results in phosphorylation of myosin light chain and myosin phosphatase. These effects are important in the mediation of calcium-independent platelet shape-change.

Thrombin

Of the soluble platelet agonists, thrombin (a serine protease) is the most potent platelet activator, and is generated, via the tissue factor pathway, at the site of vessel injury or plaque rupture. Platelets express two specific receptors for thrombin – protease activated receptor (PAR) 1 and PAR4.[Kahn *et al*, 1999] These receptors are activated via thrombin dependant cleavage of the N-terminal exodomain of the protein, which reveals a new "tethered ligand".[Brass, 2000] This can then activate the receptor to which it is attached. PAR1 is the more important receptor of the two on human platelets, whereby blocking this receptor abrogates much of the platelet activation seen with thrombin.[Kahn *et al*, 1999] Studies have shown that signalling through both receptors is mediated via Gαq and Gα12 family members,[Offermanns *et al*, 1994] through the use of specific thrombin receptor-activating peptides (TRAP). Early reports of direct thrombin-mediated signalling through Gαi, have been shown to be due to the action of secreted ADP on the P2Y12 receptor.[Kim *et al*, 2002] Stimulation of platelets with thrombin results in full activation including platelet shape change, activation of GPIIb-IIIa and platelet granule secretion. Recent studies have also shown that in addition to its effects at PAR receptors, thrombin is also implicated in activation of the GPIb-V-IX complex.[Ramakrishnan *et al*, 2001] Thrombin can cleave the GPV subunit from the GPIb-V-IX complex, and this allows thrombin to directly stimulate the receptor, in a non-proteolytic manner.

Thromboxane A2

Thromboxane A2 is a unique platelet agonist in that it is generated within platelets upon activation, and subsequently diffuses across the plasma membrane and induces further amplification of platelet activation through action on its cell-surface thromboxane GPCR, TPα.[Kinsella *et al*, 1997] The majority of platelet agonists (such as collagen, thrombin and ADP) can induce TxA2 production. The rate-limiting step in TxA2 production is the availability of arachidonic acid, which is governed by the enzyme phospholipase A2 (PLA2).[Brass, 2000] PLA2 is regulated within platelets via changes in calcium concentration, and through phosphorylation by MAPK, thus pathways

activating PLC also activate TxA2 synthesis. Arachidonic acid is metabolised in platelets via the aspirin-sensitive cyclo-oxygenase 1 (COX1) pathway.

Adenosine diphosphate

Adenosine diphosphate is considered to be a weak platelet agonist when used to stimulate platelets in isolation. The main role of ADP in thrombosis is as a secondary agonist that acts to amplify the activation response, to recruit further platelets and to help stabilise the growing thrombus.[Dorsam *et al*, 2004; Storey *et al*, 2001] It is released from the dense granules of activated platelets at the site of injury, largely in response to collagen, thrombin and TxA2 acting on their agonist receptors.[Brass, 2000; Plow *et al*, 2000a] There are two platelet ADP receptors, P2Y1 and P2Y12. The structure, function and signalling of these receptors will be discussed later in this chapter.

1.2.3 Methods of assessing platelet activation

The two most commonly used methods for determining platelet activation *ex vivo* are those based around platelet aggregometry and flow cytometry. Other methods that can be utilised include measuring adhesion to collagen or vWF coated membranes under varying conditions of shear in a flow chamber [Moroi *et al*, 1996], or through the measurement of various soluble factors, in samples of either blood or urine (such as soluble p-selectin, thromboxane B2 and β-thromboglobulin).

1.2.3.1 Platelet aggregometry

The Born method [Born GVR, 1962a] for measuring platelet aggregation is a well-established method for determining platelet activation in response to stimulation with different agonists. It is based around the changes in light transmittance that occur when platelets in suspension aggregate. Anticoagulated whole blood is gently centrifuged to sediment the red and white blood cells allowing the researcher to obtain platelet rich plasma (PRP). The PRP is incubated in a cuvette at 37°C, with continuous stirring, between a light source and a photocell within the aggregometer. On addition of an agonist, the platelets initially undergo shape-change, which results in a decrease in light transmittance, followed by aggregation, where light transmittance increases. The aggregometer must initially be calibrated with platelet-deficient plasma so that it can express the change in light transmittance as a percentage of the maximum. Thus estimations of platelet shape-change, primary aggregation, secondary aggregation

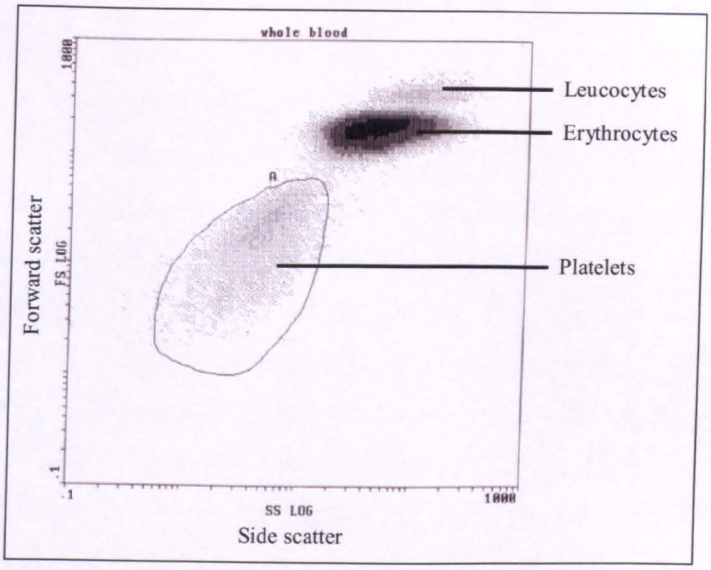
(indicative of a burst of TxA2 synthesis and release) and rate of aggregation can be made. The main disadvantages of aggregometry are that the blood has to be processed/manipulated to produce PRP, and, as a result of stirring/close platelet-platelet contact, the assay is sensitive to differences in extracellular calcium concentration (resulting in exaggerated TxA2 synthesis in response to weak agonists) [Packham *et al*, 1987].

1.2.3.2 Flow cytometry

The principle technique utilised in flow cytometry is the use of a laser radiation source, with a wavelength of 488nm, to illuminate cells passing through a flow cell in single file. This single cell resolution is achieved by "hydrodynamic focusing", whereby the sample is injected into the centre of a pressurised fluid stream of sheath fluid. The resultant forward and side scatter of the laser light is converted into voltage signals via photomultiplier tubes (PMTs), and allows inferences to be made with regard to the size and granularity respectively, of the cells passing through the flow cell. In addition fluorescent dyes can be used to label cells or cell-surface markers, through conjugation with specific antibodies. Upon excitation with the laser, these fluorophores will emit light of a certain wavelength, which can be collected and converted into a signal through the use of dichroic long-pass filters and PMTs.

When using flow cytometry for platelet analysis, unseparated whole blood is diluted with buffered saline, and incubated with the appropriate concentrations of labelled antibodies and platelet agonists/antagonists. Prior to applying to the flow cytometer, the samples must be fixed with formaldehyde [Janes *et al*, 1993]. Gating on the basis of front and side scatter is then used to select the platelet population for further analysis and detection of labelled antibody binding. Results are displayed in the form of dot density plots and histograms, which allow estimations of mean and median signal strengths, in addition to cell counts and proportions of cells carrying labelled antibody to be determined. Thus flow cytometry can be used to select cells of interest based on their size/granularity, as well as based on the expression of various markers. (Figure 6)

Figure 6 ***Dot density plot following flow cytometric analysis of whole blood***



This figure demonstrates a typical dot density plot from the flow cytometer following analysis of whole blood. Forward scatter is displayed on the y-axis and side scatter on the x-axis, thus allowing platelet populations of different size and granularity to be distinguished. Cells encompassed by the elliptical "gated" region can be subjected to further analysis.

A number of methods can be employed when using flow cytometry to analyse platelet activation. These include measuring differences in surface expression of various markers, such as p-selectin [Hjemdahl *et al*, 1994] and GPIIb-IIIa, determining levels of binding of specific proteins to the platelet surface, such as fibrinogen [Janes *et al*, 1993], and analysing binding of activation-specific antibodies, such as PAC-1.[Shattil *et al*, 1985] The binding of fibrinogen to the activated GPIIb-IIIa receptor is a specific marker of platelet activation, and was therefore one of the methods chosen to measure platelet activation in this study. A significant advantage of flow cytometry over aggregometry, in addition to the greater volume and variety of data that can be generated, is that the assay is performed on unstirred, diluted whole blood, thereby minimising any effect of preparation and extracellular calcium.[Chronos *et al*, 1994]

1.2.4 Variability in platelet reactivity

It is well recognised that there is substantial variation in platelet reactivity between different individuals. This is true when either aggregometry or flow cytometry is used

to measure platelet activation, and in response to stimulation with a variety of agonists. Heptinstall and Mulley [Heptinstall *et al*, 1977] found there to be marked variability in platelet aggregation response to ADP tested in 54 subjects, irrespective of the type of anticoagulant used. In addition, Holmes *et al* [Holmes *et al*, 1999b] found that there was significant variation (coefficient of variation, CV, 44%) in platelet activation measured by flow cytometric analysis of fibrinogen-binding to the activated GPIIb-IIIa receptor, in twelve individuals tested with 0.2 μM ADP. The cause of this variability is likely to be multifactorial, with contributions from both environmental and genetic factors. In one of the largest studies to date, O'Donnell *et al* [O'Donnell *et al*, 2001] analysed platelet aggregation following stimulation with collagen, ADP and epinephrine determined in 1969 subjects recruited as part of the Framingham Study. They also demonstrated marked interindividual variability in platelet aggregation and calculated the heritability of platelet aggregation to be between 44 and 62%.

Identifying and understanding the causes of this interindividual variation will greatly enhance our knowledge of platelet biology and thrombotic mechanisms, whilst also providing useful insights into the pathological processes in which they play an aetiological role.

Contribution of platelet volume and age

Platelet volume was linked to variation in platelet function early on in the field of platelet biology.[Mannucci *et al*, 1967] In 1969, Karpatkin showed that large-heavy platelets were metabolically [Karpatkin, 1969a] and functionally [Karpatkin, 1969b] more active when compared to small-light platelets. He hypothesised that this difference was due to large platelets representing a younger platelet population and small platelets an older one. In support of this view, Hirsh *et al* [Hirsh *et al*, 1968] showed that younger platelets had a greater capacity to adhere to collagen than older platelets. Subsequently, Haver and Gear [Haver *et al*, 1981], and Peng *et al* [Peng *et al*, 1994] have added weight to this theory through their findings in animal models.

Influence of demographic factors

Age, gender and smoking habit of an individual have all been shown to exert an effect on platelet reactivity, although the findings from different studies have not always been consistent. Terres *et al* [Terres *et al*, 1991] and Vilen *et al* [Vilen *et al*, 1989] found platelet aggregation in response to ADP to markedly increase with age, agreeing with

earlier work by Johnson *et al* [Johnson *et al*, 1975]. However, a study measuring flow cytometric fibrinogen-binding and p-selectin expression as markers of platelet activation, showed a negative correlation between platelet activation and increasing age.[Knight *et al*, 1997a]

Studies on platelet aggregation have tended to show that females have greater platelet reactivity as compared to males [Johnson *et al*, 1975], although other investigators have attributed these observations to the differing haematocrits of younger females and males causing differences in extracellular free calcium, when their blood is anticoagulated with citrate [Bell *et al*, 1990; Kelton *et al*, 1980]. More recently, Faraday *et al* [Faraday *et al*, 1997] have provided support for gender difference in platelet reactivity, through their finding of significant differences in ADP and TRAP-stimulated fibrinogen and PAC-1-binding, to washed platelets, between the sexes.

With respect to smoking, a number of studies have attempted to characterize the effect of acute and chronic smoking on platelet reactivity and have produced conflicting results [Fusegawa *et al*, 2000; Grignani *et al*, 1977; Sharp *et al*, 1995; Terres *et al*, 1991]. Grignani *et al* [Grignani *et al*, 1977] found that in a group of 10 heavy smokers aged 20 to 40 years, smoking a cigarette resulted in a significant increase in platelet aggregation. They could not, however, replicate this finding in an older aged group (43 to 72 years). Fusegawa and Handa [Fusegawa *et al*, 2000] also found that smoking enhanced platelet reactivity in a study of 90 healthy smokers compared to 141 age-matched non-smokers. After a ten hour abstinence from smoking, the smokers had significantly greater platelet aggregation in response to ADP and epinephrine. Conversely, Terres *et al* [Terres *et al*, 1991] failed to find any effect of smoking status on platelet function in 191 men, 78 of whom had angiographically determined coronary artery disease.

Genetic contribution to platelet reactivity

There is growing evidence that genetics plays an important role in determining the platelet activation response of any given individual to a variety of different agonists. This hypothesis was initially generated as a result of the observation that (i) there is a familial component to platelet reactivity, and (ii) that the response of an individual is stable over a period of time. Data from the Framingham Heart Study [O'Donnell *et al*, 2001] has shown there to be significant correlations in platelet aggregation response to

ADP, epinephrine and collagen lag-time in sibling pairs, that was absent from spouse pairs, providing weight to this theory. Fontana *et al* [Fontana *et al*, 2003a] found that platelet aggregation in response to 2 μM ADP was stable over a period of one week in 98 subjects (r^2=0.77). P-selectin expression in response to 100 μM TRAP (SFLLRN) has also been found to be stable over a one week period (r^2=0.5).[Dupont *et al*, 2003] Although the underling nature of this familial component of platelet reactivity is likely to be polygenic, a number of polymorphisms in platelet receptor genes have been identified and linked to platelet functional responses. Brief summaries of the major studies investigating these associations are described in Table 2.

Table 2 ***Platelet receptor function and polymorphism studies***

Study	Receptor	Polymorphism	Functional Effect	Mechanism
Fontana *et al* [Fontana *et al*, 2003a]	P2Y12	Haplotype i-C139T, i-T744C, i-ins801A, G52T H1=CTAG, H2=TCdelT	Increased response to ADP in carriers of H2 haplotype	Not studied
Dupont *et al* [Dupont *et al*, 2003]	PAR1	IVSn A-14T	Reduced response to TRAP in carriers of T allele	Reduced PAR1 expression in carriers of T allele
Joutsi-Korhonen *et al* [Joutsi-Korhonen *et al*, 2003]	GPVI	Haplotype T655C, A709G, A745G, A950T, C964A a=TAAAC, b=CGGTA	Reduced response to collagen related peptide in bb homozygotes	Reduced GPVI expression in bb homozygotes
Kunicki *et al* [Kunicki *et al*, 1997]	GPIa	C807T	Reduced adhesion to collagen in CC homozygotes	Reduced GPIa-IIa expression in CC homozygotes
Feng *et al* [Feng *et al*, 1999]	GPIIIa	C196T	Lower aggregation threshold to epinephrine with carriage of T allele	Not studied
Goodall *et al* [Goodall *et al*, 1999]	GPIIIa	C196T	Increased fibrinogen-binding after ADP stimulation with carriage of the T allele	Not studied
Michelson *et al* [Michelson *et al*, 2000]	GPIIIa	C196T	Increased fibrinogen-binding after ADP stimulation with carriage of the T allele	Not studied

Although a number of studies have shown an association between the GPIIb-IIIa $PL^A1/2$ (C196T) polymorphism and platelet function (see Table 2), other studies have failed to confirm this [Frey *et al*, 2003; Meiklejohn *et al*, 1999]. In addition, a detailed investigation of GPIIb-IIIa function by Bennett *et al* [Bennett *et al*, 2001] failed to

show any significant effect of the C196T polymorphism on platelet aggregation, secretion or thrombus formation, nor did they detect any effect of carriage of the 196T allele on affinity of the GPIIb-IIIa receptor for fibrinogen, using a radioligand-binding assay.

1.2.5 Variation in platelet reactivity and risk of MI

As platelet activation and thrombosis play a vital role in the development of MI, there has been extensive investigation into the possible causal relationship between factors that affect platelet reactivity, and risk of myocardial infarction.

1.2.5.1 Platelet count and volume

Increasing platelet count has been linked with increased platelet aggregation [Sharp DS *et al*, 1994], and an inverse correlation has been suggested between mean platelet volume (MPV) and platelet count, whereby the overall platelet mass between individuals remains relatively constant [O'Brien, 1974]. There is little data to link increased platelet counts with MI in healthy individuals, however, Schmuziger *et al* [Schmuziger *et al*, 1995] found that patients developing a reactive thrombocytosis post cardiopulmonary bypass had a five-fold increase in risk of post-operative MI compared to those without.

The effect of MPV on platelet reactivity has been discussed above, and a number of studies have attempted to address the effect of MPV on risk of suffering an acute MI. In 1983 Martin *et al* [Martin *et al*, 1983] found that within 12 hours of a myocardial infarction, MPV was significantly elevated in a small cohort of men, and that this persisted out to 12 weeks post-event. In a prospective follow-up study [Martin *et al*, 1991], assessing death and re-infarction in MI survivors, this group found increased MPV to significantly predict risk of either endpoint at 2 years, concluding that platelet volume was an independent risk factor for myocardial infarction. Platelet count did not have an effect in this study.

1.2.5.2 Platelet aggregation studies and coronary artery disease

The relationship between platelet aggregation and coronary artery disease (CAD) has been investigated extensively, using both prospective and retrospective means. Early studies tended to be small, of cross-sectional design and often gave strongly positive results. The Caerphilly Collaborative Heart Disease Study carried out platelet aggregometry to ADP in 308 men aged 49 to 64 years. They found odds ratios of

prevalent CAD of 3.6 for angina and 7.3 for previous MI in those patients with the most sensitive platelets to ADP compared to those with the least sensitive.[Elwood *et al*, 1990] Following on from this, Trip *et al* [Trip *et al*, 1990] attempted to characterise prospectively the risk of death and further cardiovascular events in patients with a past history of myocardial infarction, on the basis of their spontaneous platelet aggregation profiles. Their study showed a four-fold increase in risk of death in subjects positive versus subjects negative for spontaneous platelet aggregation. Two prospective studies have investigated the association of ADP induced platelet aggregation with the incidence of CAD in apparently healthy men, with conflicting results. In 1991, Thaulow *et al* [Thaulow *et al*, 1991] found that in a sample of 150 middle-aged men, the 50% of subjects with the most rapid aggregation response to ADP had significantly greater coronary heart disease mortality over the following 13.5 years. Conversely, Meade *et al* [Meade *et al*, 1997] failed to show any significant association between ADP-stimulated platelet aggregation and incidence of coronary artery disease in 740 men followed over 10.1 years. Thus the true nature of the relationship between platelet aggregation and risk of CAD has yet to be fully established.

1.2.5.3 Flow cytometry studies and coronary artery disease

The flow cytometric analysis of platelet reactivity has grown in popularity over the past decade, and as a result, a number of groups have started to investigate the association between flow cytometric determination of platelet function and coronary artery disease. The majority of these studies have looked at activation differences between patients with known coronary disease and healthy controls [Furman *et al*, 1998; Holmes *et al*, 1999a; Knight *et al*, 1997b; Murakami *et al*, 1996], or in stable versus unstable coronary disease [Chakhtoura *et al*, 2000; Lindmark *et al*, 2001; Patel *et al*, 2004], rather than analysing the risk of developing a new event after prospective stratification on the basis of their platelet reactivity.

Chakhtoura *et al* [Chakhtoura *et al*, 2000] and Lindmark *et al* [Lindmark *et al*, 2001] have both found resting levels of p-selectin expression to be increased in patients with unstable angina when compared to those with stable angina. Patel *et al* [Patel *et al*, 2004] have further defined this relationship by investigating the difference in platelet reactivity between platelets taken simultaneously from the aortic root and coronary sinus in patients with stable and unstable angina. They found that in patients with unstable disease, the proportion of platelets expressing p-selectin increased 94%

between aortic and coronary sinus blood, compared with 49% with stable patients. Thus it is likely that the presence of atherosclerotic lesions causes increased platelet activation in coronary artery disease, particularly when the lesions are unstable. This is supported by work from Keating *et al* [Keating *et al*, 2004] who found platelet reactivity to be correlated to extent of atherosclerosis in 130 patients with known vascular disease.

Other investigators have attempted to define a more causative role of platelet reactivity in ischaemic heart disease. Itoh *et al* [Itoh *et al*, 1995] measured p-selectin expression as a marker of platelet activation in 48 patients with acute coronary syndromes (ACS) and 30 healthy control subjects and found that within the ACS group, raised p-selectin expression predicted the development of further acute cardiac events. More recently, Kabbani *et al* [Kabbani *et al*, 2001a] have characterised platelet reactivity, as defined by fibrinogen-binding response to 0.2μM ADP, prospectively in 112 patients undergoing percutaneous coronary intervention (PCI), and related this to the risk of suffering an MI, or requiring urgent or repeat revascularisation in the 90 day follow-up period. They found that after separating the population into two groups defined by the median platelet response, adjusted odds of observing a negative clinical outcome was 3.78 fold higher in the high reactivity group compared to the low reactivity group.

So in addition to higher platelet reactivity being a feature of atherosclerosis *per se*, there is also emerging evidence that affected patients, with more reactive platelets, are more likely to experience an adverse clinical outcome.

1.2.5.4 Studies based on genetic polymorphisms

As discussed earlier, a number of polymorphisms in a variety of platelet receptors have been associated with differing platelet reactivity. Given the evidence for a strong genetic basis to CHD and MI (see section 1.1.3) and the potential association between platelet reactivity and CHD risk, these polymorphisms provide attractive candidates for studying to see if they influence risk of MI. Many studies have been published that have attempted to define this relationship and relative risk, often with both positive and negative results, and I will briefly discuss some of these in this section.

Glycoprotein Ibα polymorphisms

Three polymorphisms have been studied in the GPIbα gene - the human platelet antigen (HPA) 2 polymorphism, a threonine to methionine substitution at codon 145, the

variable number of tandem repeats (VNTR) polymorphism and the Kozak -5T>C promoter polymorphism. In a Finnish autopsy study [Mikkelsson *et al*, 2001], men with diagnoses of acute MI and coronary thrombosis at post mortem were found to have a higher risk of carrying the HPA-2 Met/VNTR B haplotype (OR 2.0 and 2.6 respectively) and these odds increased when taking into account only those less than 55 years old (OR 5.6 and 9.2). However, another group have not found either to be a risk factor for acute myocardial infarction [Rosenberg *et al*, 2002]. The Kozak polymorphism has also produced conflicting reports, with some investigators finding the T allele to confer increased risk of myocardial infarction [Douglas *et al*, 2002], some finding the C allele increases risk [Kenny *et al*, 2002] and others finding no association [Croft *et al*, 2000; Rosenberg *et al*, 2002].

Glycoprotein Ia polymorphisms

The GPIa 807C>T polymorphism has been extensively investigated with regard to thrombotic risk, however, the majority of these studies have been retrospective case control studies. One of the earlier reports, by Santoso *et al* [Santoso *et al*, 1999], found that in 2237 male subjects whom underwent angiography, carriage of the 807 T allele was significantly more common in subjects with a past history of MI compared to those without. This was in contrast with risk of coronary artery disease, where they failed to show any association with the GPIa 807 C>T polymorphism. Other groups have had similar positive findings [Casorelli *et al*, 2001; Moshfegh *et al*, 1999], but a number of investigators have also reported negative studies [Atherosclerosis Thrombosis and Vascular Biology Italian Study Group, 2003; Benze *et al*, 2002; Morita *et al*, 2001]. Of note, a prospective analysis of complications post coronary stent implantation failed to find an association between the incidence of thrombotic events within 30 days of PCI and carriage of the GPIa 807 T allele in 1797 consecutive patients [von Beckerath *et al*, 2000] casting further doubt on this proposed association.

Glycoprotein IIIa polymorphisms

In 2000, Zhu *et al* [Zhu *et al*, 2000] performed a meta-analysis of all published data up to October 1999, on the PLA1/2 polymorphism and risk of MI. In total, 26 reports were analysed. They came to the conclusion that there was no significant association between carriage of the PLA2 allele and risk of myocardial infarction (PLA2A2/A2A1 vs PLA1A1, OR=1.06 [0.97-1.16], p=0.2). Since then, two large studies have been

published with positive results. Mikkelsson *et al* [Mikkelsson *et al*, 2000] studied the association between the PLA1/A2 polymorphism and coronary thrombosis, MI and sudden cardiac death (SCD) in 700 male victims of sudden death from the Helsinki Sudden Death Study. Overall they found that 39.7% of SCD victims who were younger than 50 yrs carried the PLA2 allele compared with 28.3% of victims of death from other causes, who were of a similar age (OR 2.5, p=0.01). They concluded that carriage of the PLA2 allele is an important predictor of SCD in middle-aged men. Adding further support to the association, Bojesen *et al* [Bojesen *et al*, 2003] studied 9149 subjects from the prospective Copenhagen City Heart Study and found that homozygous carriage of the PLA2 allele conferred an increased risk of developing ischaemic heart disease in men compared to non-carriers (p=0.006). No association was observed in women.

Glycoprotein VI polymorphisms

To date, there has been little published data on associations between GPVI polymorphisms and risk of IHD. An initial report by Croft *et al* [Croft *et al*, 2001] identified an association between the GPVI 655 T>C polymorphism (serine to proline substitution) and risk of MI in 525 cases and 474 controls. Homozygosity for the C allele conferred an odds ratio of MI of 6.48 (1.47-28.45) compared to homozygosity for the T allele, but this was only apparent in those subjects over the age of 60 years. More recently, Ollikainen *et al* [Ollikainen *et al*, 2004] identified an association between carriage of the GPVI 655 C allele and death from coronary thrombosis in 300 middle-aged men from the Helsinki Sudden Death Study (OR 2.5 [1.05-6.20] p=0.02). It should be noted, however, that Joutsi-Kohonen *et al* [Joutsi-Korhonen *et al*, 2003] found subjects homozygous for the GPVI 655 C allele to have lower platelet GPVI expression and functional responses to collagen, and it would therefore seem unlikely that carriage of the C allele would confer an increased risk of MI or coronary thrombosis.

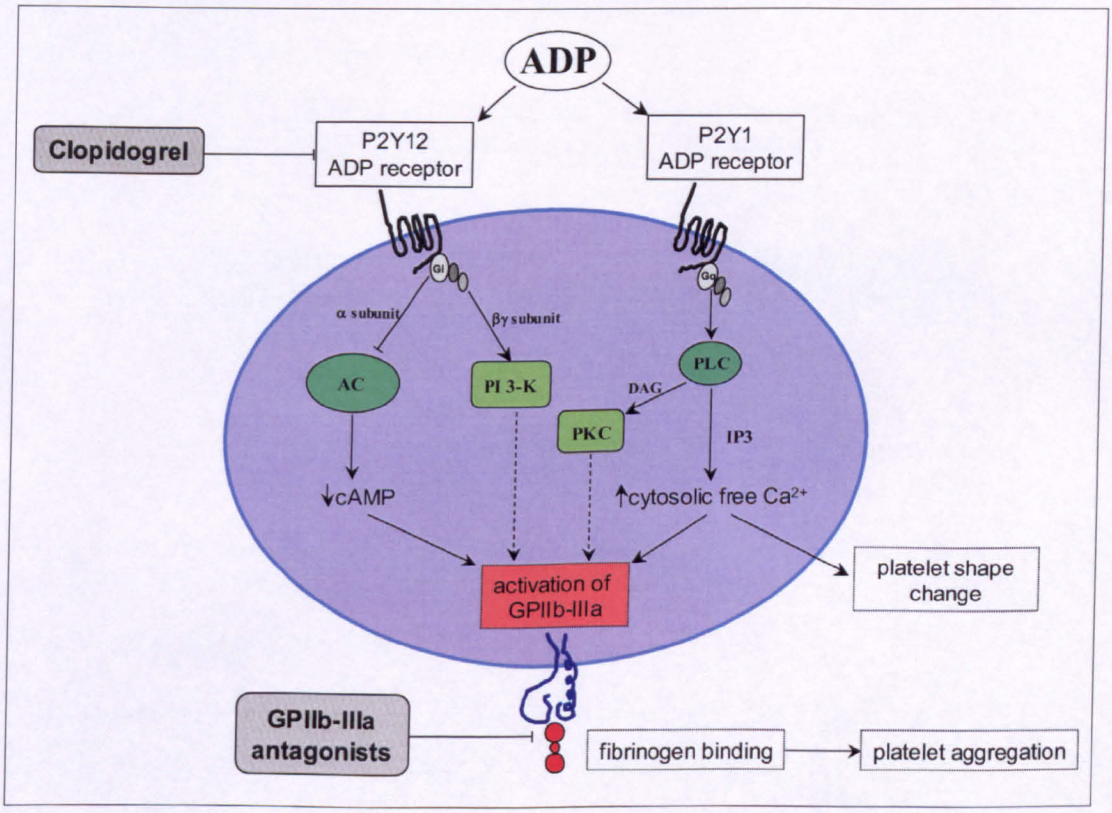
Agonist receptor polymorphisms

Unlike the *adhesion* receptor polymorphisms, data on the relationship between *agonist* receptor polymorphisms and risk of CHD or MI is relatively sparse. Thus they provide attractive targets for investigation, in terms of effects on platelet function and risk of MI.

1.3 The platelet adenosine diphosphate receptors

Adenosine diphosphate receptors belong to the family of purinergic receptors. Purinergic receptors are divided into two classes, adenosine receptors, labelled P1, and nucleotide receptors, labelled P2.[Mills, 1996] The P2 receptors are further classified into ligand-gated ion channels, P2X receptors, and G-protein coupled receptors (GPCRs), which are termed P2Y receptors. Platelets are known to express two different GPCRs, P2Y1 and P2Y12, whose physiological ligand is ADP, and one ligand-gated ion channel, P2X1, whose physiological ligand is ATP. It should be noted that although the platelet ADP receptors genes will be referred to as the P2Y1 and P2Y12 genes throughout this thesis, the author acknowledges that the approved symbols for these genes in the Human Genome Organisation Gene Nomenclature Committee (HGNC) database is P2RY1 and P2RY12 respectively. An overview platelet ADP-mediated signalling is shown in Figure 7.

Figure 7 ***Overview of ADP mediated platelet signalling and activation***



1.3.1 The P2Y1 ADP receptor

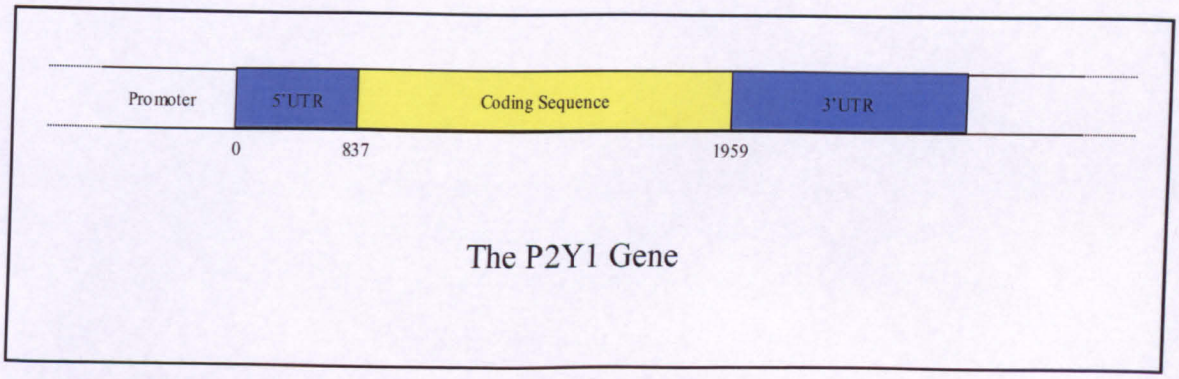
1.3.1.1 Discovery of the P2Y1 gene

The P2Y1 ADP receptor was the first of the purinergic GPCRs to be cloned. In 1996 Ayyanathan *et al* [Ayyanathan *et al*, 1996b] and Leon *et al* [Leon *et al*, 1996] independently cloned the P2Y1 receptor from a human erythroid leukaemia cell cDNA library and human placenta cDNA library respectively. They found the transcript to be widely expressed and, after initially localising the gene to chromosome 3, Ayyanathan *et al* [Ayyanathan *et al*, 1996a] went on to further map the gene to chromosome 3q25 using PCR of a subchromosomal hybrid panel.

1.3.1.2 Structure

The P2Y1 gene consists of a single exon (Figure 8), which encodes a 372-amino acid polypeptide. Northern blot analysis revealed two transcripts of 4.6 and 7.5 kilobases.[Leon *et al*, 1996] The complete receptor consists of seven hydrophobic transmembrane domains with three intracellular and three extracellular loops. The nucleotide-binding site of P2Y1 has been shown to reside in the transmembrane cleft of the second extracellular loop.[Moro *et al*, 1999]

Figure 8 ***Schematic representation of the P2Y1 ADP receptor gene***



1.3.1.3 Function and signalling

In platelets, the P2Y1 receptor couples with Gαq.[Kunapuli *et al*, 2003] As has been described earlier in this chapter (section 1.2.2.2), activation of GPCRs results in a conformational change in the Gα subunit, which causes exchange of GDP for GTP, and dissociation of the Gα from the Gβγ subunits. In the case of P2Y1, the GTP-bound Gαq

goes on to activate PLCβ with subsequent phosphotidylinositol hydrolysis to IP3 and diacylglycerol.[Jin *et al*, 1998a] IP3 acts on the dense tubular system permitting release of calcium ions, whilst diacylglycerol activates protein kinase C. This rapid increase in intracellular free calcium is the main mechanism by which P2Y1 signals further downstream intracellular events. A rise in cytosolic calcium is essential for full activation and conformational change of the GPIIb-IIIa receptor, but is also the main stimulus for platelet shape-change and plays an important role in mediating granule secretion.

With respect to platelet shape-change, calcium plays a major role through its interaction with calmodulin. The calcium-calmodulin complex activates myosin light-chain kinase, which in turn phosphorylates myosin, and facilitates its interaction with actin.[Paul *et al*, 1999] The end effect of this is cytoskeletal reorganisation and extension of filopodia. Shape-change of platelets from discoid to sphere occurs as a result of dissolution of the marginal microtubule coil. This has also been shown to be dependant on Gq-mediated activation, however in a calcium-independent manner, through the activation of the small GTP-binding protein RhoA and p160 Rho-associated coiled-coil-containing protein kinase.[Paul *et al*, 2003] Other effects of P2Y1 stimulation include activation of PLA2, through the rise in cytosolic calcium and activation of MAP kinase via diacylglycerol and PKC, which results in TxA2 synthesis and release.[Leslie, 1997]

The importance of stimulation and signalling through the P2Y1 ADP receptor in initiating platelet activation and aggregation has been extensively investigated.[Hechler *et al*, 1998; Jin *et al*, 1998a; Jin *et al*, 1998b] In addition, Leon *et al* [Leon *et al*, 1999] have shown that P2Y1 receptor-null mice are resistant to thromboembolism induced by intravenous injection of ADP, or collagen and epinephrine, further underscoring the potential importance of this receptor in the development and treatment of thrombotic disease.

1.3.2 The P2Y12 ADP receptor

1.3.2.1 Discovery of the P2Y12 gene

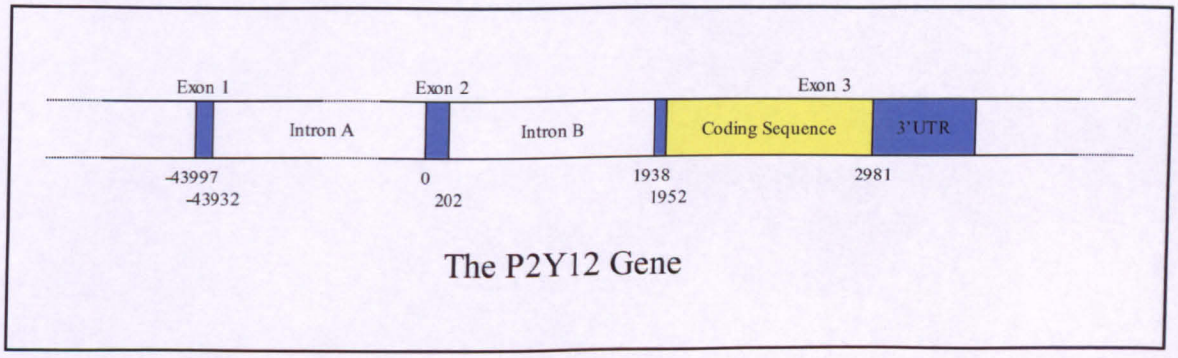
The P2Y12 ADP receptor is the most recent P2Y receptor to have been cloned. Daniel *et al* [Daniel *et al*, 1998] originally proposed that there were multiple ADP receptors on platelets in 1998, whereas prior to this, the general consensus was that all functional responses in platelets to ADP were likely to be mediated through a single type of ADP

receptor. It was not until 2001 that Hollopeter *et al* [Hollopeter *et al*, 2001] successfully cloned the P2Y12 receptor, using engineered Xenopus oocytes to allow detection of P2Y12 mediated responses using a sensitive electrophysiologic assay. Subsequent radiation hybrid analysis allowed them to map the gene to chromosome 3q24-25, a region that also contains the P2Y1 gene.

1.3.2.2 Structure

The P2Y12 gene consists of three exons (65, 202 and 1272bp) and two introns (~44kb and 1.7kb) (National Center for Biotechnology Information sequence database, accession number NT_086641.1). Exon 3 encodes the entire 342 amino acid protein (Figure 9). Hollopeter *et al* [Hollopeter *et al*, 2001] found P2Y12 receptors to be exclusively expressed by platelets and brain tissue. Northern blot analysis showed transcript species of 2.4 and 4.5 kilobases to be expressed in both tissues, whilst a shorter transcript of approximately 1000 bases was found exclusively in platelets. Sequencing of cDNA showed platelets to only express mRNA containing sequence from exons 2 and 3 (Figure 9). The receptor is of similar structure to P2Y1, with seven transmembrane domains, and three extracellular loops. There has not been any data published to date with regard to ligand binding sites on the P2Y12 receptor.

Figure 9 ***Schematic representation of the P2Y12 ADP receptor gene***



Nucleotides are numbered from the start of exon 2. Platelets have been shown to express transcripts containing sequence only from exons 2 and 3.[Hollopeter *et al*, 2001]

1.3.2.3 Function and signalling

Stimulation through the P2Y12 receptor in isolation is sufficient to cause weak activation of the GPIIb-IIIa receptor and platelet aggregation,[Kauffenstein *et al*, 2001]

however concurrent stimulation through P2Y1 and P2Y12 is required to permit full activation.[Jantzen *et al*, 1999; Jin *et al*, 1998b; Savi *et al*, 1998] The P2Y12 receptor is coupled to Giα2, which acts through the inhibition of adenylyl cyclase (AC), thereby effecting a reduction in platelet cAMP levels upon stimulation through this receptor.[Daniel *et al*, 1999]

A drop in cAMP levels alone, however, is insufficient to explain all of the intracellular events that occur following P2Y12 activation. Woulfe *et al* [Woulfe *et al*, 2002] have shown that stimulation of platelets through P2Y12 leads to activation of PI3K, which activates the small GTPase Rap1B in turn, and that this forms another important part of the signalling cascade that results in activation of the GPIIb-IIIa receptor.[Bertoni *et al*, 2002] Thomason *et al* [Thomason *et al*, 1994] had previously shown that the activation of PI3K in platelets could be stimulated through the addition of exogenous mixed Gβγ subunits, and thus it is likely that P2Y12-dependant activation of PI3K is mediated through this mechanism. Other effects of PI3K activation include potentiation of the platelet dense granule release reaction [Dangelmaier *et al*, 2001] and phosphorylation of the serine/threonine kinase Akt, which has also been shown to play an important role in platelet GPIIb-IIIa activation, aggregation and secretion.[Woulfe *et al*, 2004] A recent addition to our understanding of P2Y12 mediated responses has been the identification of G-protein-gated inward rectifying potassium (GIRK) channels as further important effectors that are activated following stimulation through this receptor. Shankar *et al* [Shankar *et al*, 2004] have shown that blockade of platelet GIRK channels results in inhibition of ADP induced platelet aggregation and Akt phosphorylation.

So, the P2Y12 receptor plays a pivotal function in mediating ADP-induced platelet activation, but there has also been extensive investigation into the role it plays in the response of platelets to other primary agonists, such as TxA2, thrombin and collagen. Storey *et al* [Storey *et al*, 2000] used a selective P2Y12 receptor antagonist to show that stimulation of P2Y12 by secreted ADP, plays a critical role in amplifying the response to other primary agonists such as TRAP, collagen and U46619 (a thromboxane analogue). Other workers have shown that P2Y12 responses also play an important part in mediating shear-induced binding, and platelet aggregation, to a vWF-collagen coated surface.[Turner *et al*, 2001] Further support for the central role of the P2Y12 receptor in platelet activation and thrombus formation has come from animal studies. Andre *et al* [Andre *et al*, 2003] generated P2Y12 receptor-null mice and investigated how its

complete absence affected the thrombotic process, using an *in vivo* mesenteric artery injury model. They showed that there was a longer delay to the development of the first thrombus following injury in P2Y12-/- mice, and thrombi that did form were smaller, unstable and non-occlusive. In humans, Hollopeter *et al* [Hollopeter *et al*, 2001] found that a patient with a bleeding diathesis and poor aggregation response to ADP carried a two base pair deletion in the coding sequence of the P2Y12 gene, which resulted in a frame shift, a premature stop codon, and a non-functional protein. Likewise, Cattaneo *et al* [Cattaneo *et al*, 2003] investigated a different patient with a bleeding tendency and poor ADP responses, and found the subject to carry two mutant alleles (nucleotide 1011G>A with Arg to Gln substitution at codon 256, and 1037C>T with Arg to Trp substitution at codon 265) as a compound heterozygote. Heterologous expression studies of the mutant P2Y12 receptors showed both polymorphisms to affect receptor function.

The importance of the P2Y12 receptor in atherothrombotic disease-states has been defined by the proven success of the non-competitive P2Y12 receptor antagonist clopidogrel in reducing morbidity and mortality in patients with stable [CAPRIE Investigators, 1996] and unstable [Yusuf *et al*, 2001a] atherothrombotic disease. In addition, blockade of the P2Y12 receptor is used routinely following intracoronary stent implantation to lower the risk of occurrence of subacute stent thrombosis.[Bertrand *et al*, 2000]

1.3.2.4 P2Y12 polymorphisms

At the current time, there has only been one published report of a systematic interrogation of the P2Y12 ADP receptor gene for sequence variation that may affect function. In 2003, Fontana *et al* [Fontana *et al*, 2003a] published data detailing sequencing of the region of the P2Y12 gene encompassing exons 2 and 3, and intron B, in 98 young, healthy adult males. They identified five polymorphisms, three in intron B, which they labelled i-C139T, i-T744C and i-ins801A, and two coding region polymorphisms, C34T and G52T, neither of which resulted in an amino acid substitution. Four of these polymorphisms were in complete linkage disequilibrium, i-C139T, i-T744C, i-ins801A and G52T, with i-139C, i-744T, i-del801 and 52G designated the major haplotype H1, and i-139T, i-744C, i-ins801A and 52T the minor haplotype H2. Haplotype frequencies for H1 and H2 were 86% and 14% respectively. Platelet aggregation in response to 1, 2 and 5 μmol/L ADP was then determined in PRP.

They found that carriers of the H2 haplotype had significantly higher maximal aggregation response to 2 μmol/L ADP (median values H1/H1=34.7%, H1/H2=67.9%, H2/H2=82.4%, p=0.0071 for the trend). The H2/H2 group, however only contained three subjects, two with a high response and one with a low response to ADP. Determination of the capacity of ADP to suppress iloprost-induced cAMP accumulation in 10 subjects each from the H1/H1 and H1/H2 groups showed carriers of the H2 allele to have a significantly greater capacity to suppress cAMP formation than non-carriers. Fontana *et al* [Fontana *et al*, 2003a] proposed that the mechanism by which the haplotype exerted its effect was likely due to linkage with promoter region polymorphisms that may affect transcription efficiency.

1.4 The Platelet Reactivity in Acute Myocardial Infarction Study (PRAMIS)

The Platelet Reactivity in Acute Myocardial Infarction Study (PRAMIS) was designed and undertaken by a previous Clinical Research Fellow within the Department of Cardiovascular Sciences at the University of Leicester (Dr Ravi Singh), as part of his MD Thesis. The purpose of the study was to recruit a large cohort of subjects who had suffered a Q wave or ST elevation myocardial infarction before the age of 50 years, and a group of healthy controls that were matched for certain demographic characteristic (such as their age, gender and current smoking status). Once the cohort had been recruited the main objectives of the study were to:

1. Determine the degree of inter-individual variation in platelet function in normal healthy individuals measured using flow cytometric techniques, to quantify the degree and identify any possible determinants of this variability

2. Assess whether platelet function and variability differs significantly between normal individuals and those with a history of premature MI

3. Analyse any contribution of the platelet GPIIIa 196C>T and GPIa 873G>A polymorphisms to the variability of platelet functional responses and to the risk of premature MI

A total of 205 premature MI cases were recruited from the Midlands Family Heart Study and the Coronary Care Unit databases from the three Leicester Hospitals. Two

hundred matched controls without a history of coronary artery disease were also recruited from two primary care practices within Leicestershire. Detailed phenotypic data was recorded on all subjects and blood was taken to determine the levels of factors that may affect thrombosis (such as plasma fibrinogen, homocysteine and full blood count), to perform flow cytometric analysis of platelet function and for DNA extraction and genotyping.

Platelet functional responses were determined by measuring fibrinogen-binding, using whole blood flow cytometry, in resting platelets and following stimulation with 0.1, 1 and 10 μmol/L ADP and 3, 10 and 30 μmol/L TRAP (SFLLRN) using previously described methods.[Janes *et al*, 1993] An additional, specific antibody to the GPIIb-IIIa receptor was used to quantify its expression in platelets. Whole blood DNA extraction was performed using a commercially available kit (Puregene DNA isolation kit, Flowgen, UK) using the manufacturers protocols. Following extraction, all samples were checked for yield and quality by measuring their optical density (OD), and working 96 well plates were created using the appropriate dilutions of the stock DNA.

1.5 Aims of work in this thesis

Given the incontrovertible evidence that platelets play a key role in the pathogenesis of acute myocardial infarction, and the wealth of data from both laboratory-based and clinical studies showing that a critical part of this pathological process is driven by the action of ADP on its specific receptors, the possibility that genetic variation in the ADP receptor genes may effect platelet activation in response to ADP, and risk of MI, warrants careful investigation. Identification of such genetic factors could have a large impact on the management of atherothrombotic disease in general, and in particular the targeting of primary and secondary prevention strategies. Furthermore, understanding the molecular mechanisms involved may lead to the development of novel therapeutic agents and diagnostic tests.

Therefore the aims of the work presented in this thesis are:

- To identify common and novel sequence variation in the P2Y1 and P2Y12 ADP receptor genes

- To determine whether the P2Y1 and P2Y12 gene polymorphisms contribute to variability in platelet function
- To investigate the mechanisms by which any polymorphisms that affect platelet function exert their effect
- To investigate whether common polymorphisms in the P2Y1 and P2Y12 genes influence risk of premature MI

Chapter 2

Methods

2.1 Overview of study

The aims of this study have been described in Chapter 1 (section 1.5). The important aspects of the strategy were:

1. The use of an efficient strategy, based around the direct sequencing of genomic DNA from subjects with divergent platelet responses, to identify common P2Y1 and P2Y12 ADP receptor gene polymorphisms
2. The use of high-throughput fluorogenic 5' nuclease TaqMan assays, in addition to conventional restriction-fragment length polymorphism analysis, to genotype the PRAMIS cohort
3. To study additional measures of platelet function (aggregometry) and signalling (calcium flux) in subjects homozygous for selected polymorphisms, allowing more robust characterisation of any effects of genotype on phenotype

In order to achieve the aims, the study is divided into two parts. The first part of the study consists of experiments undertaken to identify novel sequence variation within the P2Y1 and P2Y12 ADP receptor genes, and analyse their effects on platelet function and risk of premature MI, using DNA samples and data collected as part of PRAMIS (see Chapter 1, section 1.4 for details of PRAMIS). The specific methods used in this part of the study include direct sequencing of polymerase chain reaction (PCR)-amplified genomic DNA in subjects with extreme phenotypes, to identify novel genetic polymorphisms, and the use of restriction fragment length polymorphism (RFLP) analysis and TaqMan assays for genotyping.

The second part of the study consists of functional studies (similar to those used in PRAMIS) performed initially, to confirm the findings of the first part of the study using subjects recruited from a different cohort (namely the Genetic Regulation of Arterial Pressure of Humans in the Community (GRAPHIC) study), and thereafter to investigate possible mechanisms by which any functional polymorphisms exert their effects. The specific methods used in this part of the study include flow cytometric analysis of platelet fibrinogen-binding, light transmittance platelet aggregometry, fluorometric calcium flux determination, and quantification of platelet receptor numbers by various methods.

The GRAPHIC study was designed to investigate the gene-environment regulation of blood pressure in 500 (unselected) nuclear families consisting of both parents (40-60 years) and two adult children (20-40 years) and was recruited from three primary care practices located within Leicestershire. The methods used to select subjects from the GRAPHIC study for the second part of this study, are described below (section 2.3.2)

2.2 Ethical approval

The Leicestershire Research Ethics Committee had previously granted full ethical approval for PRAMIS (ref no. 5506) and the GRAPHIC study (ref no. 6463). This included approval to use the DNA that had been collected for other studies investigating cardiovascular disease. However, this did not include recalling patients from the GRAPHIC study on the basis of their genotype, so that they could give further samples of blood for the additional specific functional work proposed as part of this study. Additional ethical approval was sought for this, and was received on 13/01/2004 (ref no. 7195).

2.3 Subjects

The first part of the study did not require any additional subjects to be recruited as the sequencing and genotyping was performed on previously extracted DNA from subjects that had already been recruited as part of PRAMIS. The second round of functional analyses were performed *de novo* on selected subjects from the GRAPHIC study.

2.3.1 Identification of subjects for sequencing

In order to maximise the possibility of identifying functional polymorphisms in the two ADP receptor genes, subjects selected for sequencing were chosen from the PRAMIS control cohort on the basis of their platelet response to a mid-range dose of ADP (1 μmol/L). Ten subjects were chosen at random from each of the extremes of the platelet response range, i.e. from the 10% of subjects with the highest and the 10% of subjects with the lowest fibrinogen-binding response to 1 μmol/L ADP. Detailed phenotypic data was already available for all subjects recruited as part of PRAMIS.

2.3.2 Identification of subjects for functional studies

Subjects were recruited from the GRAPHIC cohort for the secondary functional studies based upon their genotype for the relevant polymorphism of interest. Only subjects with homozygous carriage of the major and minor alleles were recruited to this part of the study, so that there would be the highest likelihood of detecting a difference between the genotype groups. This effectively reduced the number of subjects that would need to be recruited to identify a significant effect.

Once a subject had been identified as eligible on the basis of their genotype, an invitation letter and patient information sheet was sent to the subject by post. After a period of approximately two weeks the subject was contacted by telephone to verify that the information had been received, to answer any questions regarding the study, and to ascertain whether the subject was happy to participate. If the subject agreed to participate, clear instructions were given to avoid aspirin for a period of two weeks before the study visit, and an appointment was arranged for the early morning (before 10am) to avoid potential effects of circadian variations.[Tofler GH *et al*, 1987]

On the day of the study visit the following was undertaken:

1. Written consent obtained

2. The following data was recorded in hard copy:

    a) GRAPHIC study unique identifier

    b) Age and gender

    c) Smoking history

    d) Personal or family history of atherothrombotic disease

    e) Blood pressure – single reading using Omron device

    f) Height (in metres) and weight (in kilograms) to calculate body mass index

3. Approximately 20ml of blood was taken from the left antecubital fossa for the following investigations:

    a) Full blood count

    b) Flow cytometric analysis of fibrinogen binding

    c) Platelet aggregation

d) Platelet calcium flux

e) Determination of platelet ADP receptor expression

A small number of subjects (n=7) were recalled for a second visit, to give a further 20ml sample of blood from which platelet messenger RNA (mRNA) was extracted and used to prepare cDNA.

2.4 Phlebotomy and sample collection

Platelets are easily activated during the process of sample collection. Because of this, blood was taken using a standardised phlebotomy technique[Hjemdahl *et al*, 1994] and only after the subject had been resting supine for at least 15 minutes. After collection, samples needed to be processed quickly to minimise leakage of intracellular components and down-regulation of surface receptors.

Blood was taken by clean venepuncture of the antecubital vein without the use of a tourniquet, via a 21 gauge butterfly needle into Vacutainers (Becton Dickinson, Oxford,UK). Whole blood was collected in a strict order. Due to the laborious nature of flow cytometric techniques and the method used to prepare washed platelets for the calcium flux experiments, the platelet samples used for receptor determinations were prepared, and then frozen for use at a later date. Table 3 details the order in which blood was taken and any special considerations relating to sample preparation. The precautions taken with phlebotomy and sample collection in this study, in particular with respect to the samples used for flow cytometry, were identical to those taken in PRAMIS. More detailed methods follow later in this chapter.

Table 3 ***Order of blood collection***

Tube Number	Analysis	Volume	Type of tube	Special Considerations
1	Full blood count and platelet volume.	5ml.	EDTA	Full blood count and mean platelet volume was measured following 2 hours at room temperature, to allow platelet volume to stabilise
2	Flow cytometric analysis of platelet function	0.5ml	Citrate	Initial sample processing within 10 minutes. Fibrinogen-binding determined within 2 hours of sample fixation
2 and 3	Platelet aggregation and calcium flux	9.5ml	Citrate	Process sample within 10 minutes. Centrifuge at 134g for 20 minutes at room temperature to prepare platelet rich plasma. Platelet aggregation determined within 30 minutes of initial centrifugation
4 and 5	Preparation of washed platelets for receptor quantification studies	10ml	Citrate, theophylline, adenosine, and dipyridamole	Process sample within 10 minutes. Centrifuge at 134g for 20 minutes at room temperature to prepare platelet rich plasma. Add aspirin, followed by further centrifugation at 535g for 15 minutes to sediment platelets. Resuspend in 500µl of hepes-buffered saline and store at -70°C

2.5 Genetic analysis

Genetic analysis was performed on DNA that had previously been extracted, quantified, diluted to 20ng per µl and aliquotted into 96 well plates, as part of PRAMIS or GRAPHIC.

2.5.1 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 and centrifugation to produce a white cell pellet. This was resuspended in a cell lysis solution to release the white cell contents, and was followed by subsequent treatment with RNAse and protein precipitation solution. Further centrifugation was performed and the supernatant containing the DNA was poured into 100% isopropanol to precipitate the DNA, which was then washed with 70% ethanol and resuspended in the hydration solution. All samples were checked for yield and quality by optical density (OD) readings on a photometer, and then diluted to 20ng per µl and aliquotted into 96 well, working stock plates.

2.5.2 Polymerase chain reaction

Polymerase chain reaction was used to amplify segments of the P2Y1 gene, including the exon and 500bp of upstream sequence, and the region of the P2Y12 gene encompassing exons 2 and 3, and intron B. Five sets of overlapping primer pairs were required for each gene to ensure complete coverage of the regions to be sequenced. Primers were designed using online software (Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and with melting temperatures (Tm's) of approximately 64°C, so that PCR cycling parameters would be similar for each primer pair. All primers were synthesised by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester. Specific details of the primers used are shown in Figure 10 and Table 4.

Figure 10 ***Schematic diagrams of the P2Y1 and P2Y12 genes showing positions of sequencing primers***

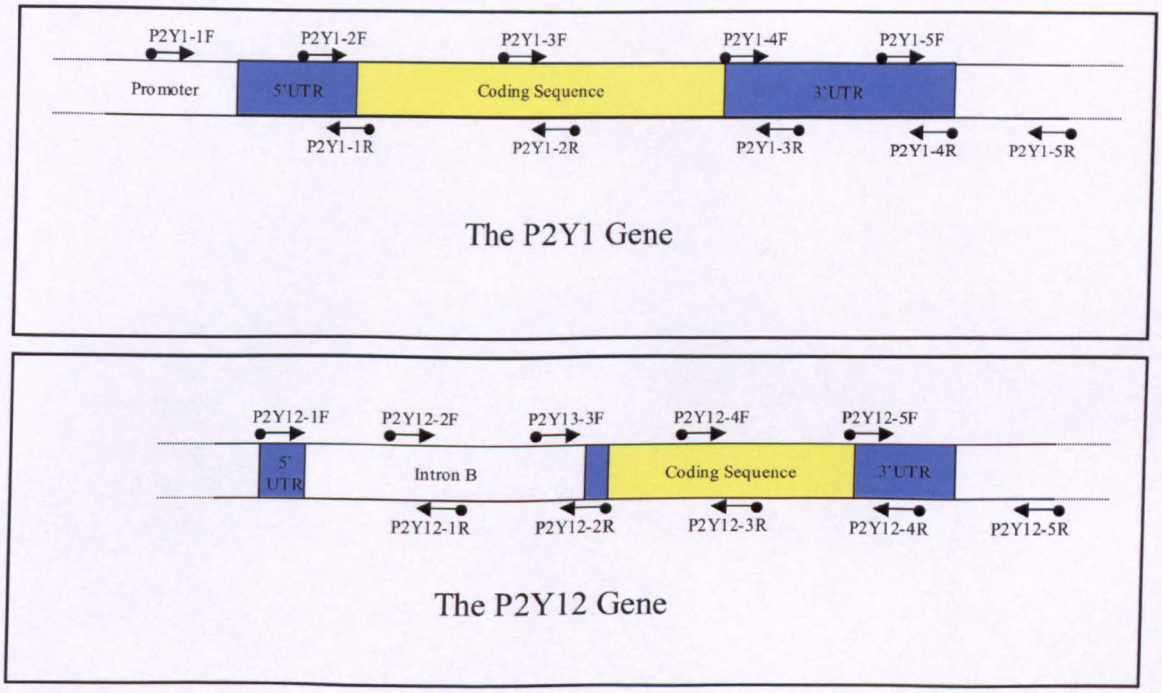


Table 4 ***Oligonucleotide primers used for amplifying and sequencing of P2Y1 and P2Y12***

Primer pairs	Position and sequence	Product size (bp)
P2Y1-1	F (-536) 5'- TTTTGGAAATTCTCACGTTGATGTT-3' (-512) R (473) 5'-GAGTTTCCCTTGACCTCGCTG-3' (453)	1009
P2Y1-2	F (345) 5'-TGAGCTGCACGTTTCTAAGGTAGG-3' (368) R (1354) 5'-GAAGAATGCGATCTGTATCAGCGT-3' (1331)	1010
P2Y1-3	F (1200) 5'-GCCATGTGTAAACTGCAGAGGTTC-3' (1223) R (2146) 5'-CTTGTTTGGGTTTGCTTTCACAGT-3' (2123)	947
P2Y1-4	F (1958) 5'-AAGGCACAAGAATCTCCAAACACC-3' (1981) R (3003) 5'-CTCAAATGTATGAAGCAACTGGGG-3' (2980)	1046
P2Y1-5	F (2744) 5'-AAAGAGCATTTACTTGCCCCACTG-3' (2767) R (3490) 5'-CCAAACAAATCCAGTGTCAAAGGA-3' (3467)	747
P2Y12-1	F (-10) 5'-TCTCTGATTGTGAAGCCCTC-3' (10) R (773)* 5'-TGGCATCTACATCTTGGGAA-3' (754)*	986
P2Y12-2	F (578)* 5'-CATTTTGGGGAATTTAAGTGC-3' (598)* R (1723)* 5'-GAGAGGATGGTTATTTTCAGCC-3' (1702)*	1146
P2Y12-3	F (1634)* 5'-TCAACTTTTAGAGGAGGCTGTG-3' (1655)* R (920) 5'-TTCACCTTTTTCCTGGGGA-3' (902)	822
P2Y12-4	F (611) 5'-CATCCAACCCCAAAAATCTC-3' (630) R (1624) 5'-TCGTTCTTCCTTAGTTGATTATGA-3' (1601)	1014
P2Y12-5	F (1203) 5'-AGAACAGGATGGTGGTGACC-3' (1222) R (2279) 5'-TGTATATGGTATGGTGAGTCATGG-3' (2256)	1077

F – forward primer, R – reverse primer. Nucleotide positions are given in relation to the transcription start site for the P2Y1 gene and the start of exon 2 for the P2Y12 gene, except those in intron B of P2Y12 (*), which are given in relation to the start of that intron.

2.5.2.1 Methods

60ng of the extracted genomic DNA was used to prime the PCR reaction for each primer pair. PCR was performed in 0.2ml thin-walled PCR tubes, on a MJ Research Peltier Thermal Cycler (MJ Research, Waltham, MA). A proof-reading DNA polymerase, Biolase Diamond (Bioline, UK), was used to amplify products for sequencing. The master mix for these reactions contained 10x Reaction Buffer (160 mmol/L $(NH_4)_2SO_4$, 670 mmol/L Tris-HCl pH 8.8, 0.1% Tween-20), 2 mmol/L $MgCl_2$, 1.25 mmol/L dNTP mix, 100-250 nmol/L each primer and Biolase Diamond. Specific primer requirements were determined through optimisation of each individual reaction. The PCR program is detailed below, and took approximately 3 hrs to complete.

PCR Program:

HOLD:	95°C for 3 min.
CYCLE:	95°C for 1 min, 58-60°C for 1 min, 72°C for 2 min x 33 cycles.
HOLD:	72°C for 2 min.
HOLD:	10°C until tubes were removed.

Annealing temperature requirements were again determined following optimisation of specific reactions.

Following amplification, PCR products were resolved using 1% agarose gel electrophoresis following staining with ethidium bromide and gel-purified using TaKaRa Recochips (Cambrex, UK). Recochips allow PCR products to be reclaimed from agarose gels without the use of additional reagents. In principle, they work by eluting the PCR product from the gel onto cellulose dialysis membrane, which is housed within the recochip. The product can then be recovered from the recochip by centrifugation at 5000rpm for 5 seconds. The resultant highly purified PCR product is supplied in a small volume of electrophoresis buffer (Tris-HCl, sodium acetate, EDTA) and could be used directly for priming sequencing reactions after quantification against a suitable mass ladder, such as Hyperladder I (Bioline,UK).

2.5.3 Sequencing

Direct sequencing of purified PCR products was performed using a two-step cycle sequencing reaction and Big Dye Terminator v3 chemistry (Applera, UK). In brief, 40-60ng of PCR product was used per reaction, depending on amplicon length (5ng per 100bp of product). This was carried out using the same primers as for the PCR reaction,

with 0.5µl of Big Dye reaction mix per 10µl reaction. Cycle sequencing was performed in ABI PRISM 96 well optical plates on an MJ Research Peltier Thermal Cycler using the program detailed below.

Sequencing program:

HOLD: 96°C for 2 min.

CYCLE: 96°C for 30 sec, 60°C for 4 min x 40 cycles.

HOLD: 14°C until tubes were removed.

Following completion of the reaction the sequencing products were precipitated in 50% isopropanol, 5 mmol/L $MgCl_2$ for one hour at room temperature, washed in 70% ethanol, and resuspended in 10µl formamide Hi-Di (Applera, UK). The sequencing products were then resolved on an ABI PRISM® 3700 Gene Analyzer, and analysed using Sequencing Analysis v3.7 software (Applera, UK).

Certain P2Y12 amplicons required additional internal primers to allow full sequencing of the fragments.

2.5.4 Genotyping

Following the identification of ADP receptor gene polymorphisms through sequencing, genotyping strategies were designed to allow selected polymorphisms to be typed in the full PRAMIS cohort of approximately 400 subjects. In addition, a high throughput method for genotyping the GRAPHIC cohort was also required.

2.5.4.1 Restriction fragment length polymorphism analysis

Two polymorphisms were genotyped using RFLP analysis. This method utilises restriction endonucleases to cleave PCR amplification products, which are then resolved on agarose gels stained with ethidium bromide. Restriction endonucleases recognise specific base sequences within the DNA, and cleave the DNA as a result. Thus, once resolved on agarose gels, the specific distribution of DNA bands allows the reader to determine which alleles are carried by an individual, for the polymorphism under investigation.

The method used was similar for the two polymorphisms genotyped using this technique. Essentially a region of gene was amplified that flanked the polymorphism to be investigated, using the appropriate primer pair that had been used for initial sequencing, and the PCR method described above. Following completion of the PCR

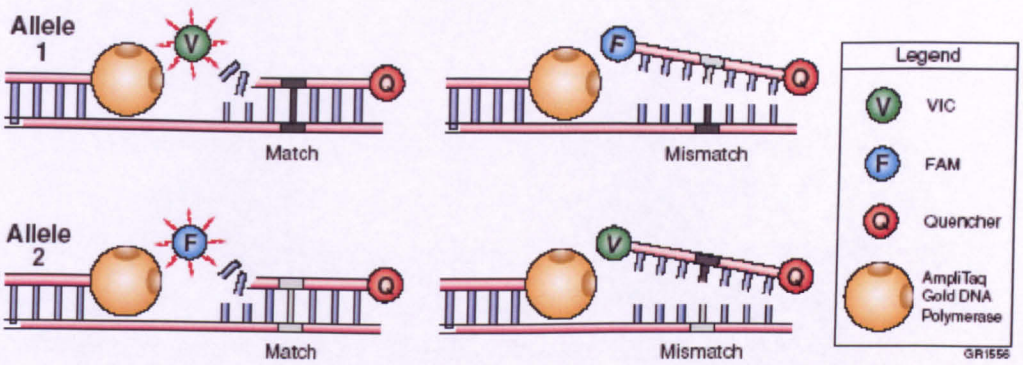
reaction, the PCR products were incubated with the appropriate restriction endonuclease and its buffer for 16 hours following the manufacturers standard conditions (supplied by New England BioLabs, UK in both cases). The cleaved products were then resolved on agarose gels stained with ethidium bromide. Specific details of endonucleases used, reaction conditions and size of cleaved products are given in Chapter 4.

2.5.4.2 TaqMan assay for allelic discrimination

Livak originally described the TaqMan assay for allelic discrimination.[Livak, 1999] The principle of this method is the use of fluorescent dye-labelled, oligonucleotide probes specific for the two alleles of the polymorphism under investigation, in a 5' nuclease PCR assay. The probes contain different reporter dyes to allow the differentiation of the two alleles, which are covalently linked to the 5' nucleotide. A non-fluorescent quencher is bound to the 3' nucleotide, in addition to a minor groove binder (MGB). The MGB moiety raises the Tm of the probe allowing shorter probes to be designed.[Kutyavin *et al*, 2000] As a consequence the TaqMan MGB probes have a greater Tm difference between matched and mismatched bound probes, resulting in greater specificity.

During PCR, each probe anneals specifically to complementary sequences between the forward and reverse primer sites. The Taq DNA polymerase used in the assay, AmpliTaq Gold®, can cleave only probes that hybridise completely to the DNA strand. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter dye. As a result, the fluorescence signals generated by PCR amplification indicate the alleles that are present in the sample. Mismatched probes reduce the hybridization efficiency of that probe to the DNA, and mismatched probes are more readily displaced by AmpliTaq Gold®, rather than cleaved, so resulting in specific increases in fluorescence only with matched probes. Figure 11 illustrates the effect of matched and mismatched probe hybridisation in the allelic discrimination assay.

Figure 11 ***Matched and mismatched probe hybridisation in the TaqMan allelic discrimination assay***



TaqMan assays were designed for the remaining polymorphisms, and also for the screening of the GRAPHIC cohort. All allelic discrimination assays were performed on an ABI PRISM 7900HT Sequence Detection System (SDS) (Applera, UK). TaqMan MGB probes and primers were designed in-house using Primer Express v2.0 software using the standard protocols. In particular, probes were designed with a Tm of between 65-67°C and primers between 58-60°C and amplicon size was kept as small as possible, without the primers overlapping the probes. Probes specific for the more common allele were labelled with 6-FAM whilst probes specific for the less common allele were labelled with VIC. 20ng of genomic DNA was used per 15µl reaction and reactions were performed in ABI PRISM 96-well optical plates. The PCR master mix consisted of 2x TaqMan Universal PCR Mix (no amp-Erase UNG) (Applera, UK), which contained the appropriate buffer, dNTPs and AmpliTaq Gold®, with 800 nmol/L of each primer and 130 nmol/L of each probe. Primer and probe concentrations were derived through optimization/titration. Specific details of the primers and probes used for each polymorphism are given in Chapter 4.

A two-step PCR program was used, and is described below:

TaqMan program:

HOLD: 95°C for 10 min. - required to activated AmpliTaq Gold®

CYCLE: 92°C for 15 sec, 60°C for 1 min x 45 cycles.

HOLD: 10°C until plate-read performed.

Following completion of the PCR program, a final plate read was performed on the ABI PRISM 7900HT and the output data was analysed using the SDS software. Auto-calling was used where possible, however in the event of an indeterminate call, the real-time amplification plots were analysed to allow confidence in manual calling.

2.5.5 Messenger RNA extraction

A small number of subjects recruited from the GRAPHIC study for the secondary functional analyses were called back to give blood on a second occasion, for the purpose of mRNA extraction. The Dynabead method of extracting mRNA was used instead of total RNA preparation to improve the mRNA yield when attempting extraction from cell preparations that are of a relatively small volume.

2.5.5.1 Method

As mRNA is easily degraded by RNases that are relatively abundant in most environments, exceptional care was taken to prevent contamination and degradation of samples through the use of dedicated pipettes, frequent gloves changes and keeping samples on ice where appropriate.

20ml of whole blood was collected into Vacutainer tubes containing a combination of anticoagulants and platelet inhibitors (citrate, theophylline, adenosine and dipyridamole) whose presence was necessary to prevent platelet activation during the centrifugation steps. The blood was centrifuged at 134g at room temperature for 20 minutes to prepare PRP. The PRP was then removed with care taken to avoid disturbing the buffy coat and avoid the walls of the Vacutainer tubes (measures to minimise white cell contamination). PRP containing approximately 10^9 platelets was then centrifuged at 535g for 15 minutes at room temperature to sediment the platelets. The supernatant was carefully removed and 1ml of lysis buffer was added (100mmol/L Tris-HCl pH 8.0, 500mmol/L LiCl, 10mmol/L EDTA, % LiDS, 5mmol/L DTT). The platelet pellet was then lysed by pipetting.

100µl of washed Oligo $dT_{(25)}$ Dynabeads (Dynal, UK) were then added to the platelet lysate and incubated at room temperature for 5 minutes with continuous rotation. The Dynabeads with bound platelet mRNA were then separated from the aqueous phase using an MPC-2 magnetic device (Dynal, UK), and the supernatant was removed with a pipette. The Dynabeads were washed twice in wash buffer A (10mmol/L Tris-HCl pH 8.0, 150mmol/L LiCl, 1mmol/L EDTA, 0.1% LiDS), followed by a further two washes

in wash buffer B (10mmol/L Tris-HCl pH 8.0, 150mmol/L LiCl, 1mmol/L EDTA). The Dynabead bound mRNA was separated from the aqueous phase after each wash using the MPC-2 magnetic device. Finally the mRNA (Dynabead bound) was resuspended in RNase free TE buffer (10mmol/L Tris-HCl pH 8.0, 1mmol/L EDTA) and used immediately for cDNA synthesis.

2.5.6 Reverse transcriptase polymerase chain reaction to quantify P2Y1 mRNA levels

Reverse transcriptase PCR consists of two steps. The initial step is first strand synthesis, whereby a reverse transcriptase is used to synthesise a single strand of complementary DNA (cDNA) for each molecule of mRNA that is present in the reaction. Usually oligo-dT is used to prime this reaction, however, when using Dynabead prepared mRNA, additional oligo-dT is not required as this is already found as an integral part of the Dynabeads themselves. The second step consists of conventional PCR primed using the cDNA synthesised in the initial step. Multiple optimisations are required to standardise the PCR reaction, followed by titration of the quantity of priming cDNA for each subject using primers specific for a constitutively expressed, house-keeping gene, such as the GAPDH gene, to normalise the quantity of cDNA of each subject that is analysed. This then allows the researcher to quantify the cDNA, and hence mRNA expression, specific to his gene of interest, relative to the expression of the house-keeping gene.

2.5.6.1 First strand cDNA synthesis

A commercial kit was used to perform first strand cDNA synthesis (Superscript II RT, Invitrogen, UK) using platelet mRNA that was extracted as detailed in the preceding section. In brief, 12μl of Dynabead-bound mRNA was mixed with 2μl 10mmol/L dNTP mix and 10μl of RNase-free water, and incubated at 65°C for 5 minutes. After this time the reaction was rapidly chilled on ice. Subsequently, 8μl of 5X First-Strand Buffer (250 mmol/L Tris-HCl pH 8.3, 375 mmol/L KCl, 15 mmol/L MgCl2), 4μl of 0.1M DTT and 2μl of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen, UK) was added to the reaction, followed by a further incubation at 42°C for 2 minutes. Finally, 2μl (400 units) of Superscript II RT was added, and the reaction was incubated for 50 minutes, at 42°C. After this time, a final incubation step of 70°C for 15 minutes was performed to inactivate the reverse transcriptase. This reaction protocol produced 40μl

of platelet cDNA for each subject, which was further diluted to 160μl with RNase and DNase-free water, and stored frozen at -70°C in 40μl aliquots.

2.5.6.2 Polymerase chain reaction of platelet cDNA

Quantification of P2Y1 mRNA was performed relative to the level of GAPDH expression, which was chosen as the constitutively expressed house-keeping gene. Initially, the PCR reaction used to amplify GAPDH cDNA was optimised by adjusting the number of cycles, so that the reaction was terminated whilst still in the linear phase of amplification. During this phase, doubling the quantity of cDNA used to prime the reaction will result in a doubling of the intensity of the DNA band visualised following agarose gel electrophoresis, as measured by spot densitometry, allowing an estimation of expression to be inferred. Once the reaction had been refined so that this was the case, the quantity of cDNA added to the GAPDH PCR reaction was adjusted so that approximately the same intensity of band was observed for each subject. These quantities would then be used when amplifying cDNA specific to the ADP receptor genes, allowing relative differences between subjects to be estimated. In the case of GAPDH, 250nM of each primer was used and 1.0-4.1μl of cDNA. The PCR program used for GAPDH amplification is detailed below.

GAPDH RT-PCR Program:

HOLD:	95°C for 3 min.
CYCLE:	95°C for 1 min, 58°C for 1 min, 72°C for 1 min x 24 cycles.
HOLD:	72°C for 2 min.
HOLD:	10°C until tubes were removed.

Details of the specific primers and reaction conditions used for the ADP receptor genes are given in Chapter 5.

2.6 Platelet count and mean platelet volume

Platelet count and, in particular, MPV have previously been found to have an effect on platelet activation. In view of this, they were determined as part of the secondary studies to complement the other tests of platelet function utilised, and to ensure that any differences in these measures between genotype groups could be assessed and compensated for as necessary, when performing the statistical analysis.

White cell count, red cell count, platelet count and MPV were measured in citrated whole blood that had been left to stand at room temperature for 2 hours, to allow stabilisation of MPV, using a Coulter® A$^{C\cdot}$T diff™ analyser, that was calibrated each day.

2.7 Flow cytometry

Flow cytometric determination of fibrinogen-binding to the activated GPIIb-IIIa receptor was one of two methods used to measure platelet activation in the secondary functional studies in subjects recruited from the GRAPHIC study. This was chosen as it is the final common pathway that occurs following activation of platelets through any means, and it is this interaction which must occur for platelet aggregation to take place.

In the following sections I will only describe the flow cytometry methods relevant to the secondary functional assays undertaken in the subjects recruited from the GRAPHIC study. The methods used in PRAMIS can be found in Chapter 1, and are essentially the same as those described below, with the exception that TRAP was used in PRAMIS without the addition of ADP receptor antagonists.

2.7.1 Reagents

2.7.1.1 Agonists

The agonists used for the flow cytometry studies were ADP (Sigma, UK) and TRAP-SFLLRN (PNACL, University of Leicester, UK). Titrations were performed for each agonist to determine the optimum concentrations to use. Three concentrations of ADP were used (0.1, 1 and 10 μmol/L) to give low, intermediate and maximum platelet responses, whilst a single concentration of TRAP was used (30 μmol/L). The various concentrations were produced in one batch by serial dilution, aliquoted in appropriate volumes, and stored at -70°C for single use. Platelet function was quantified as fibrinogen-binding to activated GPIIb-IIIa receptors in resting samples, and following stimulation with ADP and TRAP. The rationale of using TRAP as an agonist, was so that a comparison could be made between TRAP and ADP responses between the genotype groups, allowing an inference to be made as to whether any difference seen in ADP response is due to a specific effect through the ADP receptor pathway, or as a result of generalised hyper-responsiveness of those subjects' platelets.

2.7.1.2 Antagonists

A significant proportion of the platelet activation induced by TRAP is mediated via the release of ADP from dense granules within the platelets, which then act upon the P2Y1 and P2Y12 ADP receptors.[Storey *et al*, 2001] Thus, in order to measure the TRAP response specifically, it was felt that it would be important to remove any contribution from P2Y1 and P2Y12 receptor stimulation. To achieve this, a specific P2Y1 receptor antagonist, MRS2179 (Sigma, UK) and a specific P2Y12 receptor antagonist, AR-C69931MX (Astra-Zeneca, UK) were used to block the effect of released ADP. The final concentrations, determined by titration, were 10 μmol/L and 0.1 μmol/L respectively.

2.7.1.3 Antibody

Fibrinogen bound to activated GPIIb-IIIa receptors on platelets was detected using polyclonal rabbit anti-fibrinogen, which was conjugated to fluoroscein isothiocyanate (FITC) (DakoCytomation, UK). The antibody was used at its optimal concentration, giving the best signal-to-background ratio, which had been determined previously as part of PRAMIS.

2.7.2 Assay procedure

The assay procedure was essentially the same as that which had been used in PRAMIS. This was based around the whole blood flow cytometric method described by Janes *et al.*[Janes *et al*, 1993] Care was taken to avoid artificial platelet activation during the process of phlebotomy and sample preparation, and blood was used within ten minutes of collection.

5μl of citrated whole blood was added to each of eleven LP3 tubes containing 2μl of anti-fibrinogen, 5μl each of agonists and antagonists where appropriate and made up to a final reaction volume of 50μl with hepes buffered saline (HBS) pH 7.4. After gentle mixing, the tubes were incubated at room temperature for 25 minutes. Following this, 450μl of 0.2% (v/v) formyl saline was added to each tube to fix the sample. After a further ten minutes at room temperature a 50μl aliquot of each sample was diluted 10-fold with 450μl formyl saline into LP4 tubes, and applied to the flow cytometer within 2 hours. The running order and specific contents of each tube is given in Table 5. Samples were run in duplicate, except for the negative control.

A Profile EPICS-XL MCL flow cytometer (Coulter Electronics Limited, UK) was used to analyse the samples in an automated fashion. The flow cytometer was aligned on a daily basis with Immunocheck beads (Coulter Immunology, UK) to ensure standardised calibration throughout the study. The platelet population was identified from its light scatter characteristics, and gated for single colour analysis. In total, 5000 platelets were analysed from each sample. Fibrinogen-binding was recorded as the percentage of platelets positive for bound antibody and median fluorescence.

Table 5 ***Sample running order and specific tube contents***

Sample Number	Agonist	Antagonist	Volume of HBS (μl)
1	-	(6 mmol/L EDTA)	41
2/3	-	-	43
4/5	0.1 μmol/L ADP	-	38
6/7	1 μmol/L ADP	-	38
8/9	10 μmol/L ADP	-	38
10/11	30 μmol/L TRAP	10 μmol/L MRS2179 0.3 μmol/L AR-C69931MX	28

Sample 1 was the negative control, used to estimate non-specific fibrinogen-binding in the absence of platelet agonist, and samples 2 and 3 were resting samples, giving an indirect measure of artificial platelet activation induced by sample handling and preparation.

2.8 Aggregometry

Light transmittance aggregometry was performed essentially as described by Born,[Born GVR, 1962a] in stirred PRP, normalised to 2 x 10^5 platelets/μl.

2.8.1 Reagents

A single agonist, 2-methylthio-ADP (2MeSADP) (Sigma, UK), was used to stimulate platelet aggregation. This potent ADP analogue is stable in aqueous solution and non-hydrolysable. It was chosen for the aggregation assay for this reason, and also for consistency when comparing the aggregation data with calcium flux data, where it was not possible to use ADP due to the use of apyrase. A titration was performed in a single

subject to determine the optimum concentration of 2MeSADP to use. Three concentrations of 2MeSADP were employed (40, 80 and 160 nmol/L) to give low, intermediate and maximum platelet aggregation responses.

100 μmol/L aspirin (acetylsalicylic acid) was also used in the aggregation assay to block the synthesis of TxA2, as it is widely recognised that when stimulating platelets with "weak" agonists (such as ADP), in conditions where there is close cell-cell contact and a relatively low extracellular calcium concentration (such as occurs following citrate anticoagulation), TxA2 is rapidly generated and augments the platelet activation response.[Born GVR, 1962a; Packham *et al*, 1987] This close cell contact-dependant TxA2 generation has been shown to require "outside-in" signalling through the GPIIb-IIIa receptor.[Jin *et al*, 2002]

2.8.2 Assay procedure

PRP was prepared by centrifugation at room temperature of citrated whole blood, within 10 minutes of collection, at 134g for 20 minutes. Autologous platelet poor plasma (PPP) was prepared by centrifugation of whole blood for 10 minutes at 1500g. The platelet count of the PRP was determined using a Coulter® A$^{C.}$T diff™ analyser (Coulter Electronics Limited, UK), following which the PRP was normalised to 2 x 10^5 platelet/μl by adding the appropriate volume of autologous PPP. Aspirin was added to the normalised PRP to block TxA2 production. 450μl aliquots were then incubated at 37°C for 3 minutes prior to stimulation with 2MeSADP. Platelet aggregation was measured in a PAP4 platelet aggregation profiler (Bio/Data Corporation, USA), for ten minutes and expressed as the percentage increase in light transmittance, compared to autologous PPP. Maximal platelet aggregation and initial rate of aggregation were recorded for analysis.

2.9 Calcium flux

The platelet calcium flux assay used in this study was adapted from the method originally described by Sage *et al.*[MacKenzie *et al*, 1996; Sage *et al*, 1987] The main difference in the method was that Fluo-3 was chosen instead of fura-2 because its absorption spectrum was compatible with laser excitation at 488nM, it has a very large fluorescence intensity increase in response to calcium binding, and because of the lack of the spectral shift that occurs with UV-light excited indicators.

2.9.1 Reagents

Fluo-3 AM (Molecular Probes, Europe) was selected as the fluorescent indicator for the calcium flux assay. The acetoxymethyl (AM) ester of fluo-3 is cell permeable, thus allowing adequate loading of cells, such as platelets. Other reagents used in the assay were aspirin, probenecid (Sigma, UK) and apyrase (Sigma, UK). Probenecid, an organic anion-transport inhibitor, was used to reduce leakage of the de-esterified indicator from the fluo-3 loaded platelets. Apyrase is a potato-derived ADP/ATPase, which was added to breakdown any ADP released from platelet dense granules during incubation and processing, thereby minimising any risk of ADP receptor desensitisation. Aspirin was used to block TxA2 synthesis. During initial optimisation, 6β-prostaglandin I_1 was used to help prevent platelet activation during processing. However, it became apparent that even at relatively low concentrations (1 μmol/L) its presence was having a variable effect on measured calcium flux. It was therefore not used in the final assay protocol.

The agonist used to maximally stimulate the platelets was 2MeSADP (the presence of apyrase prevented the use of ADP). AR-C69931MX was used to completely antagonise the P2Y12 receptor, so that isolated calcium flux following stimulation only through the P2Y1 receptor could be determined. Optimum concentrations of 2MeSADP and AR-C69931MX were determined by titration, using flow cytometric measurement of fibrinogen-binding as an indicator of platelet activation. The concentration of 2MeSADP used to stimulate maximal calcium flux was verified by dose titration, using the calcium flux assay itself.

2.9.2 Assay procedure

PRP was prepared essentially as described earlier in this chapter, but without normalisation of the platelet count. 1ml of PRP was incubated for 30 minutes at 37°C following addition of 5 μmol/L fluo-3 AM, 100 μmol/L aspirin, 2.5 mmol/L probenecid and 4 units/ml apyrase. After this the PRP was centrifuged at 535g for 15 minutes, at room temperature. The supernatant was carefully removed and the platelet pellet was gently resuspended in 400 μl of resuspension buffer (10 mmol/L Hepes, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgSO_4.7H_2O$, 5 mmol/L glucose, 2.5 mmol/L probenecid, 4 units/ml apyrase, pH 7.4). The platelet count was adjusted to 4×10^5 platelets/μl by the addition of resuspension buffer, and the washed platelet suspension

was incubated at 37°C for a further 10 minutes prior to testing. This final incubation was performed to ensure all internalised fluo3-AM had been fully de-esterified. Calcium flux was measured in duplicate samples of 4 x 10^7 platelets using a Fluoroskan Ascent fluorometer (Thermo Labsystems, UK), in the presence or absence of a maximum inhibiting concentration of the specific P2Y12 receptor antagonist AR-C69931MX (1 mmol/L), following stimulation with 1 μmol/L 2MeSADP. The fluorometer was calibrated to excite at a wavelength of 485nm, with emission detected at 520nm.

A baseline measurement of fluorescence was taken immediately prior to addition of agonist, followed by the collection of data for a total of 40 seconds, commencing simultaneously with the addition of agonist, at a sampling rate of 5 Hz and an integration time of 100 milliseconds. The increase in fluorescence due to calcium flux was calculated by subtraction of the baseline reading from the measured data post agonist addition and expressed as change in fluorescence (ΔF), in relative fluorescence units (RFU). The rate of initial calcium flux (initial ΔF/Δt) was calculated from the peak measurement divided by the time to peak.

2.10 Quantification of P2Y1 receptor numbers

The preparation of frozen, washed platelets for use in the receptor quantification assays is detailed in Chapter 2 (section 2.4, Table 3). Two methods were used to attempt to quantify platelet ADP receptor numbers in the frozen samples of washed platelets. These will be detailed in the following subsections.

2.10.1 Quantification of platelet protein

Before attempting to quantify receptor numbers either by radioligand binding or immunoblotting, it was necessary to quantify the amount of platelet protein in each thawed sample of frozen platelets, so that the appropriate quantities of protein could be used in each assay that would allow direct comparisons between subjects to be made. The Bradford method for protein determination was performed, using BioRad Coomassie Reagent (BioRad, UK).[Bradford, 1976]

A standard curve was initially prepared using serial dilutions of bovine serum albumin (BSA) in the range of 2-30μg, so that the study samples could be quantified relative to

this. 1ml of Coomassie reagent was added to each dilution of BSA. Absorbance was then determined at 595nm using a spectrophotometer. The standard curve was analysed using SPSS v12 statistical software (SPSS Inc., USA) to calculate the regression equation. The study samples were then analysed in duplicate and the protein concentration was calculated from the mean absorbance using the regression equation for the standard curve.

2.10.2 Radioligand-binding assay

Three different radioligands have previously been used, by various groups, to quantify the number of ADP receptors found on platelets, namely [^{3}H]MRS2279,[Waldo *et al*, 2002] [^{33}P]2MeSADP [Savi *et al*, 2004] and [^{35}S]dATPαS.[Oras *et al*, 2002] MRS2279 is a specific P2Y1 receptor antagonist, dATPαS has partial P2Y1 receptor agonist properties whilst 2MeSADP is a potent agonist at both P2Y1 and P2Y12 receptors. Attempts were made to obtain [^{3}H]MRS2279 from the group responsible for its synthesis,[Waldo *et al*, 2002] but insufficient stocks were available. [^{33}P]2MeSADP was only available by custom synthesis and therefore financial constraints prevented its use. Thus [^{35}S]dATPαS was chosen for use in this study to quantify platelet P2Y1 receptor expression.

[^{35}S]dATPαS with a specific activity of 1000 Ci/mmol was supplied by Amersham Biosciences, UK, as a 10 μmol/L aqueous solution, and was stored at -80°C prior to use to minimize chemical decomposition. The radioactive half-life of [^{35}S] is 87.4 days and the fractional activity was determined on each day of use. 240 nmol/L [^{35}S]dATPαS was added to 50μg of platelet protein in 25μl of HBS. This concentration of hot ligand was chosen to allow an estimate of saturation binding to be made. The reaction was incubated at 25°C for 20 minutes to allow equilibrium-binding to be achieved. 975μl of ice cold HBS was then added to terminate the reaction by dilution and to minimise dissociation of labelled ligand from the receptor-ligand complexes, followed by immediate rapid filtration of two 400μl aliquots over Whatman GF/C glass-fibre filters under vacuum. The filters were then washed twice with 5ml of ice cold HBS to remove unbound radioligand. Following this, the filters, with platelet membranes attached, were then placed in scintillation tubes with BCS Scintillation Cocktail (Amersham Biosciences, UK), and radioactivity was measured by scintillation counting. Non-specific binding was determined by incubation in the presence of either 1 mmol/L

MRS2179 or 1 μmol/L 2MeSADP. Counting efficiency was determined by measuring the radioactivity (in counts per minute, CPM) of 100μl of the unfiltered reaction mixture and dividing this by the predicted number of disintegrations per minute (DPM) for that volume of reaction mixture, calculated from the initial specific activity of the radioligand, and its fractional activity at the time of use.

2.10.3 Immunoblotting of platelet ADP receptor protein

Immunoblotting (Western blotting) of platelet ADP receptor protein was the second method used to attempt to quantify platelet ADP receptor expression. The four main steps in Western blotting consist of 1) resolving of platelet proteins on a denaturing polyacrylamide gel, 2) blotting of the resolved protein bands from the gel onto a PVDF membrane, 3) blocking of the PVDF membrane with dried milk solution followed by binding of the specific primary antibody, and secondary antibodies, 4) visualisation of specific antibody binding using enhanced chemiluminescence (ECL) reagent and autoradiography. The standard method used is detailed below, with specific details of the P2Y1 receptor antibody used given in Chapter 5.

50μg of platelet protein (as determined by the experiments detailed in section 2.10.1) was made up to 20μl with 3x denaturing buffer (Tris, SDS, sucrose, DTT, bromophenol blue) and incubated at 95°C for 5 minutes to facilitate full denaturing of the platelet protein. The samples were then loaded onto 8% SDS-PAGE Minigels (resolving gel - 360 mmol/L Tris-HCl pH 8.8, 8% acrylamide, 0.1% SDS, 0.6 mg/ml ammonium persulphate, 0.0015% TEMED; stacking gel - 125 mmol/L Tris-HCl pH 6.8, 4% acrylamide, 0.1% SDS, 0.6mg/ml ammonium persulphate, 0.0015% TEMED). Two lanes were reserved for the addition of high and low molecular weight markers. The gels were then run in gel-running buffer (Tris, Glycine, SDS) at a constant voltage of 120V for 90 minutes.

Once the platelet proteins had been resolved on the polyacrylamide gel, they were then wet-blotted onto PVDF membrane at a constant current of 150mA for 2 hours using chilled blot solution (Tris-HCl pH 7.5, Glycine, Methanol). The PVDF membrane was soaked in 100% methanol prior to use, and special care was taken when assembling the blot apparatus to prevent the inclusion of air bubbles that would interfere with the blotting process. After blotting, the lanes in which the protein standards had been run were cut from the membrane, stained with amido-black, and destained with a solution

of 50% methanol and 10% acetic acid. The blotted gel was stained with Fairbank I Stain (280 μg/ml Coomassie blue, 25% isopropanol, 10% acetic acid) and destained with 10% acetic acid solution. This allowed verification that all of the protein had been successfully blotted.

The PVDF membrane was then blocked in TBST (10 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) containing 1% dried milk for 60 minutes. Following this, the membrane was incubated overnight at 4°C in TBST containing 1% dried milk and a 1:400 dilution of the primary, anti-ADP receptor antibody. After the overnight incubation with the primary antibody, the membrane was washed twice in TBST, for 10 minutes each. The secondary antibody, a 1:1000 dilution of swine anti-rabbit IgG in TBST containing 1% dried milk, was applied for a period of 1 hour, followed by two 10 minute washes in TBST. Finally, the membrane was incubated with a third antibody (a 1:1000 dilution of rabbit peroxidase/anti-peroxidase (PAP) in TBST containing 1% dried milk) for a further 1 hour period. After this, the membrane was washed in TBST a further six times, for 10 minutes each, to remove any PAP that was non-specifically bound to the membrane.

At this point, the ECL solution (100 mmol/L Tris-HCl pH 8.5, 200 μmol/L coumaric acid, 1.25 mmol/L Luminol, 0.006% hydrogen peroxide) was prepared from stock solutions of coumaric acid and Luminol (both Sigma, UK). The membrane was then incubated in the ECL solution for 2 minutes, prior to wrapping in cling-film, and placing in a film cassette with a suitably sized piece of x-ray film. The film was then exposed to the blot membrane for variable lengths of time to determine the optimum exposure and developed using an automated machine. All of these final steps were undertaken in a dark room.

2.11 Data storage and statistical analysis

All collected data, including demographic details and measured platelet function data, were anonymised and recoded into numeric format where necessary, and entered onto a single spreadsheet created using Microsoft Office 2000 (Microsoft Corp., USA).

All statistical analyses were performed using SPSS version 12 statistical software (SPSS Inc., USA). Graphs, charts and histograms were created using either Microsoft Office 2000, SPSS v12 or GraphPad PRISM v3.02 for Windows (GraphPad Software

Inc., USA). Professor John Thompson from the University of Leicester Department of Statistics provided guidance on the appropriate analysis of the data.

Detailed descriptions of the statistical methods used are given in Chapters 4 and 5. Briefly, unless stated otherwise, data are presented as mean ± SD. Observed allele frequencies were compared with the Hardy-Weinberg equilibrium prediction using the chi-squared test where appropriate. All response data from PRAMIS were transformed prior to analysis (see Chapter 4, section 4.4.1 for details). The association between genotype and phenotype was tested by one-way ANOVA and associations were further analysed after adjustment for other parameters using multiple linear regression models. Logistic regression analysis was performed on case-control data to calculate odds ratios and 95% confidence intervals.

Chapter 3

Analysis of Sequence Variation in the P2Y1 and P2Y12 Genes

3.1 Introduction

This chapter describes the work undertaken to identify polymorphisms in the P2Y1 and P2Y12 genes. Specifically it includes (i) an outline of the factors taken into consideration when designing PCR primers; (ii) the methods utilised to optimise the individual PCR reactions; (iii) single nucleotide polymorphisms (SNPs) identified in the P2Y1 and P2Y12 genes; (iv) linkage disequilibrium between SNPs that were identified.

3.2 Amplification of the P2Y1 and P2Y12 genes

The focus of this work was to amplify primarily the coding sequence of each gene, in order to identify polymorphisms that affect the protein structure of each receptor. As the P2Y1 gene consists of a single exon, primers were designed to encompass this entire region. With respect to the P2Y12 gene, Hollopeter *et al* [Hollopeter *et al*, 2001] have found platelets to express only the shorter transcript variant containing sequence from exons 2 and 3, with the entire coding sequence residing in exon 3. Thus amplification primers were designed to flank this region of the P2Y12 gene.

The method used to amplify segments of the P2Y1 and P2Y12 ADP receptor genes is discussed in Chapter 2 (section 2.5.2). In this section I will describe in more detail the principles that need to be considered when designing primers and optimising PCR reactions.

3.2.1 Primer design

All PCR primers were designed using a free online resource (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). This site uses a complex algorithm to determine the melting temperatures (Tm, the temperature at which 50% of an oligonucleotide will be hybridised to its complementary sequence, with the remaining 50% still unbound) for any sequences of DNA that is entered. The user must input a base sequence from which the primer is to be created, select whether the program should create a forward or reverse primer, and then the user can specify desirable characteristics for the primer, such as optimum length, Tm, %GC base composition and tolerable degree of 3'- and self-complementarity. For this study, all primers were designed using identical characteristics, the most important being the primer should consist of at least 20 bases (to reduce the risk of non-specific binding),

and the Tm should be as close to 64°C as possible. High levels of complementarity were also avoided as this can either result in excessive primer dimer formation (primer annealing to itself), or the formation of secondary structure within the primer, which impedes its hybridisation to the target DNA sequence. Both of these can result in low product yield.

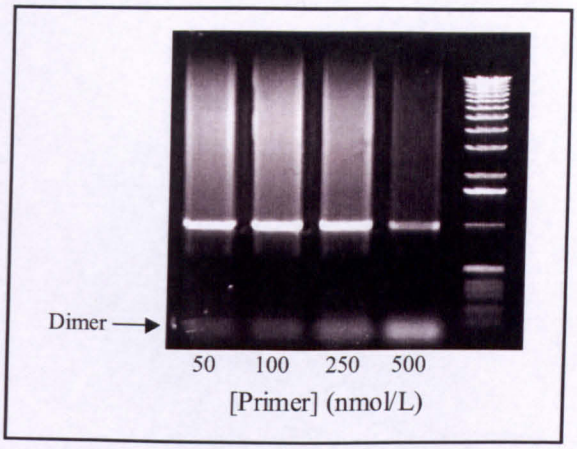
Once the required fields had been inputted, the program then identifies any suitable primers from within the target sequence, and lists them in rank order, based around a score determined from how closely the primer matches the desirable characteristics. The user can then choose whichever primer he feels to be best suited to his application. Target sequences from within the P2Y1 and P2Y12 genes were selected so that there was complete coverage of the regions of interest, and primers were designed to ensure there was adequate overlapping of amplified products, such that the full sequence of the genes could be obtained, without gaps. The primers that were used for amplification of the P2Y1 and P2Y12 genes are listed in Chapter 2 (section 2.5.2).

3.2.2 Assay optimisation

As the primers used for PCR amplification were designed to exhibit similar properties, particularly in terms of Tm, it was possible to use a relatively standard set of primer and magnesium concentrations, and PCR cycling conditions.

When first setting up the PCR reaction, primers at 1 μmol/L were used, and with magnesium concentration of 5 mmol/L. At these initial concentrations there was excessive primer dimer formation and smearing through the agarose gel lanes following electrophoresis. The first optimisation, performed on a single primer pair, was a primer titration, using primer concentrations ranging from 50 nmol/L through to 500 nmol/L (Figure 12). It was found that reducing the primer concentration reduced the quantity of primer dimer formed, whilst also increasing the yield of specific product. The optimum primer concentration determined by this titration was 100 nmol/L of each primer. However, there still remained significant smearing, and because the PCR product was going to be used in downstream sequencing reactions following gel purification, it was necessary to try and minimise this also.

Figure 12 ***Agarose gel showing primer titration for primer pair P2Y12-3, ranging from 50 to 500 nmol/L of each primer***

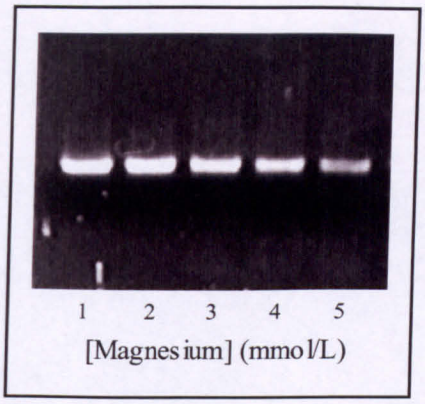


The second optimisation performed was a magnesium titration, varying the magnesium concentration in the PCR reaction between 1 and 5 mmol/L. An increasing magnesium concentration in PCR results in a more efficient reaction, leading to improved yield, but at the expense of specificity. This was demonstrated with the magnesium titration, where increasing magnesium concentrations resulted in increased smearing and the appearance of non-specific products. The optimum magnesium concentration determined by this titration was 2 mmol/L (Figure 13). A primer concentration of 100 nmol/L and magnesium concentration of 2 mmol/L was then used as the starting conditions for each set of primers designed. The use of these conditions resulted in sufficient yield and specificity for the majority of primer pairs used. Only the first primer pair for the P2Y12 gene (P2Y12-1) required a different primer concentration (250 nmol/L) to achieve a good yield.

In terms of PCR cycling conditions, annealing temperature adjustment can have the biggest impact on yield and specificity. Increasing the annealing temperature for oligonucleotide hybridisation, results in increased specificity of the PCR reaction, but to the detriment of product yield, whereas reducing the annealing temperature has the opposite effect. Convention states that the optimum annealing temperature tends to lie between 3°C and 5°C below the Tm of the primers used. In this study the standard annealing temperature used was 60°C (4°C below Tm) in all cases, except the P2Y1

gene primer pairs 3 and 5, where an annealing temperature of 58°C was found to improve yield without affecting specificity.

Figure 13 ***Agarose gel showing magnesium titration for primer pair P2Y12-3, ranging from 1 to 5 mmol/L***



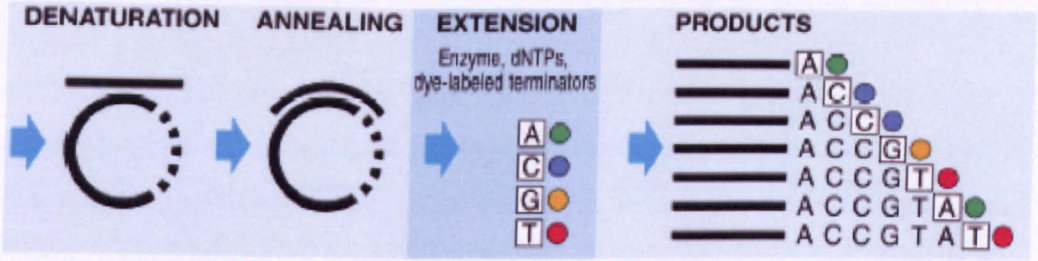
3.3 Sequencing of the ADP receptor genes

3.3.1 Dye terminator sequencing

The P2Y1 gene and a region encompassing exons 2 and 3 and intron B of the P2Y12 gene were screened for single nucleotide polymorphisms by automated dye terminator sequencing, using ABI BigDye Terminator v3.1 chemistry, with sequencing products being resolved on an ABI PRISM 3700 Gene Analyzer. This method is based around the chain-termination method developed by Sanger *et al* in 1977.[Sanger *et al*, 1977] Briefly, this method involves using the PCR product as template for the synthesis of new DNA starting from a defined primer-binding site. A mixture of deoxynucleotides and dideoxynucleotides are present in the ready reaction mixture at a concentration that creates a finite probability that a dideoxynucleotide will be incorporated into the growing DNA chain at every nucleotide position instead of a deoxynucleotide. Since the incorporation of a dideoxynucleotide results in termination of chain elongation, a mixture of DNA fragments of varying lengths results, with a dideoxynucleotide capping the 3' end of each fragment (Figure 14). BigDye v3.1 contains 4 different dideoxynucleotides (ddATP, ddGTP, ddCTP and ddTTP), each labelled with a different fluorescent dye, so that when resolved on high-resolution polymer (Performance Optimised Polymer 6- POP6, Applera, UK) in a 3700 Gene Analyzer, the sequence in

which the labelled fragments pass through its laser can be used to automatically call the actual sequence of the original priming PCR product. The detailed method of the sequencing method used, including the method used to clean up the sequencing products and remove unincorporated dye terminators prior to running on the 3700 Gene Analyzer, is given in Chapter 2 (section 2.5.3).

Figure 14 ***Illustration showing one cycle of a dye-terminator cycle sequencing reaction***



3.3.2 Identification of single nucleotide polymorphisms

Twenty subjects from the PRAMIS cohort were selected for sequencing of the two platelet ADP receptor genes on the basis of the response of their platelets to 1 μmol/L ADP, as measured by fibrinogen-binding to activated GPIIb-IIIa receptors. Ten subjects were studied with high responses to ADP and ten subjects with low responses, to maximise the chance of identifying a functional polymorphism, and those with a minor allele frequency (MAF) of >10% (sequencing 20 subjects provides 95% power to identify alleles with a MAF of 0.1). The high responders had a mean age of 49.3 years, 90% were male and the mean fibrinogen-binding in response to 1 μmol/L ADP was 91.7%. Conversely, the low responders had a mean age of 43.7 years, 50% were male and the mean fibrinogen-binding was 20.4%. Sequencing 20 subjects (40 chromosomes) would provide 64% power to identify polymorphisms with a MAF of 2.5%.

The sequence files generated by the 3700 Gene Analyzer were studied off-line using Chromas v1.45 (freeware courtesy of Conor McCarthy, Griffith University, Queensland, Australia) to ensure good quality sequence was obtained. Sequence, in FASTA format, was exported from Chromas to text file and imported into BioEdit Sequence Alignment Editor [Hall, 1999] to allow further analysis and alignment with

the reported sequence for each gene, downloaded from GenBank (NCBI Sequence Database, U.S. National Library of Medicine).

3.4 Results

Figure 15 shows an example of the how the sequence file is displayed within Chromas when the subject is heterozygous for a SNP (P2Y12 IntB742T>C substitution). Figure 16 demonstrates the divergence of sequence that is seen when a subject is homozygous for a single base deletion (P2Y12 IntB798del/A) that results in inability to read the downstream sequence. A further problem encountered within the same amplicon of the P2Y12 gene as the deletion, was the presence of a homopolymeric region (consisting of an 11 base polyT), which caused slippage/stuttering of the Taq polymerase and loss of downstream sequence resolution (Figure 17). The consequence of this in the subjects heterozygous for the deletion was that two further sequencing primers were needed, lying between the polyT and deletion, to allow the intervening sequence to be determined.

Figure 15 ***Sequence file generated by an ABI PRISM 3700 Gene Analyzer and viewed in Chromas, showing heterozygous carriage of a T>C substitution***

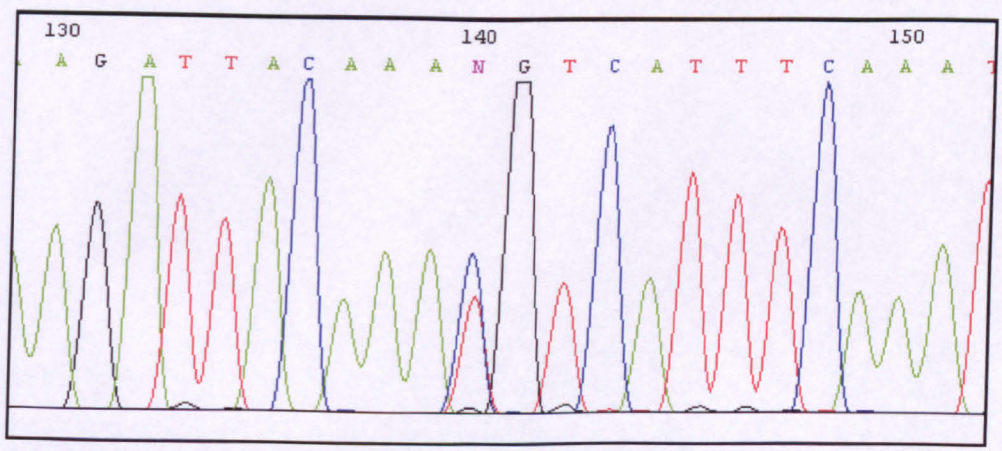


Figure 16 ***Sequence file generated by an ABI PRISM 3700 Gene Analyzer and viewed in Chromas, showing heterozygous carriage of an AA>A- deletion***

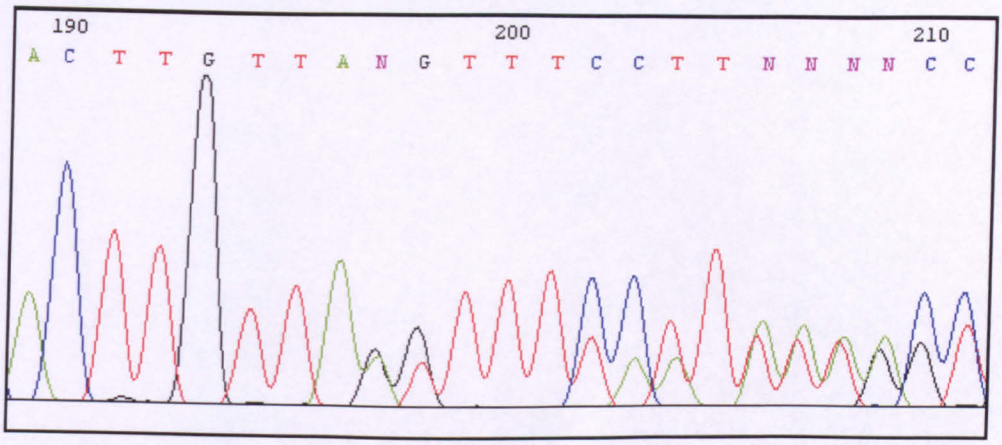
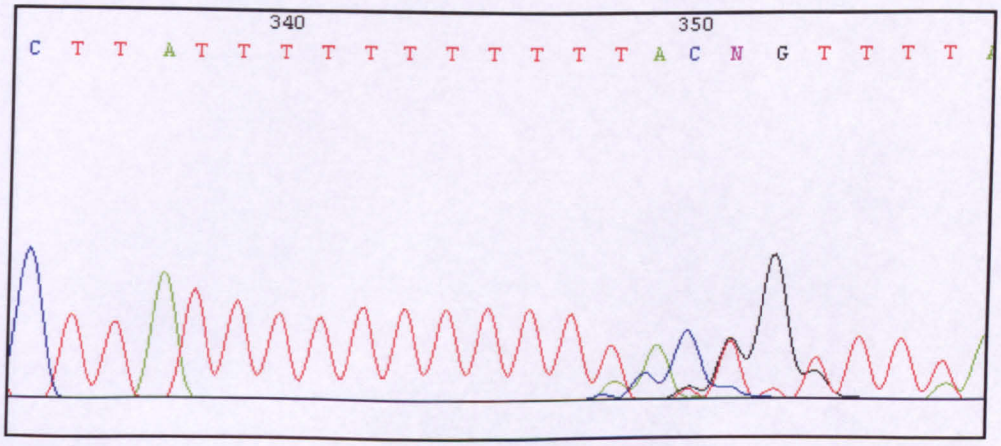


Figure 17 ***Sequence file generated by an ABI PRISM 3700 Gene Analyzer and viewed in Chromas, showing loss of sequence resolution downstream of a homopolymeric region***



Sixteen polymorphisms were identified in total, 5 in the P2Y1 gene and 11 in the P2Y12 gene. These are listed in Table 6 and in Figures 18 and 19. Two SNPs in the P2Y1 gene were found to lie in the coding region of the gene, as compared to three in the P2Y12 gene. None of the coding region SNPs were found to affect the amino acid composition in either of the two receptors. Ten of the polymorphisms had previously been identified, and were annotated with reference numbers in the NCBI Sequence Database. However, the other 6 polymorphisms, had not previously been described.

Table 6 ***Polymorphisms identified in the P2Y1 and P2Y12 genes***

Gene	Location	Nucleotide position	NCBI dbSNP Reference	Observed alleles (approximate frequencies*)
P2Y1	5'UTR	179	-	C/A (0.98/0.02)
	5'UTR	190	rs3755711	G/C (0.85/0.15)
	Coding	893	rs1065776	C/T (0.98/0.02)
	Coding	1622	rs701205	A/G (0.82/0.18)
	3'UTR	2717	-	C/G (0.98/0.02)
P2Y12	5'UTR	145	rs3821667	C/T (0.87/0.13)
	Intron B	137	-	C/T (0.87/0.13)
	Intron B	742	rs2046934	T/C (0.87/0.13)
	Intron B	798	rs5853517	del/A (0.87/0.13)
	Intron B	1209	rs1388626	T/C (0.95/0.05)
	Coding	234	rs6785930	C/T (0.67/0.33)
	Coding	252	rs6809699	G/T (0.87/0.13)
	Coding	676	-	T/C (0.98/0.02)
	Downstream	1622	-	C/T (0.87/0.13)
	Downstream	2014	rs6803224	C/T (0.75/0.25)
	Downstream	2087	-	A/del (0.5/0.5)

NCBI dbSNP – National Centre for Biotechnology Information single nucleotide polymorphism database, 5'UTR – 5' untranslated region, 3'UTR – 3' untranslated region.
*derived from sequencing of 40 chromosomes.

On initial examination of genotype versus phenotype, based on the results from sequencing of the ten high and ten low responders to ADP, there did not appear to be clear segregation of alleles into either response group for the majority of SNPs identified (Table 7). However, a partial segregation was observed with the P2Y1 190G>C and 1622A>G polymorphisms, and the P2Y12 1622C>T polymorphism. For the P2Y1 190G>C and 1622A>G polymorphisms there appeared to be an excess of minor alleles carried in the high responders (5 vs 1 and 6 vs 1 in high vs low responders respectively), whereas for the P2Y12 1622C>T polymorphism the opposite was true (1 vs 4 high vs low responders).

Figure 18 ***Schematic representation of P2Y1 gene with polymorphisms annotated***

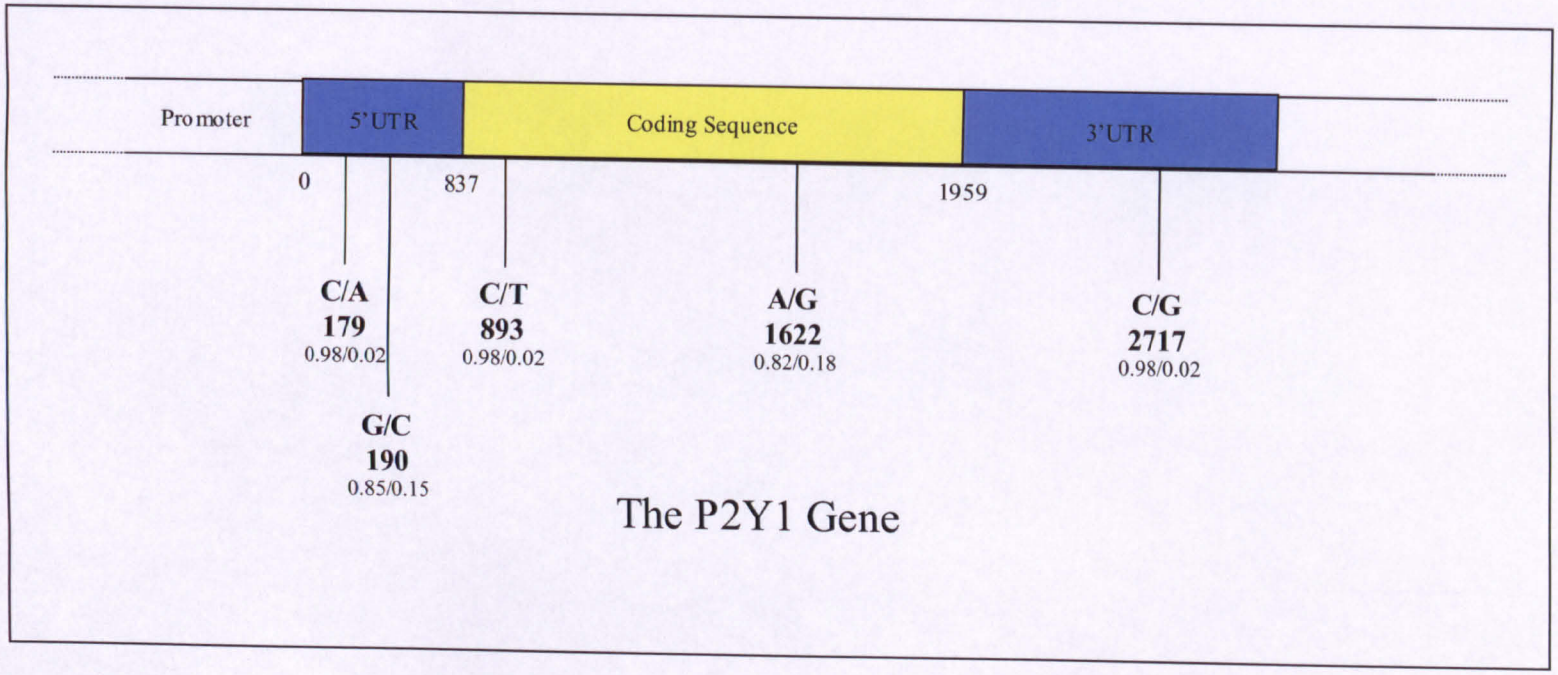


Figure 19 ***Schematic representation of P2Y12 gene with polymorphisms annotated***

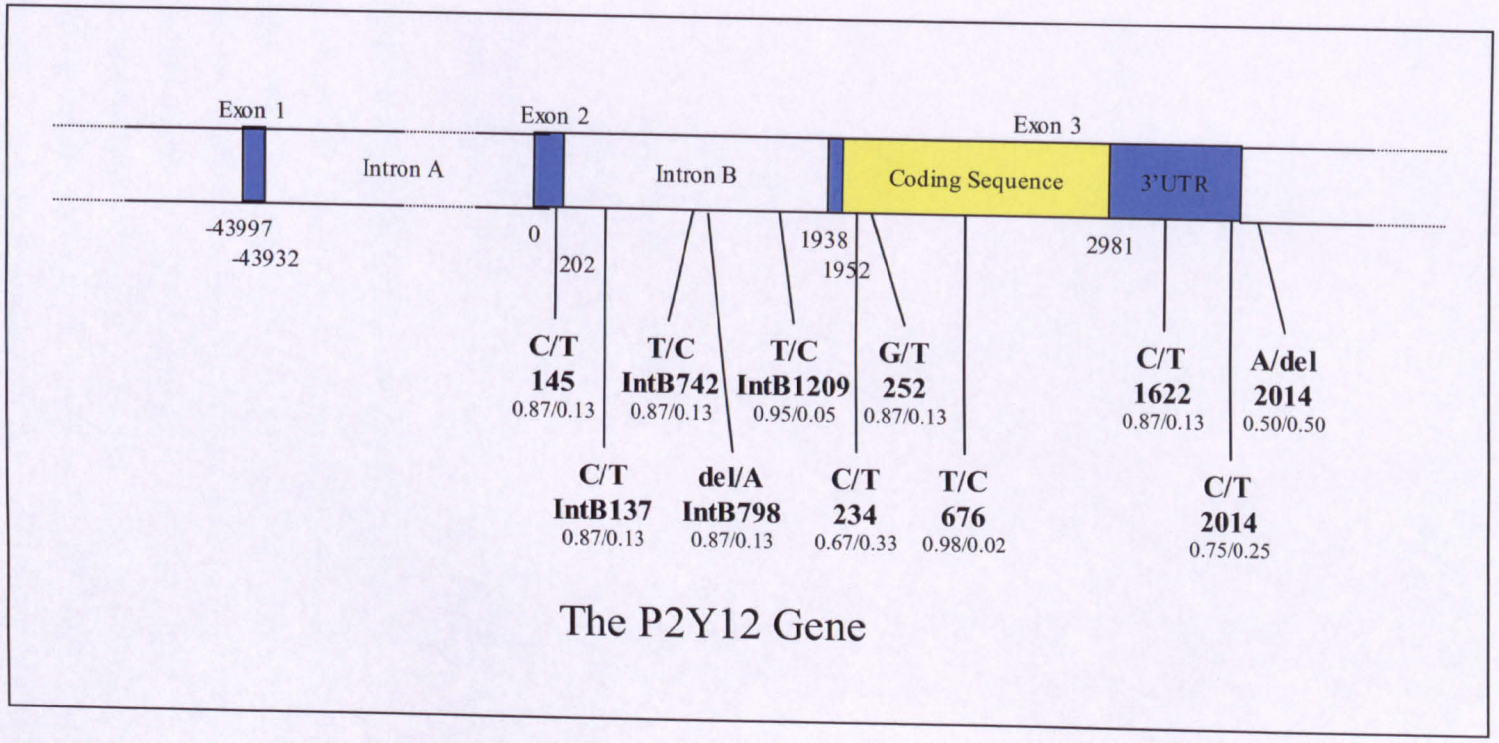


Table 7 ***Segregation of less common alleles between high and low responders to ADP***

Gene	Location	Nucleotide position	Number of less common alleles	
			High responders	Low responders
P2Y1	5'UTR	179	1	0
	5'UTR	190	5	1
	Coding	893	1	0
	Coding	1622	6	1
	3'UTR	2717	1	0
P2Y12	5'UTR	145	2	3
	Intron B	137	2	3
	Intron B	742	2	3
	Intron B	798	2	3
	Intron B	1209	2	0
	Coding	234	8	7
	Coding	252	2	3
	Coding	676	0	1
	Downstream	1622	1	4
	Downstream	2014	5	5
	Downstream	2087	9	11

3.5 Linkage disequilibrium of polymorphisms

Linkage disequilibrium is a term used to describe the co-segregation of alleles within a population, whereby alleles that lie in close proximity on the same chromosome are often inherited together, and form haplotypes. This occurs as a result of ancestral recombination events, and is perpetuated by the lack of crossing over of maternal and paternal chromosomes within the region encompassing the SNPs during meiosis. Complete linkage disequilibrium occurs when each allele of one SNP is always inherited with a particular allele of another SNP. Partial linkage is found when certain alleles from different SNPs are often inherited together (i.e. more commonly than by chance), but not always.

3.5.1 P2Y1 gene

The distribution of P2Y1 gene alleles in the twenty sequenced subjects is shown in Table 8. Less common alleles are highlighted in red. From this it can be seen that the P2Y1 190G>C and 1622A>G polymorphisms are in partial linkage disequilibrium, whereby the 190C allele is always carried with the 1622G allele, but the 1622G allele can, rarely, be carried with the 190G allele (subject 084). It would also appear that P2Y1 179A, 893T and 2717G co-segregate, however, as each of these alleles is only carried in one of the subjects, it is difficult to be conclusive regarding this.

Table 8 ***Distribution of P2Y1 gene alleles by subject***

Subject	ADP response	P2Y1 gene polymorphisms				
		179 C>A	190 G>C	893 C>T	1622 A>G	2717 C>G
016	High	CC	GG	CC	AA	CC
052	Low	CC	GG	CC	AA	CC
054	High	CC	CC	CC	GG	CC
061	High	CC	GG	CC	AA	CC
062	High	CC	GG	CC	AA	CC
084	High	CA	GG	CT	AG	CG
093	Low	CC	GG	CC	AA	CC
143	Low	CC	GG	CC	AA	CC
163	Low	CC	GG	CC	AA	CC
186	Low	CC	GC	CC	AG	CC
508	Low	CC	GG	CC	AA	CC
509	Low	CC	GG	CC	AA	CC
521	High	CC	GC	CC	AG	CC
532	Low	CC	GG	CC	AA	CC
534	Low	CC	GG	CC	AA	CC
540	Low	CC	GG	CC	AA	CC
580	High	CC	GG	CC	AA	CC
635	High	CC	GG	CC	AA	CC
652	High	CC	CC	CC	GG	CC
702	High	CC	GG	CC	AA	CC

3.5.2 P2Y12 Gene

Table 9 shows the genotypes of the 20 sequenced subjects with respect to the 11 polymorphisms identified in the P2Y12 gene. From this, it can be seen that 5 of the polymorphisms appear to be in complete linkage disequilibrium with each other, with a further polymorphism partially linked to those 5. The completely linked SNPs are P2Y12 145C>T, intron B (IntB) 137C>T, IntB742T>C, IntB798delA and 252G>T, where the co-inherited alleles are 145C, IntB137C, IntB742T, IntB798del, 252G (from henceforth labeled the H0 haplotype) and 145T, IntB137T, IntB742C, IntB798A, 252T (labeled the H1 haplotype). The additional downstream SNP partially linked to the H0/H1 haplotype is P2Y12 2014C>T. With respect to this SNP and the previously described haplotype, the H1 haplotype is always inherited with 2014T, however 2014T may also be found to co-segregate with the H0 haplotype, but less frequently.

Table 9 ***Distribution of P2Y12 gene alleles by subject***

Subject	ADP response	P2Y12 gene polymorphisms										
		145 C>T	IntB137 C>T	IntB742 T>C	IntB798 delA	IntB1209 T>C	234 C>T	252 G>T	676 T>C	1622 C>T	2014 C>T	2087 Adel
016	High	CT	CT	TC	delA	TC	CC	GT	TT	CC	CT	deldel
052	Low	CC	CC	TT	deldel	TT	CT	GG	TT	CT	CC	Adel
054	High	CC	CC	TT	deldel	TT	CT	GG	TT	CC	CC	Adel
061	High	CC	CC	TT	deldel	TT	CT	GG	TT	CT	CC	Adel
062	High	CC	CC	TT	deldel	TT	CT	GG	TT	CC	CT	AA
084	High	CC	CC	TT	deldel	TT	CT	GG	TT	CC	CC	Adel
093	Low	CT	CT	TC	delA	TT	CT	GT	TT	CT	CT	Adel
143	Low	CC	CC	TT	deldel	TT	CT	GG	TT	CC	CC	Adel
163	Low	CC	CC	TT	deldel	TT	CC	GG	TT	CC	CT	Adel
186	Low	CT	CT	TC	delA	TT	CC	GT	TT	CC	CT	deldel
508	Low	CC	CC	TT	deldel	TT	CC	GG	TT	CC	CT	Adel
509	Low	CT	CT	TC	delA	TT	CC	GT	TT	CC	CT	deldel
521	High	CT	CT	TC	delA	TC	CC	GT	TT	CC	CT	deldel
532	Low	CC	CC	TT	deldel	TT	TT	GG	TT	TT	CC	AA
534	Low	CC	CC	TT	deldel	TT	CC	GG	TC	CC	CC	deldel
540	Low	CC	CC	TT	deldel	TT	TT	GG	TT	CC	CC	AA
580	High	CC	CC	TT	deldel	TT	CC	GG	TT	CC	TT	AA
635	High	CC	CC	TT	deldel	TT	CT	GG	TT	CC	CC	Adel
652	High	CC	CC	TT	deldel	TT	TT	GG	TT	CC	CC	AA
702	High	CC	CC	TT	deldel	TT	CT	GG	TT	CC	CC	Adel

3.6 Discussion

In this chapter I have described the methods employed to identify common sequence variation within the P2Y1 and P2Y12 ADP receptor genes. Direct sequencing of genomic DNA was employed to screen a subset of the PRAMIS cohort for common and novel polymorphisms. The main advantage to using this method is that it is recognised to be the "gold standard" for identifying genetic polymorphisms in DNA samples, due to it providing single base resolution. Accuracy was improved further by the use of a proof-reading Taq polymerase (Biolase Diamond) with very high sequence fidelity to amplify the genomic DNA prior to sequencing. The main disadvantages of using direct sequencing are its expense and also it being a relatively time-consuming process, in terms of post-amplification sample preparation and processing.

Twenty subjects, 10 with a high response to ADP and 10 with a low response, were selected for sequencing. This strategy was employed to try and maximise the possibility of identifying polymorphisms with a MAF of >2.5% and those that affect function. Another potential strategy that could have been used to identify polymorphisms within the P2Y1 and P2Y12 genes would have been to utilise the HapMap and SNP consortium resources. However, these were not complete at the time, and as the geographical location of the populations screened for SNPs in these databases may be very different from the PRAMIS cohort, there would be no guarantee that common polymorphisms in these databases would necessarily be common in PRAMIS. In addition, the depth of sequencing in HapMap was lower than in this thesis (5 vs 20 subjects). This is borne out by the identification of 6 novel polymorphisms in this study.

The focus of this study was to identify polymorphisms in the P2Y1 and P2Y12 ADP receptor genes that may affect function. Coding region polymorphisms that result in an amino acid substitution are most likely to generate a functional consequence, therefore sequencing was targeted to the exonic regions of the two genes, and in particular those that contain coding sequence. As the P2Y1 gene consists of a single exon, this region was sequenced in the screening phase. With regard to P2Y12, although the NCBI Sequence Database reports that there are two transcript variants, differing by the presence or absence of exon 1 (see Figure 8, Chapter 1, section 1.3.2.2), Hollopeter [Hollopeter *et al*, 2001] found platelets only to express the shorter transcript variant, therefore sequencing was restricted to this region (encompassing exons 2 and 3).

Identification of sequence variation in the P2Y1 and P2Y12 ADP receptor genes through the use of PCR amplification and direct sequencing was successfully accomplished in 20 subjects with divergent platelet ADP responses. Using this method, 16 polymorphisms were identified in total, 5 in the P2Y1 gene and 11 in the P2Y12 gene. 6 of these polymorphisms had not previously been identified in the NCBI SNP database.

Potential limitations of the method used to identify polymorphisms in this work mainly relate to the number of subjects screened. This study was designed to identify common polymorphisms within the P2Y1 and P2Y12 genes, so that, given the overall sample size involved, it would be possible to identify polynmorphisms that have a significant overall effect on platelet reactivity in the population. As a limited number of subjects were screened for sequence variation, it is not inconceivable that a number of rare polymorphisms that may affect platelet function have been missed using this method.

None of the polymorphisms resulted in any change in the amino acid structure of either receptor, despite the identification of 5 coding region polymorphisms. From this, it can be concluded that there are no common polymorphisms that directly contribute to the variation observed in platelet response to ADP in this cohort, by affecting the amino acid structure of either the P2Y1 or the P2Y12 receptor.

Since completion of the work for this thesis, a single additional polymorphism has been idenitifed and annotated in the NCBI SNP database that resides in the coding region of the P2Y12 gene. The P2Y12 1256A>G polymorphism (dbSNP reference no. rs16846673) produces a non-synonymous amino-acid change, from glutamic acid to glycine, at amino-acid position 330 of the P2Y12 receptor protein. This polymorphism exhibits genotype frequencies of 0.83, 0.13 and 0.04 respectively for the AA, AG and GG genotypes. However, this polymorphism was only identified in a panel of 23 subjects of African-American descent from the Coriell Cell Repository (Cambden, NJ, USA), but not in panels of subjects from European-American or Chinese-American descent. Thus it is very unlikely that this polymorphism has been missed by chance in this study. There has been no published work on this polymorphism in the literature at the present time.

Several polymorphisms were found to be in varying degrees of linkage disequilibrium within the two genes. Of note, 5 polymorphisms in P2Y12 (145C>T, IntB137C>T,

IntB742T>C, IntB798delA and 252G>T) were found to be in complete linkage disequilibrium. The H0/H1 haplotype found in this study is equivalent to the four SNP haplotype identified by Fontana *et al.*[Fontana *et al*, 2003a] However, due to the placement of their amplification primers, they sequenced a smaller region of the P2Y12 gene and consequently failed to identify either the P2Y12 145C>T or 2014C>T polymorphisms, which were also found to be in linkage in this study.

Finding this sequence variation provided a basis for looking at the functional consequences of variation within the platelet ADP receptor genes on platelet reactivity and cardiovascular system phenotypes (Chapter 4).

3.7 Conclusions

In this Chapter I have:

- Described an efficient strategy to identify common sequence variation within the P2Y1 and P2Y12 platelet ADP receptor genes using direct sequencing of PCR amplified genomic DNA
- Identified 5 polymorphisms in the P2Y1 gene and 11 polymorphisms in the P2Y12 gene, none of which affect their amino-acid structure
- Confirmed the presence of the haplotype previously identified by Fontana *et al* [Fontana *et al*, 2003a] in the PRAMIS cohort, and identified additional linked polymorphisms

Chapter 4

The Relationships between P2Y1 and P2Y12 Genotypes, Platelet Function and Myocardial Infarction

4.1 Introduction

This chapter describes the genotyping of the complete PRAMIS cohort, including data relating to both premature MI cases and normal controls, to examine relationships between P2Y1 and P2Y12 genotypes, platelet functional responses and risk of premature myocardial infarction.

4.2 Selection of common polymorphisms for genotyping

The 16 polymorphisms identified in the two platelet ADP receptor genes are detailed in Chapter 3. Polymorphisms were chosen for genotyping in the full PRAMIS cohort based on their allele frequencies and the linkage disequilibrium data. Only polymorphisms with a minor allele frequency of >5% were selected for genotyping. These selection criteria were used to ensure a high possibility that there would be sufficient statistical power to determine an effect of the polymorphisms on platelet function, given the sample size available. In addition, where a number of polymorphisms were found to be in strong linkage disequilibrium, a single SNP would essentially provide information on all of them.

In total 5 SNPs were chosen for genotyping, namely the 1622A>G polymorphism in the P2Y1 gene, and IntB742T>C, 234C>T, 1622C>T and 2014C>T polymorphisms from the P2Y12 gene. The P2Y1 190G>C polymorphism was not typed as it was in near complete linkage disequilibrium to the P2Y1 1622 A>G polymorphism, but less common, and it was therefore felt that typing it was unlikely to yield any extra biological information. The IntB742T>C polymorphism in P2Y12 was selected to be representative of the 5 completely linked SNPs within that gene. The P2Y12 2087Adel polymorphism was not typed, despite being the most common polymorphism identified, because the nature of the sequence around the deletion (a run of seven or eight A's) prevented an efficient method for genotyping being developed.

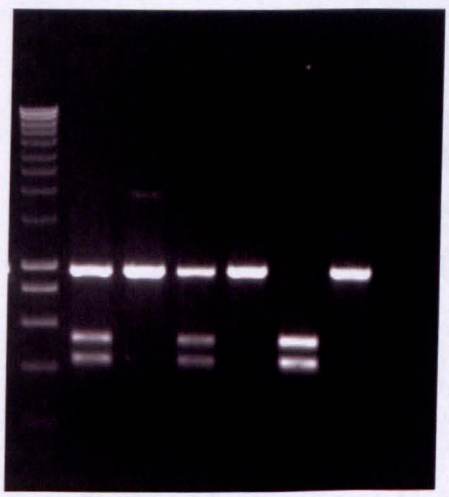
Two of the polymorphisms, P2Y1 1622A>G and P2Y12 IntB742T>C, were typed by RFLP analysis, whereas the remaining three SNPs were genotyped using TaqMan assays for allelic discrimination. The restriction endonucleases used for RFLP analysis were Bcl I and HpyCH4 IV for the P2Y1 1622A>G and P2Y12 IntB742T>C polymorphisms respectively (New England BioLabs, UK). The primer pairs used for

PCR amplification were P2Y1-3 and P2Y12-2 respectively (see Chapter 2, section 2.5.2 for details), and using the PCR method given in Chapter 2.

4.2.1 Bcl I restriction digestion for the P2Y1 1622A>G polymorphism

Restriction digestion with Bcl I was performed in 15μl reaction volumes containing 100 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.9, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 2.5 units Bcl I and 10μl of completed PCR reaction. The samples were incubated at 50°C for 16 hours, after which they were resolved on agarose gels and stained with ethidium bromide. The recognition sequence for Bcl I is 5'…T/GATCA…3', thus the PCR product produced using primer pair P2Y1-3 was cleaved once in the presence of the G allele, yielding fragments of 422 and 525 base pairs length, but was left uncleaved in the presence of the A allele. Complete digestion was ensured by including samples with known genotypes (through direct sequencing), for cross-referencing purposes, in each run. The 20 subjects that underwent sequencing were included in each genotyping assay, so that accuracy could be assessed. An example gel demonstrating the pattern of bands observed with the various P2Y1 1622 polymorphism genotypes is shown in figure 20. Genotype calling was undertaken manually following inspection of resolved gels.

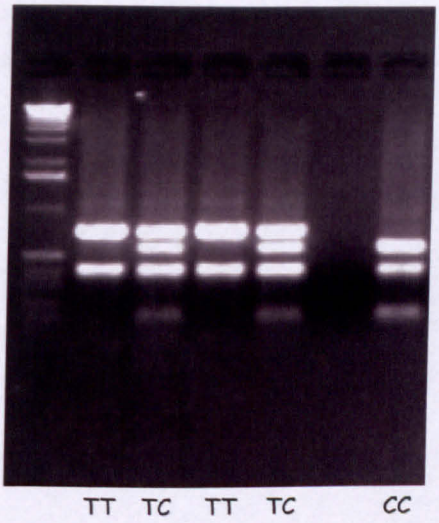
Figure 20 ***Restriction endonuclease digestion of PCR products using Bcl I***



4.2.2 HpyCH4 IV restriction digestion for the P2Y12 IntB742T>C polymorphism

Restriction digestion with HpyCH4 IV was performed in 25µl reaction volumes containing 10 mmol/L Bis Tris Propane-HCl pH 7.0, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 4 units HpyCH4 IV and 20µl of completed PCR reaction. The samples were incubated at 37°C for 16 hours, after which they were resolved on agarose gels and stained with ethidium bromide. The recognition sequence for HpyCH4 IV is 5'…A/CGT…3', thus the PCR product produced using primer pair P2Y12-2 was cleaved once in the presence of the P2Y12 IntB742T allele, yielding fragments of 744 and 402 base pairs length, and was cleaved twice in the presence of the C allele, yielding fragments of 164, 580 and 402 base pairs length. Similar control methods to those used for the Bcl I digestion were performed. An example gel demonstrating the pattern of bands observed with the different P2Y12 IntB742 polymorphism genotypes is shown in figure 21. Genotype calling was undertaken manually following inspection of resolved gels.

Figure 21 ***Restriction endonuclease digestion of PCR products using HpyCH4 IV***



4.2.3 TaqMan assays for allelic discrimination

Allelic discrimination assays using TaqMan MGB probes were designed for the remaining three SNPs – P2Y12 234C>T, 1622C>T and 2014C>T (see Chapter 2, section 2.5.4.2 for the detailed method used). Primers and probes were designed using Primer Express v2.0 software following the standard protocols. The primers and probes

used in the three assays are shown in Table 10, where the polymorphic alleles are indicated by bold type.

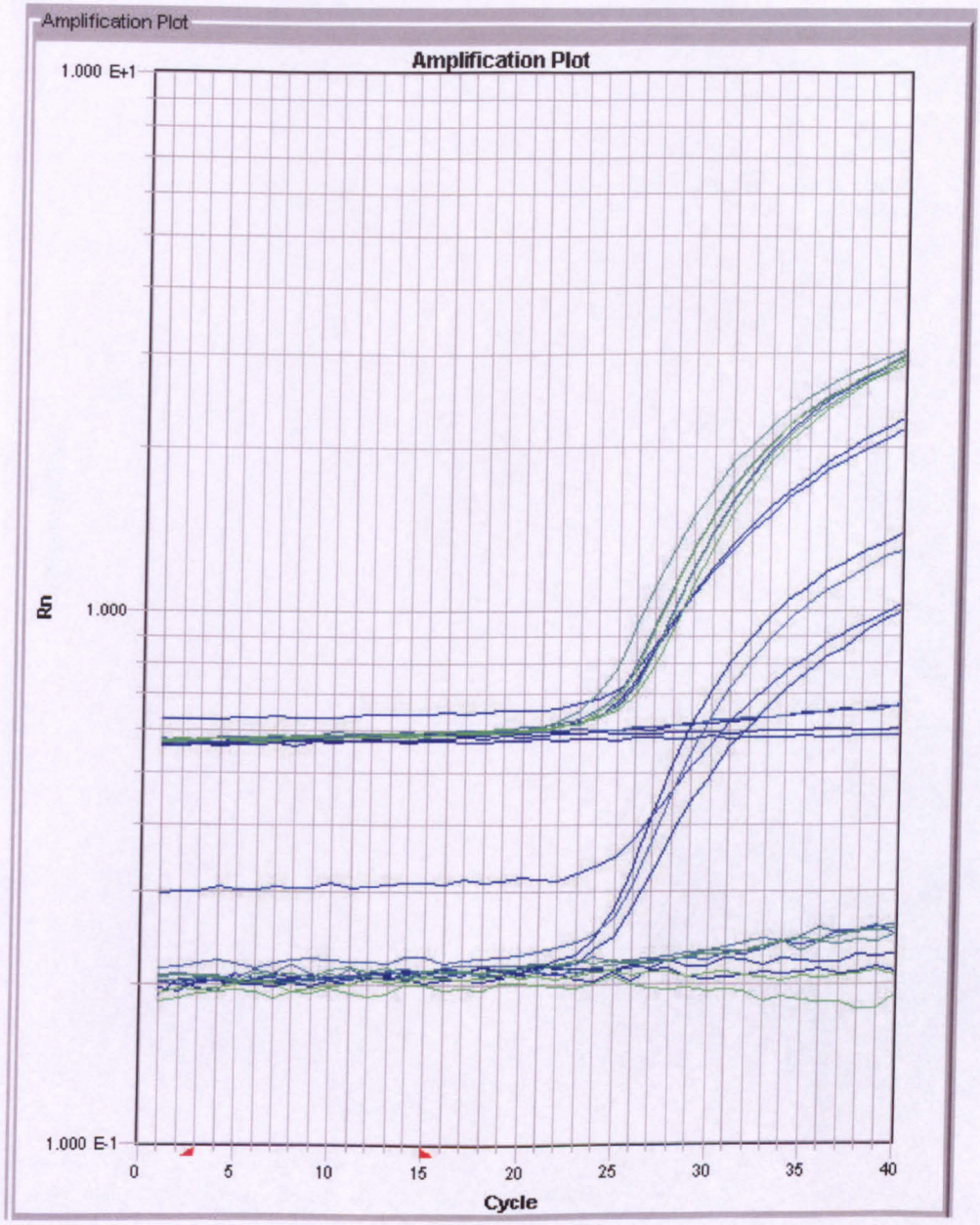
Output data from the ABI PRISM 7900HT Sequence Detection System can be viewed into two formats, either as real-time amplification plots which are predominantly used for troubleshooting indeterminate calls, or as an end plate read, where the normalized relative fluorescence of the two dyes for each sample are displayed on the X and Y axes, and a scatterplot is produced. This allows calling of the sample genotypes dependant on the recorded fluorescence of each dye. The SDS software would attempt to call genotypes automatically, and any indeterminate calls were verified following inspection of the real-time amplification plots. Examples of a real-time amplification plot and end plate read scatterplot are shown in Figures 22 and 23 respectively.

Table 10 ***Oligonucleotide primers and fluorescent dye-labelled probes used in TaqMan assays***

Polymorphism	Description	Oligonucleotide sequence
P2Y12 234 C>T	C allele-specific probe	*6FAM*-TCGACAA**C**CTCACCTC
	T allele-specific probe	*VIC*-CGACAA**T**CTCACCTCT
	Forward primer	TCTCTAGGTAACCAACAAGAAATGCA
	Reverse primer	GGACCTGGGTGATTTTGTAGTCTCT
P2Y12 1622 C>T	C allele-specific probe	*6FAM*-ATTGATC**G**TTCTTCC
	T allele-specific probe	*VIC*-AATTGATC**A**TTCTTCC
	Forward primer	TGCCAAAATGAGTAATCATATAATATTTACTG
	Reverse primer	GTTAATAGTTATTATTCTTTGTGTATCCCACTTAC
P2Y12 2014 C>T	C allele-specific probe	*6FAM*-ACCATCA**C**TGACACTT
	T allele-specific probe	*VIC*-ACCATCA**T**TGACACTTA
	Forward primer	AAGACCTTTACAATAAAGAGAAGAAATATCG
	Reverse primer	TAGCAGCTATTTTCTAGAAGCTAATTAATAAAG

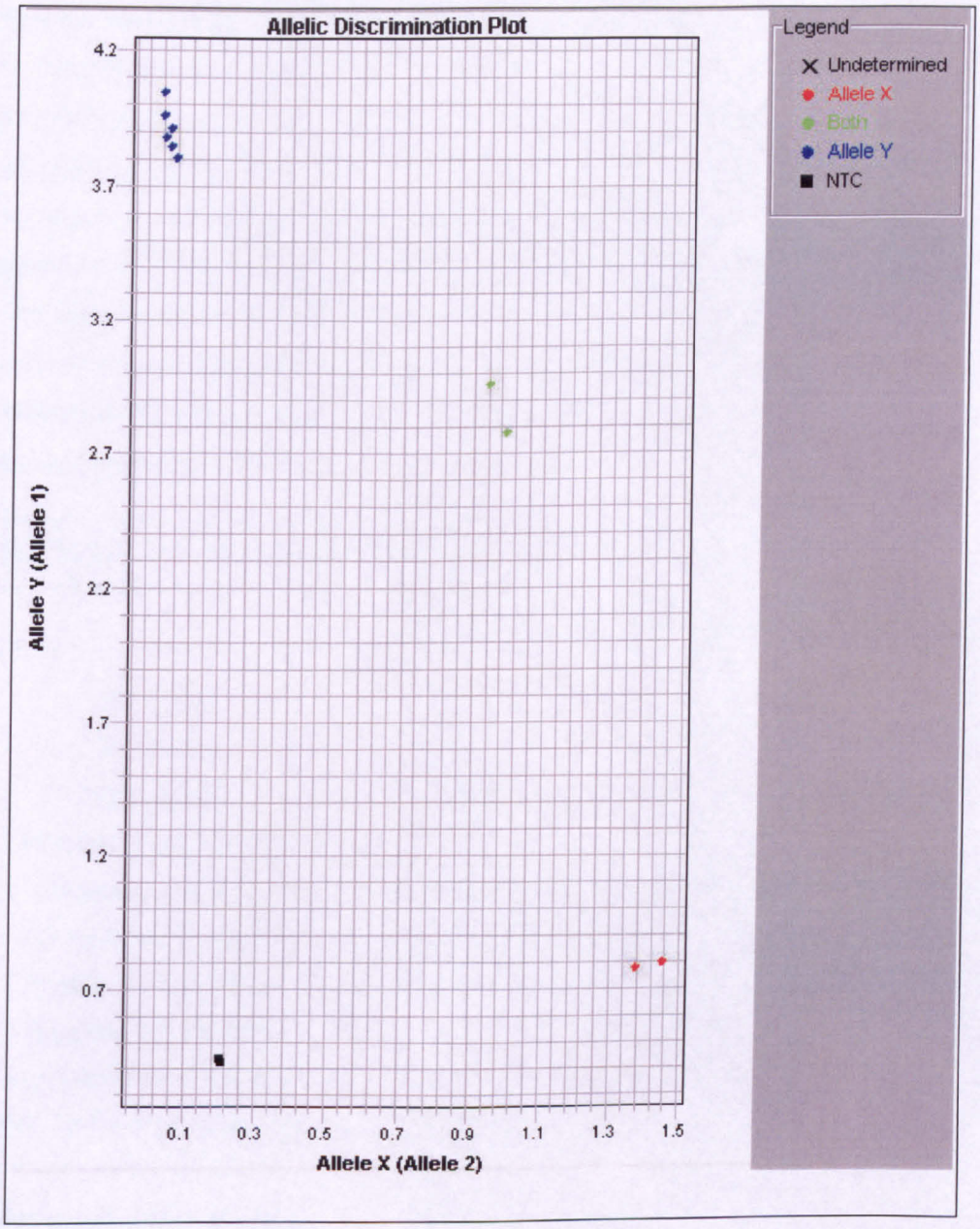
Collected genotype data was recoded into numeric format (i.e. homozygotes for major allele = 1, heterozygotes = 2, homozygotes for minor allele = 3), and was then inputted into the PRAMIS master database to permit formal statistical analysis.

Figure 22 ***TaqMan real-time amplification plot produced on the ABI PRISM 7900HT SDS***



Coloured lines represent relative fluorescence (Rn, y-axis, on a log scale) following normalisation to correct for any differences in sample volumes. Amplification is demonstrated as an increase in sample relative fluorescence. Note that fluorescence only starts to increase significantly after the 20th PCR cycle.

Figure 23 ***TaqMan end read scatterplot produced on the ABI PRISM 7900HT SDS***



Y-axis represents relative fluorescence for the major allele marker and x-axis relative fluorescence for the minor allele marker. Thus homozygotes for the major allele (blue dots) are found at the top left of the scatterplot, homozygotes for the minor allele (red dots) at the bottom right, and heterozygotes (green dots) in between. The negative controls (black dots) can be seen at the bottom left corner.

4.3 Demographic characteristics of the PRAMIS population

The basic demographic characteristics of the PRAMIS population are shown in Table 10. The case and control cohorts were matched for age, sex and current smoking status upon entry to the study, thus there were no significant differences between the groups with respect to these. There were, however, significant differences between the groups with regard to other common cardiovascular risk factors (see Table 11). Of note, the significant differences in systolic and diastolic blood pressure and total cholesterol are in the opposite direction to that anticipated. However, this can be readily explained by the fact that a large proportion of the cases were taking anti-hypertensive and/or cholesterol-lowering medications for the secondary prevention of ischaemic heart disease, compared to few in the control cohort.

Table 11 ***Demographic characteristics of the PRAMIS population***

	Cases (n=204)	Controls (n=200)	p-value
Mean age	46.8 ± 6.2	47.3 ± 6.0	0.363
Event age	42.3 ± 5.7	-	-
Male/female	175/30	174/26	0.634
Diabetes mellitus (%)	22 (10.7%)	2 (1%)	<0.001
Hypertension (%)	58 (28.3%)	18 (9%)	<0.001
Current smokers (%)	42 (20.5%)	36 (18%)	0.526
Systolic BP (mmHg)	128 ± 18	133 ± 14	0.004
Diastolic BP (mmHg)	82 ± 12	86 ± 10	0.002
Total cholesterol (mmol/L)	4.93 ± 1.27	5.22 ±1.04	0.014
HDL cholesterol (mmol/L)	1.13 ± 0.35	1.35 ± 0.34	<0.001
Triglyceride (mmol/L)	1.93 ± 0.99	1.50 ± 0.84	<0.001
Body mass index (kg/m^2)	29.8 ± 5.3	26.9 ± 3.9	<0.001
Plasma fibrinogen (g/L)	3.15 ± 0.74	2.84 ± 0.61	<0.001
Platelet count ($x10^3/\mu l$)	244 ± 72	240 ± 61	0.634
Mean platelet volume (fl)	9.0 ± 1.1	9.2 ± 1.2	0.178

4.4 Genotyping results and allele frequencies

The results of genotyping of the five selected polymorphisms in the premature MI cases (n=204) and matched controls (n=200) showing observed genotype frequencies and allele frequencies, and expected genotype frequencies assuming the presence of Hardy-Weinberg equilibrium are given in Table 12.

All genotype frequencies were in Hardy-Weinberg equilibrium in both cases and controls (Chi-square test, $p>0.05$).

4.5 Platelet function and its association with P2Y1 and P2Y12 sequence variation

Following collection, the genotyping data was recoded into numeric format, so that it was in a format suitable for statistical analysis, and entered into the PRAMIS master database. This database contains all of the platelet function data recorded as part of PRAMIS, in addition to the PRAMIS demographic data. The following sections describe the analysis of the genotyping data with respect to platelet functional responses in the cases and controls. As all of the premature MI cases were taking aspirin and often other drug therapies (such as β-blockers, angiotensin converting enzyme inhibitors and nitrates), which could potentially influence platelet responsiveness, it was not felt that it would be appropriate to perform the statistical analyses on the pooled case and control data. In view of this, the relationships between the polymorphisms and platelet responses in case and control subjects were assessed separately.

Table 12 ***Genotyping results***

Gene	Polymorphism	Genotype	Controls			Cases		
			Genotype frequency n(%) Observed	Expected*	Allele frequency	Genotype frequency n(%) Observed	Expected*	Allele frequency
P2Y1	1622	AA	147 (73.5)	145 (72.5)	A/G 0.85/0.15	138 (67.6)	137 (67.2)	A/G 0.82/0.18
		AG	46 (23.0)	51 (25.5)		59 (28.9)	64 (31.4)	
		GG	7 (3.5)	4 (2.0)		7 (3.4)	3 (1.4)	
P2Y12	IntB742	TT	138 (69.0)	138 (69.0)	T/C 0.83/0.17	138 (67.6)	141 (69.1)	T/C 0.83/0.17
		TC	56 (28.0)	56 (28.0)		61 (29.9)	57 (27.9)	
		CC	6 (3.0)	6 (3.0)		5 (2.5)	6 (3.0)	
	234	CC	88 (44.0)	90 (45.0)	C/T 0.67/0.33	86 (42.0)	87 (42.4)	C/T 0.65/0.35
		CT	91 (45.5)	88 (44.0)		94 (45.9)	93 (45.4)	
		TT	21 (10.5)	22 (11.0)		25 (12.2)	25 (12.2)	
	1622	CC	183 (91.5)	184 (92.0)	C/T 0.96/0.04	190 (92.7)	189 (92.2)	C/T 0.96/0.04
		CT	16 (8.0)	16 (8.0)		14 (6.8)	16 (7.8)	
		TT	1 (0.5)	0 (0)		1 (0.5)	0 (0)	
	2014	CC	119 (59.5)	122 (61.0)	C/T 0.78/0.22	113 (55.1)	112 (54.6)	C/T 0.74/0.26
		CT	69 (34.5)	68 (34.0)		79 (38.5)	79 (38.5)	
		TT	12 (6.0)	10 (5.0)		13 (6.3)	14 (6.9)	

* expected genotype frequencies assuming the presence of Hardy-Weinberg equilibrium (calculated using the Hardy-Weinberg equation $a^2 + 2ab + b^2$, where a=major allele frequency and b=minor allele frequency)

4.5.1 Data considerations

In PRAMIS, platelet functional responses were measured using flow cytometric analysis of fibrinogen-binding to platelets at rest, and following stimulation with 0.1, 1 and 10 μmol/L ADP and 3, 10 and 30 μmol/L TRAP. A FITC-labeled rabbit anti-fibrinogen was used to identify platelets that had fibrinogen bound to their surface-expressed GPIIb-IIIa receptors, and thus represented activated platelets. In addition, the expression of GPIIb-IIIa was determined using a different FITC-labeled specific antibody. The flow cytometer can express data as either the percentage of platelets positive for bound antibody (% positive), or as the median fluorescence intensity (measured in arbitrary units). For fibrinogen-binding, where activated platelets bind fibrinogen, and hence antibody, and non-activated platelets do not, the % positive measure is the most appropriate to use. Conversely, in the case of GPIIb-IIIa expression, where virtually all platelets will bind antibody but to varying extents depending on receptor expression, it is more appropriate to use the median fluorescence measure.

The distributions of platelet reactivity in the PRAMIS control and case cohorts are shown in Figures 24 and 25 respectively. It can be seen that there is marked inter-individual variation in fibrinogen-binding in resting and stimulated platelets, at all concentrations of ADP and TRAP, and in both the premature MI cases and matched controls. The horizontal bar represents the median value for each agonist dose.

Figure 24 ***Variation in platelet activation (% platelets positive for fibrinogen-binding) in 200 healthy control subjects, in resting and stimulated samples***

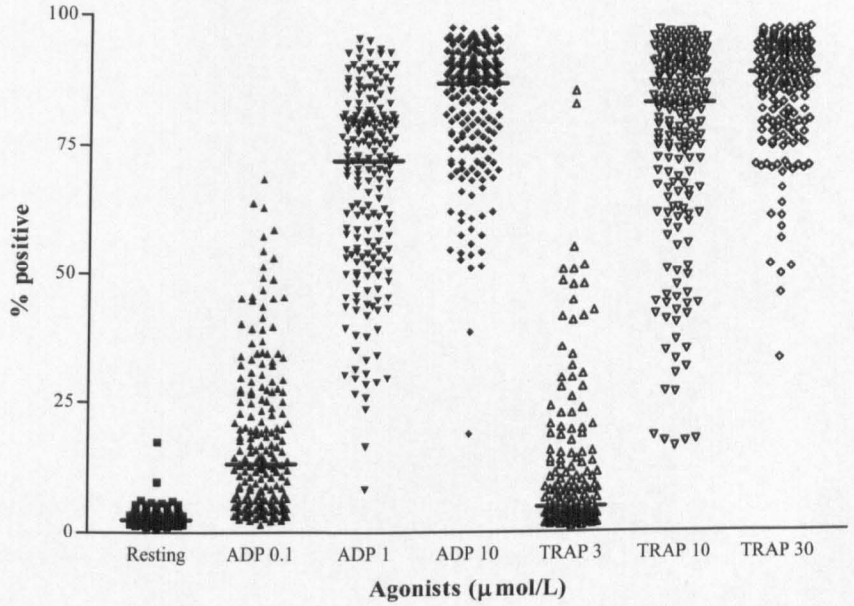
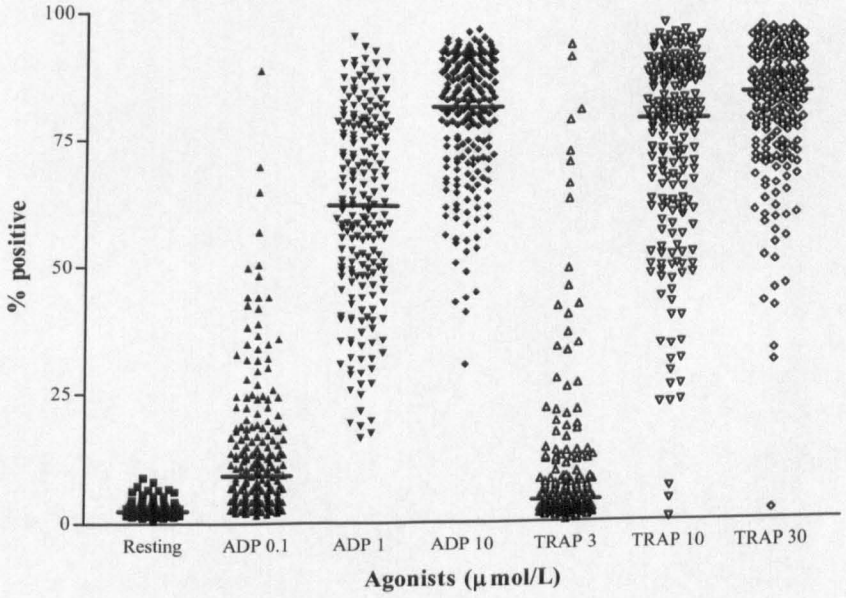


Figure 25 ***Variation in platelet activation (% platelets positive for fibrinogen-binding) in 205 subjects that have suffered a premature myocardial infarction, in resting and stimulated samples***



From these figures it is also possible to appreciate that platelet reactivity is not normally distributed, rather that there is extreme skewing of the distributions at low and high response. In the case of the resting samples and following low dose stimulation with ADP and TRAP, there is skewing towards low response, and following stimulation with intermediate and high dose ADP and TRAP, there is skewing towards high response. As the values for % positive must lie somewhere between 0 and 100%, it is possible to perform a logit transformation to normalise the data sets. As a result of this, it is then appropriate to use parametric tests to analyse the relationship between genotype and phenotype, thus improving statistical power and accuracy.

The formula used to transform the platelet response data was as follows:

$$\textbf{Transformed response (z)} = \log\left[\frac{\text{response}}{(100-\text{response})}\right]$$

Following statistical analysis, means and 95% confidence intervals were back-transformed, to allow more meaningful interpretation of the results, using the following formula:

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

The one sample Kolmogorov-Smirnov test was used to analyse whether the distributions of platelet fibrinogen-binding in resting and stimulated samples were significantly different from a normal distribution. Tables 13 and 14 show the results of these tests, in addition to the descriptive statistics, in the controls and cases respectively, before and after transformation. From these tables it can be seen that the distributions of all the raw data deviate from normality, and following transformation, all data sets, except those for resting platelets and platelets stimulated with low dose TRAP, become normally distributed. However, after transformation, the resting and low dose TRAP datasets are closer to normality than before transformation (indicated by a numerically smaller Kolmogorov-Smirnov Z value). This is further demonstrated graphically in Figure 26, where histograms of raw and transformed control data, from platelets stimulated with 0.1 μmol/L ADP are shown, overlaid with normal curves.

Table 13 ***Raw and transformed platelet function test results in the control cohort***

Agonist (μmol/L)	Fibrinogen binding (% positive)			
	Raw data		Transformed data	
	Median (range)	K-S test for normal distribution*	Mean (95% CI for mean)	K-S test for normal distribution*
Resting	2.4 (1.0-17.3)	Z=2.49, p<0.001	2.6 (2.5-2.7)	Z=1.45, p=0.030
ADP 0.1	13.1 (1.5-68.6)	Z=1.97, p=0.001	13.5 (11.9-15.3)	Z=1.09, p=0.183
ADP 1	72.1 (8.3-95.5)	Z=1.59, p=0.013	69.9 (67.0-72.6)	Z=0.77, p=0.594
ADP 10	87.0 (18.8-97.5)	Z=2.20, p<0.001	85.4 (83.9-86.9)	Z=1.04, p=0.232
TRAP 3	4.8 (1.2-85.5)	Z=3.62, p<0.001	6.6 (5.7-7.7)	Z=1.81, p=0.003
TRAP 10	83.3 (16.1-97.2)	Z=2.27, p<0.001	80.8 (78.2-83.2)	Z=0.98, p=0.294
TRAP 30	89.0 (33.2-97.7)	Z=2.17, p<0.001	88.3 (87.1-89.5)	Z=0.77, p=0.591

* the K-S test determines whether the distribution of a set of variables differs significantly from normality, thus a p-value of <0.05 means that the distribution is NOT normally distributed

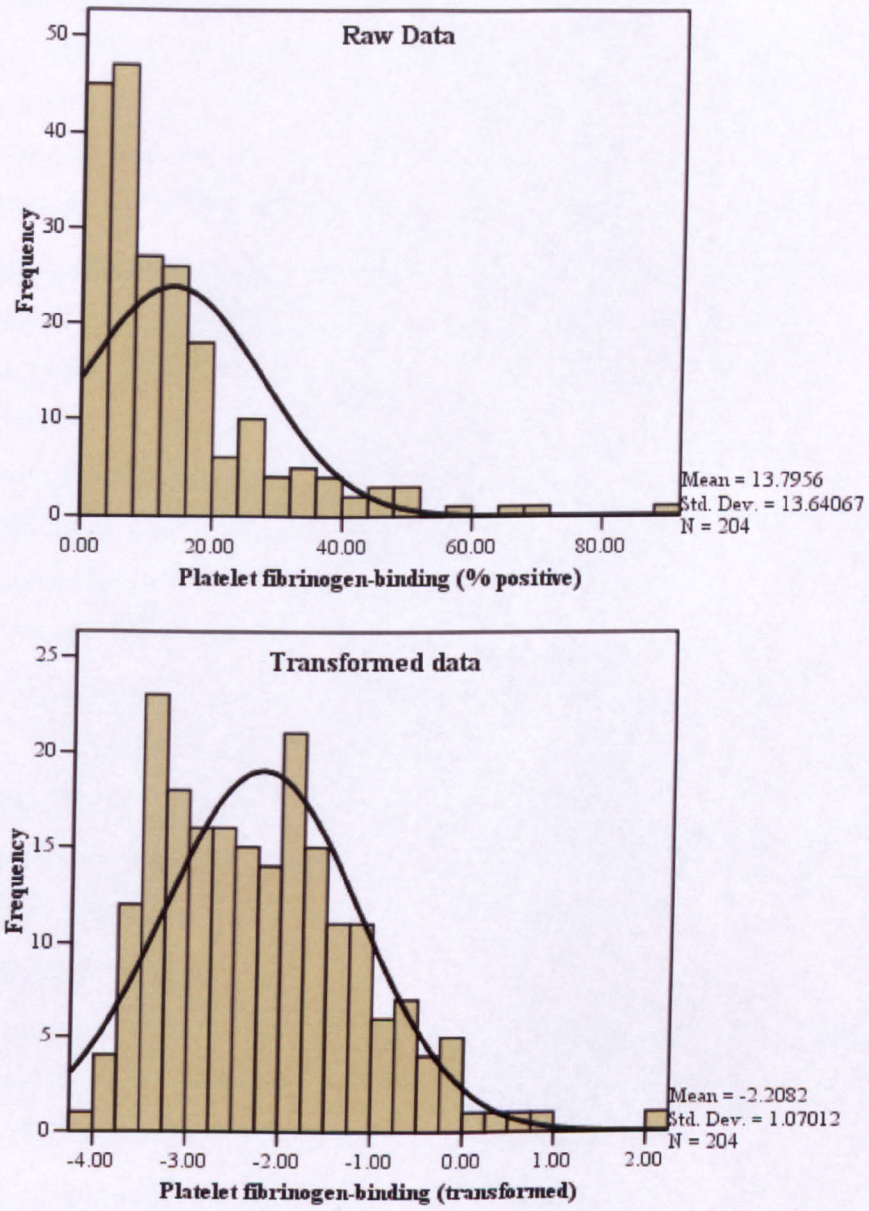
Table 14 ***Raw and transformed platelet function test results in the case cohort***

Agonist (μmol/L)	Fibrinogen binding (% positive)			
	Raw data		Transformed data	
	Median (range)	K-S test for normal distribution*	Mean (95% CI for mean)	K-S test for normal distribution*
Resting	2.4 (0.7-8.9)	Z=3.43, p<0.001	2.5 (2.3-2.6)	Z=2.25, p<0.001
ADP 0.1	9.2 (1.6-88.8)	Z=2.65, p<0.001	9.9 (8.7-11.3)	Z=1.06, p=0.213
ADP 1	62.3 (16.5-95.5)	Z=1.10, p=0.182	63.5 (60.5-66.4)	Z=0.59, p=0.877
ADP 10	81.9 (31.0-96.9)	Z=1.61, p=0.011	82.0 (80.3-83.6)	Z=0.64, p=0.811
TRAP 3	4.2 (0.4-93.8)	Z=4.29, p<0.001	6.0 (5.1-7.0)	Z=2.28, p<0.001
TRAP 10	79.2 (0.5-97.8)	Z=2.05, p<0.001	76.5 (73.5-79.3)	Z=0.99, p=0.280
TRAP 30	84.4 (1.9-97.3)	Z=1.75, p=0.004	83.8 (82.0-85.5)	Z=0.72, p=0.684

* the K-S test determines whether the distribution of a set of variables differs significantly from normality, thus a p-value of <0.05 means that the distribution is NOT normally distributed

The distributions of the raw data for glycoprotein IIb-IIIa receptor expression in both the cases and control cohorts did not differ significantly from normality (Kolmogorov-Smirnov Z=0.61 p=0.854 and Z=0.77 p=0.589 for cases and controls respectively), therefore this data did not require transformation prior to use.

Figure 26 ***Histograms of platelet fibrinogen-binding following stimulation with 0.1 μmol/L ADP, showing raw and transformed data***



4.5.2 Associations in healthy subjects

4.5.2.1 The ADP response

Associations between the five platelet ADP receptor gene polymorphisms, and platelet response to ADP in the control cohort are shown in Table 15. One-way analysis of variance (ANOVA) was used to compare the differences in mean fibrinogen-binding between the genotypes groups of each polymorphism, using a co-dominant model, in the transformed data set. Results were back transformed for presentation purposes.

None of the polymorphisms had a significant effect on the resting level of bound fibrinogen to unstimulated platelets, and there was no significant association between any of the P2Y12 gene polymorphisms and platelet reactivity to any dose of ADP. The P2Y1 1622A>G polymorphism, however, was found to have a significant effect on platelet activation in response to ADP, where subjects homozygous for the G allele have significantly greater platelet responses to ADP as compared to those subjects homozygous for the A allele. The effect is seen at all doses of ADP, but at the 1 μmol/L concentration just fails to reach statistical significance (p=0.067, Table 15).

The major difference was between the AA and the GG genotype. For example, at the 0.1 μmol/L ADP concentration, there was a 130% greater response in GG homozygotes when compared to AA homozygotes (p=0.011). The response of the AG genotype was also different from that of the AA genotype at all doses, but this difference failed to reach statistical significance before adjustment for other covariates.

4.5.2.2 The TRAP response

Associations between the five platelet ADP receptor gene polymorphisms, and platelet responses to TRAP in the control cohort are shown in Table 16. Similar statistical tests were used for the TRAP data, as those used for the ADP data.

There were no statistically significant associations observed between any of the P2Y12 gene polymorphisms and platelet functional responses following stimulation with TRAP. However, a similar pattern was seen with the P2Y1 1622A>G polymorphism and TRAP response, to that observed with ADP, i.e. homozygous carriage of the G allele conveyed numerically greater platelet reactivity, although this did not reach statistical significance.

Table 15 ***Platelet activation in response to ADP by P2Y1 and P2Y12 genotypes in control subjects***

Gene	Polymorphism	Genotype	Frequency n (%)	Mean platelet response (95% C.I.) to ADP (% positive for bound fibrinogen)				p-value			
				Resting	0.1 μmol/L	1 μmol/L	10 μmol/L	Resting	0.1	1	10
P2Y1	1622	AA	147 (73.5)	2.7 (2.5-2.8)	12.5 (10.8-14.5)	68.4 (65.0-71.7)	84.5 (82.6-86.3)				
		AG	46 (23.0)	2.4 (2.2-2.6)	15.0 (11.5-19.4)	71.9 (66.3-76.9)	86.5 (83.5-89.1)	0.129	0.025	0.067	0.020
		GG	7 (3.5)	3.1 (2.5-3.8)	28.8 (16.7-45.0)	82.8 (74.8-88.8)	93.0 (90.4-94.6)				
P2Y12	IntB742	TT	138 (69.0)	2.7 (2.5-2.9)	14.6 (12.5-17.0)	70.7 (67.3-73.9)	85.7 (83.9-87.4)				
		TC	56 (28.0)	2.5 (2.3-2.7)	11.1 (8.8-13.9)	67.2 (61.5-72.4)	84.0 (80.6-86.8)	0.463	0.170	0.495	0.376
		CC	6 (3.0)	2.5 (1.8-3.4)	12.6 (6.5-22.9)	73.2 (63.3-81.3)	89.2 (85.1-92.2)				
	234	CC	88 (44.0)	2.7 (2.5-2.9)	13.0 (10.7-15.8)	69.3 (65.0-73.4)	85.5 (83.2-87.5)				
		CT	91 (45.5)	2.6 (2.4-2.8)	14.1 (11.8-16.8)	71.1 (67.3-74.6)	86.0 (83.9-87.9)	0.598	0.784	0.613	0.313
		TT	21 (10.5)	2.4 (2.1-2.8)	12.5 (7.7-19.8)	66.5 (54.0-77.0)	81.8 (73.5-87.9)				
	1622	CC	183 (91.5)	2.6 (2.5-2.8)	13.7 (12.0-15.5)	70.5 (67.7-73.2)	85.8 (84.2-87.2)				
		CT	16 (8.0)	2.2 (1.9-2.7)	11.5 (6.7-19.1)	62.2 (47.9-74.7)	80.6 (71.0-87.6)	0.074†	0.470†	0.120†	0.090†
		TT	1 (0.5)	-	-	-	-				
	2014	CC	119 (59.5)	2.6 (2.4-2.7)	14.2 (12.0-16.8)	70.9 (67.2-74.3)	85.8 (83.8-87.6)				
		CT	69 (34.5)	2.6 (2.4-2.9)	11.7 (9.6-14.1)	66.8 (62.1-71.2)	83.8 (81.0-86.3)	0.641	0.237	0.216	0.193
		TT	12 (6.0)	2.9 (2.1-3.8)	17.0 (8.3-31.9)	75.7 (61.2-86.1)	89.0 (81.8-93.6)				

p-values represent overall genotype effects using a co-dominant model. † p-value tested after pooling CT and TT group.

Table 16 ***Platelet activation in response to TRAP by P2Y1 and P2Y12 genotypes in control subjects***

Gene	Polymorphism	Genotype	Frequency n (%)	Mean platelet response (95% C.I.) to TRAP (% positive for bound fibrinogen)			p-value		
				3 μmol/L	10 μmol/L	30 μmol/L	3	10	30
P2Y1	1622	AA	147 (73.5)	6.2 (5.3-7.3)	79.9 (76.7-82.8)	87.7 (86.2-89.1)			
		AG	46 (23.0)	7.6 (5.2-11.2)	81.9 (76.4-86.5)	89.4 (86.8-91.5)	0.295	0.209	0.105
		GG	7 (3.5)	10.1 (4.8-20.1)	89.4 (80.9-94.4)	93.0 (88.1-96.0)			
P2Y12	IntB742	TT	138 (69.0)	6.9 (5.8-8.4)	81.7 (78.7-84.3)	88.9 (87.5-90.2)			
		TC	56 (28.0)	5.7 (4.2-7.5)	78.0 (71.6-83.3)	86.9 (83.7-89.5)	0.379	0.389	0.360
		CC	6 (3.0)	9.1 (4.6-17.1)	84.7 (74.6-91.2)	88.6 (81.0-93.4)			
	234	CC	88 (44.0)	6.2 (5.1-7.4)	81.0 (77.2-84.3)	88.3 (86.4-90.0)			
		CT	91 (45.5)	7.0 (5.5-8.9)	81.8 (78.0-85.1)	88.9 (87.1-90.5)	0.713	0.365	0.390
		TT	21 (10.5)	7.0 (3.7-13.0)	75.1 (61.0-85.4)	85.9 (79.4-90.5)			
	1622	CC	183 (91.5)	6.7 (5.7-7.9)	81.3 (78.6-83.7)	88.7 (87.4-89.8)			
		CT	16 (8.0)	5.6 (3.2-9.7)	75.1 (62.0-84.8)	84.3 (76.8-89.7)	0.521†	0.213†	0.073†
		TT	1 (0.5)	-	-	-			
	2014	CC	119 (59.5)	6.8 (5.6-8.3)	82.0 (78.9-84.7)	89.0 (87.5-90.2)			
		CT	69 (34.5)	6.0 (4.7-7.7)	78.1 (72.9-82.6)	87.0 (84.3-89.2)	0.493	0.343	0.283
		TT	12 (6.0)	8.6 (4.4-16.3)	83.1 (64.8-92.9)	89.6 (81.2-94.5)			

p-values represent overall genotype effects using a co-dominant model. † p-value tested after pooling CT and TT group.

4.5.3 Associations in subjects with a history of premature MI

4.5.3.1 The ADP response

Associations between the P2Y1 and P2Y12 genotypes and platelet function following stimulation with ADP, and in subjects that have suffered a previous premature MI, are shown in Table 17.

None of the five polymorphisms showed any significant association with platelet activation induced following stimulation with ADP at any concentration. This was even true for the P2Y1 1622A>G polymorphism that had shown a significant effect on platelet function in the control cohort.

4.5.3.2 The TRAP response

Data comparing platelet activation in response to stimulation with TRAP, with P2Y1 and P2Y12 genotypes in the cases is shown in Table 18. There was no significant association between the TRAP response in the cases and any of the P2Y1 or P2Y12 gene polymorphisms, except in the case of the P2Y12 234C>T polymorphism at 3 μmol/L, where carriage of the T allele appeared to be associated with significantly lower platelet activation ($p=0.039$).

On inspection of the raw data for the P2Y12 2014C>T polymorphism, subjects homozygous for the T allele appeared to have numerically greater fibrinogen-binding following stimulation with 10 and 30 μmol/L TRAP (Table 18). However, this relationship was not found to be statistically significant even when the data was analysed as a recessive model.

Table 17 ***Platelet activation in response to ADP by P2Y1 and P2Y12 genotypes in case subjects***

Gene	Polymorphism	Genotype	Frequency n (%)	Mean platelet response (95% C.I.) to ADP (% positive for bound fibrinogen)				p-value			
				Resting	0.1 μmol/L	1 μmol/L	10 μmol/L	Resting	0.1	1	10
P2Y1	1622	AA	138 (67.5)	2.5 (2.3-2.6)	9.8 (8.3-11.5)	63.4 (59.7-67.0)	82.0 (79.8-83.9)				
		AG	59 (29.1)	2.4 (2.2-2.6)	9.8 (7.7-12.4)	63.5 (58.0-68.7)	81.8 (78.6-84.7)	0.541	0.597	0.947	0.845
		GG	7 (3.4)	2.8 (1.9-4.2)	14.2 (4.0-39.5)	66.1 (2.6-84.5)	84.4 (69.3-92.9)				
P2Y12	IntB742	TT	138 (67.6)	2.5 (2.3-2.7)	9.2 (7.8-10.7)	61.9 (58.2-65.4)	81.2 (79.0-83.1)				
		TC	61 (29.9)	2.4 (2.2-2.6)	11.9 (9.1-15.6)	66.9 (61.2-72.2)	83.7 (80.4-86.5)	0.684	0.197	0.275	0.362
		CC	5 (2.5)	2.2 (1.7-2.9)	9.1 (4.5-17.3)	67.0 (49.1-81.1)	83.7 (72.8-90.8)				
	234	CC	86 (42.0)	2.5 (2.3-2.7)	10.1 (8.1-12.4)	64.1 (59.4-68.6)	82.5 (79.9-84.9)				
		CT	94 (45.9)	2.4 (2.3-2.6)	10.2 (8.4-12.4)	63.3 (58.9-67.6)	82.0 (79.3-84.3)	0.894	0.705	0.940	0.745
		TT	25 (12.2)	2.4 (2.1-2.8)	8.5 (5.9-12.2)	62.3 (53.3-70.6)	80.4 (74.5-85.1)				
	1622	CC	190 (92.7)	2.5 (2.4-2.6)	10.1 (8.8-11.6)	64.1 (61.1-67.1)	82.3 (80.6-83.9)				
		CT	14 (6.8)	2.2 (1.9-2.6)	8.2 (4.7-13.9)	55.9 (41.8-69.1)	77.8 (66.7-85.9)	0.284†	0.443†	0.166†	0.190†
		TT	1 (0.5)	-	-	-	-				
	2014	CC	113 (55.1)	2.5 (2.3-2.6)	9.0 (7.6-10.7)	62.1 (58.1-65.9)	81.1 (78.7-83.2)				
		CT	79 (38.5)	2.5 (2.3-2.7)	11.1 (8.8-13.9)	64.7 (59.7-69.5)	82.7(79.8-85.4)	0.951	0.279	0.441	0.330
		TT	13 (6.3)	2.4 (2.1-2.9)	11.5 (6.5-19.7)	68.6 (56.2-78.8)	85.4 (79.2-90.0)				

p-values represent overall genotype effects using a co-dominant model. † p-value tested after pooling CT and TT group.

Table 18 ***Platelet activation in response to TRAP by P2Y1 and P2Y12 genotypes in case subjects***

Gene	Polymorphism	Genotype	Frequency n (%)	Mean platelet response (95% C.I.) to TRAP (% positive for bound fibrinogen)			p-value		
				3 μmol/L	10 μmol/L	30 μmol/L	3	10	30
P2Y1	1622	AA	138 (67.5)	6.3 (5.0-7.8)	75.6 (71.6-79.3)	83.4 (81.0-85.6)			
		AG	59 (29.1)	5.2 (4.1-6.5)	77.4 (72.3-81.8)	84.3 (81.1-87.0)	0.469	0.596	0.819
		GG	7 (3.4)	7.9 (2.5-22.4)	82.7 (63.6-92.8)	85.9 (72.1-93.5)			
P2Y12	IntB742	TT	138 (67.6)	5.6 (4.6-6.7)	74.8 (71.1-78.2)	83.0 (80.9-84.9)			
		TC	61 (29.9)	7.3 (5.1-10.4)	79.4 (73.2-84.5)	85.3 (81.0-88.7)	0.186	0.298	0.456
		CC	5 (2.5)	3.4 (2.2-5.2)	81.5 (63.2-91.8)	86.1 (72.9-93.5)			
	234	CC	86 (42.0)	6.8 (5.1-9.1)	78.7 (74.3-82.6)	84.8 (82.1-87.2)			
		CT	94 (45.9)	5.3 (4.3-6.5)	74.8 (69.7-79.4)	83.4 (80.4-86.0)	0.334	0.369	0.495
		TT	25 (12.2)	6.0 (3.7-9.6)	74.2 (65.2-81.5)	81.7 (75.7-86.4)			
	1622	CC	190 (92.7)	6.3 (5.3-7.4)	76.9 (73.7-79.8)	84.0 (82.1-85.8)			
		CT	14 (6.8)	3.2 (2.4-4.3)	69.7 (55.8-80.7)	80.4 (70.9-87.4)	0.039	0.236	0.322
		TT	1 (0.5)	-	-	-			
	2014	CC	113 (55.1)	5.5 (4.5-6.8)	75.2 (71.2-78.7)	83.1 (80.8-85.2)			
		CT	79 (38.5)	6.6 (4.9-8.7)	76.7 (70.9-81.7)	83.9 (80.3-87.0)	0.603	0.278	0.343
		TT	13 (6.3)	6.5 (2.9-13.8)	84.2 (75.4-90.3)	88.1 (82.7-92.0)			

p-values represent overall genotype effects using a co-dominant model. † p-value tested after pooling CT and TT group.

4.5.4 Effects of other subject characteristics

In order to assess the effects of other subject characteristics on the platelet responses measured as part of PRAMIS a stepwise linear regression was performed, with the following variables entered into the model: age, gender, current smoking status, systolic and diastolic blood pressure, GPIIb-IIIa expression, platelet count, MPV, plasma fibrinogen and resting level of bound fibrinogen in unstimulated platelets. Resting fibrinogen-binding, which is an indicator of the degree of platelet activation induced during phlebotomy and sample preparation, was included in the model as there was modest, but highly significant correlations observed between this variable and all stimulating concentrations of ADP and TRAP (Pearson correlations, r=0.47, 0.41, 0.37, 0.42, 0.31 and 0.35 respectively for ADP 0.1, 1 and 10 μmol/L and TRAP 3, 10 and 30 μmol/L. All p-values <0.001). Thus it was felt to be important to adjust for this influence if necessary.

Following the stepwise regression, multiple linear regression modelling was used to adjust the genotype effects for the important independent predictors identified, and to estimate partial effects.

The consistent, significant predictors of fibrinogen-binding in response to stimulation with ADP in the control population were age, current smoking status, GPIIb-IIIa expression and the resting level of bound fibrinogen in unstimulated platelets. The predictors of platelet response following stimulation with TRAP were similar but did not include current smoking status. The unstandardised coefficients, p-values and estimates of partial effects for these variables, at each stimulating concentration of ADP and TRAP are given in Table 19. All predictors are positively correlated with platelet responses, except smoking status, where current smokers were found to have lower response than non-smokers.

Table 19 ***Significant independent predictors of platelet responses in the controls***

Agonist (μmol/L)	Independent predictor	Unstandardised coefficient (B)	p-value	Partial effect (%)
ADP 0.1	Resting fibrinogen-binding	1.229	<0.001	24.5
	Age	0.060	<0.001	15.0
	GPIIb-IIIa expression	0.296	0.006	4.1
	Smoking status	-0.530	0.002	5.5
ADP 1	Resting fibrinogen-binding	0.968	<0.001	18.5
	Age	0.045	<0.001	10.4
	GPIIb-IIIa expression	0.346	0.001	6.2
	Smoking status	-0.382	0.015	3.3
ADP 10	Resting fibrinogen-binding	0.770	<0.001	15.1
	Age	0.033	<0.001	6.1
	GPIIb-IIIa expression	0.380	<0.001	8.7
	Smoking status	-0.378	0.009	4.6
TRAP 3	Resting fibrinogen-binding	1.230	<0.001	17.8
	Age	0.034	0.006	3.8
	GPIIb-IIIa expression	0.180	0.150	1.1
TRAP 10	Resting fibrinogen-binding	0.861	<0.001	9.6
	Age	0.041	0.001	5.3
	GPIIb-IIIa expression	0.529	<0.001	8.4
TRAP 30	Resting fibrinogen-binding	0.724	<0.001	13.8
	Age	0.022	0.009	3.4
	GPIIb-IIIa expression	0.552	<0.001	17.5

In the controls, following adjustment for other important independent predictors of the platelet reponse to ADP and TRAP, the associations between the P2Y12 gene polymorphisms and platelet responses to either agonist remained non-significant. The p-values and partial effects for the associations between the P2Y1 1622A>G polymorphism and platelet reactivity at each concentration of agonist, both before and after adjustment in the controls, is shown in Table 20.

Table 20 ***Associations between the P2Y1 1622A>G polymorphism and platelet function, before and after adjustment for other covariates in the control cohort***

Agonist (μmol/L)	p-value		Partial effect (%)	
	unadjusted	adjusted	unadjusted	adjusted
ADP 0.1	0.025	0.005*	3.3	5.6*
ADP 1	0.067	0.019*	2.5	4.2*
ADP 10	0.020	0.004*	3.8	5.7*
TRAP 3	0.295	0.169†	0.9	1.9†
TRAP 10	0.209	0.104†	1.4	2.4†
TRAP 30	0.105	0.015†	2.1	4.4†

* after adjusting for resting level of bound fibrinogen, age, smoking status and GPIIb-IIIa expression

† after adjusting for resting level of bound fibrinogen, age and GPIIb-IIIa expression

Stepwise and multiple linear regression analyses for the case cohort is detailed in Table 21, in a similar format to that used for the controls. In contrast to the controls, the independent predictors of platelet responses to ADP and TRAP in the cases were resting fibrinogen-binding, plasma fibrinogen concentration and mean platelet volume for both agonists, in addition to systolic blood pressure for ADP and GPIIb-IIIa expression for TRAP. All predictors were positively correlated with platelet responses, except plasma fibrinogen concentration, which was found to be negatively correlated.

Adjusting for the other independent predictors of platelet responses to ADP and TRAP in the case cohort did not alter the finding of a lack of significant association between any of the platelet ADP receptor gene polymorphisms and platelet reactivity.

Table 21 ***Significant independent predictors of platelet responses in the cases***

Agonist (μmol/L)	Independent predictor	Unstandardised coefficient (B)	p-value	Partial effect (%)
ADP 0.1	Resting fibrinogen-binding	1.543	<0.001	28.3
	Plasma fibrinogen	-0.288	0.001	6.2
	Mean platelet volume	0.164	0.005	4.5
	Systolic blood pressure	0.008	0.015	2.0
ADP 1	Resting fibrinogen-binding	1.054	<0.001	18.3
	Plasma fibrinogen	-0.226	0.003	4.7
	Mean platelet volume	0.203	<0.001	8.2
	Systolic blood pressure	0.008	0.014	3.3
ADP 10	Resting fibrinogen-binding	0.852	<0.001	15.4
	Plasma fibrinogen	-0.226	0.001	6.1
	Mean platelet volume	0.190	<0.001	9.1
	Systolic blood pressure	0.006	0.034	2.4
TRAP 3	Resting fibrinogen-binding	1.331	<0.001	14.4
	Mean platelet volume	0.105	0.207	1.1
	Plasma fibrinogen	-0.140	0.209	0.7
	GPIIb-IIIa	0.158	0.277	0.5
TRAP 10	Resting fibrinogen-binding	0.779	0.001	5.4
	Mean platelet volume	0.189	0.018	5.8
	Plasma fibrinogen	-0.260	0.014	3.0
	GPIIb-IIIa	0.325	0.019	2.4
TRAP 30	Resting fibrinogen-binding	0.703	<0.001	8.1
	Mean platelet volume	0.171	0.005	8.0
	Plasma fibrinogen	-0.280	0.001	6.2
	GPIIb-IIIa	0.104	0.007	3.5

4.5.5 Combined effects of different polymorphisms

Data from the above analyses shows that the P2Y1 1622 A>G polymorphism has a significant effect on platelet reactivity measured as fibrinogen-binding to the activated GPIIb-IIIa receptor, when analysed in the 200 normal subjects that make up the control cohort from PRAMIS. However, this study was unable to confirm the earlier findings of Fontana *et al*,[Fontana *et al*, 2003a] with regard to the effect of the P2Y12 gene haplotype (represented by the P2Y12 IntB742 T>C polymorphism in this study) on platelet function. To ascertain whether there may be an interaction between the P2Y1 1622 polymorphism and the P2Y12 IntB742 polymorphism that underlies this, further statistical analysis was performed on the control cohort, which is detailed below.

As the absolute number of homozygotes for the less common allele with both the P2Y1 1622 polymorphism and P2Y12 IntB742 polymorphism was small (7 GG and 6

CC subjects respectively), the PRAMIS control subjects were re-grouped into carriers and non-carriers of the minor allele for each polymorphism. This manipulation did not affect the finding of a statistically significant association between the P2Y1 1622A>G polymorphism and platelet function (p-values, after adjustment for the resting level of bound fibrinogen, GPIIb-IIIa expression, age and smoking, = 0.004, 0.015 and 0.007 following stimulation with 0.1, 1 and 10 μmol/L ADP respectively). General linear modelling was then used to assess the main effects of carriage of either the P2Y1 1622 G allele or P2Y12 IntB742 C allele following adjustment for the other independent predictors (resting level of bound fibrinogen, GPIIb-IIIa expression, age and smoking) and a term was added to the model to analyse for any possible interaction between the two polymorphisms. Data from this analysis is shown in Tables 22 and 23.

Table 22 ***Platelet function partitioned by genotype after adjustment for other independent covariates in the control cohort***

P2Y12 IntB742 C allele carriage	P2Y1 1622 G allele carriage (n)	Mean platelet response (95% confidence intervals) to ADP (% positive for bound fibrinogen)		
		0.1 μmol/L	1 μmol/L	10 μmol/L
No	No (100)	12.9 (11.1-14.8)	68.2 (64.7-71.5)	84.4 (82.4-86.2)
	Yes (38)	18.4 (14.7-22.7)	75.1 (70.1-79.6)	88.0 (85.2-90.2)
Yes	No (47)	11.3 (9.1-13.9)	67.9 (62.8-72.7)	84.2 (81.2-86.8)
	Yes (15)	14.3 (9.8-20.3)	72.7 (64.0-80.0)	88.5 (84.1-91.8)

Table 23 ***General linear modelling output showing tests of between-subjects effects***

Model term	p-values		
	0.1 μmol/L	1 μmol/L	10 μmol/L
Resting bound fibrinogen	<0.001	<0.001	<0.001
GPIIb-IIIa expression	0.003	<0.001	<0.001
Age	<0.001	<0.001	0.001
Smoking status	0.001	0.012	0.003
P2Y12 IntB742 C allele carriage	0.131	0.626	0.877
P2Y1 1622 G allele carriage	0.020	0.043	0.010
Genotype interaction	0.597	0.686	0.791

This data shows that, after adjustment for the resting level of bound fibrinogen, GPIIb-IIIa expression, age and smoking status, there is no significant interaction between the effects of the P2Y12 IntB742 polymorphism and the P2Y1 1622 polymorphism (p-values >0.05 at all concentrations of ADP) on platelet function in the controls.

4.5.6 Discussion

Five of the sixteen polymorphisms identified by direct sequencing were selected for genotyping in the full PRAMIS cohort, one from the P2Y1 gene and four from the P2Y12 gene. One of these, the P2Y1 1622A>G polymorphism, was found to be significantly associated with platelet activation, measured by flow cytometric analysis of fibrinogen-binding, following stimulation with ADP. Carriage of the P2Y1 1622 G allele conferred greater platelet reactivity to all doses of ADP. This effect persisted after adjustment for other variables that were significant predictors of the platelet ADP response. In fact, adjusting for other important predictors of the platelet response to ADP merely served to strengthen the associations observed between genotype and phenotype. Adjusting the platelet responses to the highest dose of TRAP for other significant covariates also renders the association between the P2Y1 1622 polymorphism and TRAP-stimulated platelet activation significant. Associations at the lower doses of TRAP, however, remained non-significant.

Because of the nature of this type of genetic association study, where the effects of multiple polymorphisms are analysed against a single quantitative trait, a criticism would be that it is possible to identify associations that may have occurred by chance. However, in this work, it remains likely that the observed effect of the P2Y1 1622A>G polymorphism on platelet ADP responses is genuine, for a number of reasons. First, the fact that the association was observed at all ADP doses strongly indicates a true genotype effect. Furthermore, the effect of the polymorphism on platelet response to ADP is further underscored by the fact that 6 out of the 7 individuals with the GG genotype had a response in the top 50% of the range at all doses of ADP. Second, in addition to its association with the platelet response to ADP, the P2Y1 1622A>G polymorphism also showed an association with the response to TRAP, but to a lesser extent than was observed with the ADP response. This is important because up to 50% of the response to TRAP is due to the secondary

effect of ADP released from the platelet dense granules, acting through the P2Y1 and P2Y12 receptors.[Storey *et al*, 2001] Thus any polymorphisms within these genes that affect platelet function following stimulation with ADP, will also have an effect on TRAP responses. Reproduction of the genotype effect with a second agonist that is heavily reliant on secondary ADP release indicates that the observed effect with ADP is genuine.

In the controls, the proportion of the interindividual variation in platelet reactivity to ADP explained by the P2Y1 1622 polymorphism was between 4.2% and 5.7% dependant on the stimulating concentration of agonist, and after adjustment for other significant covariates. The degree of platelet activation in unstimulated platelets explained the largest proportion of the variation observed (15.1% to 24.5%), followed by age (6.1% to 15.0%) and GPIIb-IIIa expression (4.1% to 8.7%). Smoking status explained the least proportion of this variation (3.3% to 5.5%). Thus the magnitude of the P2Y1 1622A>G polymorphism's effect is comparable to other factors that are accepted to affect platelet reactivity.

The mechanism by which the P2Y1 1622 polymorphism is associated with increased platelet response to ADP is not immediately obvious. Given that the polymorphism is itself silent, the likelihood is that this is through an effect on P2Y1 receptor numbers. Although silent, the base change could affect the stability of the mRNA message,[Duan *et al*, 2003; Shen *et al*, 1999] thereby leading to alterations in the number of P2Y1 receptors expressed. Alternatively, the polymorphism may be linked with one or more polymorphisms upstream of the gene that may affect its transcriptional regulation. Associations between receptor expression and silent polymorphisms have been observed in studies on other platelet receptors.[Dupont *et al*, 2003; Kunicki *et al*, 1997] In addition, a study by Hechler *et al* [Hechler *et al*, 2003] has found that over-expression of the P2Y1 receptor in a transgenic mouse model resulted in platelet hyper-reactivity to ADP, supporting this possibility. Further investigation of the P2Y1 1622 polymorphism in relation to platelet aggregation, signalling and attempts to establish its mechanism of action are described in Chapter 5.

This study failed to find any significant association between sequence variation in the P2Y12 gene and platelet function. This is in contrast to work published by Fontana *et al* [Fontana *et al*, 2003a] who found that a common haplotype in the P2Y12 gene was

associated with increased platelet aggregation in response to ADP and greater inhibition of iloprost-stimulated platelet cAMP accumulation. Fontana recruited 98 healthy male subjects (aged 18 to 35 yrs), and determined platelet aggregation in platelet rich plasma following stimulation with a range of concentrations of ADP. Following stimulation with 2 μmol/L ADP, the minor haplotype (termed H2) was associated with higher maximal aggregation in response to ADP, with median values of 34.7% in subjects carrying none of the H2 alleles (n=74), 67.9% in subjects carrying one H2 allele (n=21), and 82.4% in the three subjects carrying two H2 alleles (p=0.0071). However, of the three subjects homozygous for H2, although two subjects had high responses (82% and 86%), the response of the third subject was only 27%.[Fontana *et al*, 2003a]

The Fontana haplotype was successfully identified in the PRAMIS cohort. A single SNP representative of this haplotype, P2Y12 IntB742T>C, was genotyped in this work, with T/C allele frequencies of 0.83/0.17, compared with H1/H2 haplotype frequencies of 0.86/0.14. There was no significant association between this and platelet activation in response to either ADP or TRAP, before or after adjustment for other independent predictors. There was also no evidence for an interaction between this polymorphism and the P2Y1 1622 polymorphism with respect to platelet function. The reason behind this discrepancy remains unclear, but may reflect the different methodologies used in the two studies (platelet aggregation vs. fibrinogen-binding), although one would expect the two measures of platelet activation to correlate. An alternative explanation, given the finding of a significant effect of age on fibrinogen-binding in response to ADP in the control cohort and the known effect of age on aggregometry responses,[Terres *et al*, 1991] is the difference in the demographics of the subjects studied. In contrast to the PRAMIS cohort, Fontana *et al*'s subjects were all males and significantly younger. A third explanation is that either the results of Fontana are an example of a type 1 statistical error (i.e. false positive) or the results of this study are an example of a type 2 error (i.e. a false negative). It is difficult to be certain which of the above is true, but given that this work studied a cohort twice the size of Fontana's, and as fibrinogen-binding is a more direct measure of platelet activation compared to platelet aggregation, one can speculate that the data presented in the current work is more robust.

A discrepancy arises, however, when comparing the data from the control subjects to the case subjects for the P2Y1 1622A>G polymorphism, as it only appears to exhibit an effect in the control population. A number of possibilities exist that may account for this difference. In particular, the effects of medications taken by the cases (for example aspirin, nitrates, β-blockers and statins) may negate any effect on platelet reactivity that can be attributed to the P2Y1 1622 polymorphism. In a small study of 9 healthy volunteers, Knight *et al* [Knight CJ *et al*, 1997] measured platelet fibrinogen-binding at rest and following stimulation with ADP and TRAP before and after treatment with β-blockers, calcium channel antagonists and nitrates. They found that intravenous nitrate (GTN) reduced platelet fibrinogen-binding at rest and following stimulation with ADP. The calcium channel antagonist amlodipine was found to increase degranulation, and the β-blocker atenolol was found to increase platelet aggregation, both following stimulation with TRAP.

Aspirin has also been shown to have an effect on platelet fibrinogen-binding. Payne *et al* [Payne DA *et al*, 2002] found a single, 150mg dose of aspirin to reduce ADP-stimulated fibrinogen-binding by 21% in a group of seven healthy subjects. In PRAMIS, all of the cases were taking aspirin, whilst the majority of the controls were not. As aspirin undoubtedly has an effect on platelet reactivity, it is conceivable that its effect on platelet reactivity may not be consistent between subjects with differing P2Y1 1622 genotypes. In support of this hypothesis, Jefferson *et al* [Jefferson *et al*, 2005] have recently shown that carriage of the P2Y1 893 T allele is associated with a significant, 3-fold increase in aspirin resistance, determined by analysis of platelet aggregation following stimulation with 0.5mg/ml arachidonic acid in 330 white men with a prior history of myocardial infarction.

A further explanation for the differing results between the cases and controls is that the disease process present in the cases may alter the control mechanisms that are responsible for initiating platelet activation by this route. Knight *et al* [Knight *et al*, 1997a] measured platelet fibrinogen-binding in 12 subjects with coronary heart disease and 12 age and sex matched controls, and found that fibrinogen-binding following stimulation with ADP was significantly lower in those subjects with CHD, concluding that atherosclerosis impaired platelet responsiveness, thus providing support for this theory.

4.6 Associations between the ADP receptor gene polymorphisms and risk of premature myocardial infarction

4.6.1 Introduction

As platelet activation plays a critical role in the development of myocardial infarction and ADP is an important mediator of platelet activation, genetic variation within the platelet ADP receptor genes may impact upon risk of myocardial infarction, and in particular, those polymorphisms that have been shown to affect platelet function.

At the current time, there has been no published systematic investigation of associations between P2Y1 and P2Y12 ADP receptor gene polymorphisms and risk of MI. In this section, I will describe the analysis of the data-set for associations between platelet ADP receptor polymorphisms and risk of premature MI.

4.6.2 Results of univariate analysis

4.6.2.1 P2Y1 gene polymorphisms

A single polymorphism, P2Y1 1622A>G, was analysed in the P2Y1 gene. The genotype distribution of this polymorphism in the PRAMIS population has been detailed earlier in this chapter (section 4.3, Table 12).

There were no significant differences between the case and control populations for the P2Y1 1622A>G genotype distributions, Chi-square=1.85, p=0.396. The G allele frequency was 0.18 and 0.15 in the cases and controls respectively. The odds ratio (OR) for the risk of premature MI associated with the G allele, using a co-dominant model, was 1.22 (95% confidence intervals, C.I., 0.85-1.76), p=0.281. The results of dominant and recessive models are shown in Table 24. There was no significant association between the P2Y1 1622A>G polymorphism and risk of MI, irrespective of the model used.

Table 24 ***The P2Y1 1622A>G polymorphism and risk of premature MI***

Model	Odds ratio (95% CI)	p-value
Co-dominant	1.22 (0.85-1.76)	0.281
Dominant	1.33 (0.86-2.04)	0.198
Recessive	0.98 (0.33-2.85)	0.980

4.6.2.2 P2Y12 gene polymorphisms

Four common polymorphisms in the P2Y12 gene were genotyped in the full PRAMIS population. The genotype distributions of these polymorphisms in the PRAMIS population have been described earlier in this chapter (section 4.3, Table 12). The effects of these polymorphisms on risk of premature MI are considered in the following subsections.

P2Y12 IntB742T>C polymorphism

The P2Y12 IntB742T>C polymorphism was chosen to be representative of the haplotypes defined by the five P2Y12 polymorphisms in complete linkage disequilibrium (see Chapter 3, section 3.5.2).

There were no significant differences between the case and control populations for the P2Y12 IntB742 genotype distributions, Chi-square=0.27, p=0.876. The C allele frequency was 0.17 in both the cases and controls. The OR for the risk of premature MI associated with the C allele, using a co-dominant model, was 1.03 (95% C.I., 0.71-1.49), p=0.879. The results of dominant and recessive models are shown in Table 25. There was no significant association between the P2Y12 IntB742T>C polymorphism and risk of MI, irrespective of the model used.

Table 25 ***The P2Y12 IntB742T>C polymorphism and risk of premature MI***

Model	Odds ratio (95% CI)	p-value
Co-dominant	1.03 (0.71-1.49)	0.879
Dominant	1.07 (0.70-1.62)	0.770
Recessive	0.81 (0.24-2.71)	0.735

P2Y12 234C>T polymorphism

There were no significant differences between the case and control populations for the P2Y1 234 genotype distributions, Chi-square=0.36, p=0.836. The T allele frequencies were 0.35 and 0.33 in the cases and controls respectively. The OR for the risk of premature MI associated with the T allele, using a co-dominant model, was 1.09 (95% C.I., 0.81-1.46), p=0.572. The results of dominant and recessive models are shown in

Table 26. There was no significant association between the P2Y12 234C>T polymorphism and risk of MI, irrespective of the model used.

Table 26 ***The P2Y12 234C>T polymorphism and risk of premature MI***

Model	Odds ratio (95% CI)	p-value
Co-dominant	1.09 (0.81-1.46)	0.572
Dominant	1.09 (0.73-1.61)	0.677
Recessive	1.18 (0.64-2.19)	0.591

P2Y12 1622C>T polymorphism

There were no significant differences between the case and control populations for the P2Y1 1622 genotype distributions, Chi-square=0.20, p=0.903. The T allele frequency was 0.04 in both the cases and controls respectively. The OR for the risk of premature MI associated with the T allele, using a co-dominant model, was 0.87 (95% C.I., 0.45-1.69), p=0.871. The results of dominant and recessive models are shown in Table 27. There was no significant association between the P2Y12 1622C>T polymorphism and risk of MI, irrespective of the model used.

Table 27 ***The P2Y12 1622C>T polymorphism and risk of premature MI***

Model	Odds ratio (95% CI)	p-value
Co-dominant	0.87 (0.45-1.69)	0.871
Dominant	0.85 (0.41-1.75)	0.659
Recessive	0.98 (0.06-15.7)	0.986

P2Y12 2014C>T polymorphism

There were no significant differences between the case and control populations for the P2Y1 2014 genotype distributions, Chi-square=0.81, p=0.667. The T allele frequency was 0.26 and 0.22 in the cases and controls respectively. The OR for the risk of premature MI associated with the T allele, using a co-dominant model, was 1.14 (95% C.I., 0.83-1.56), p=0.437. The results of dominant and recessive models are shown in Table 28. There was no significant association between the P2Y12 2014C>T polymorphism and risk of MI, irrespective of the model used.

Table 28 ***The P2Y12 234C>T polymorphism and risk of premature MI***

Model	Odds ratio (95% CI)	p-value
Co-dominant	1.14 (0.83-1.56)	0.437
Dominant	1.20 (0.81-1.77)	0.373
Recessive	1.06 (0.47-2.38)	0.886

4.6.3 Multiple logistic regression analysis accounting for other risk factors

To further test for associations between the P2Y1 1622A>G and P2Y12 IntB742T>C polymorphisms and risk of premature MI, binary logistic regression modelling was used to adjust the OR's for age, gender, smoking and other demographic variables that are risk factors for MI (history of diabetes mellitus and hypertension, HDL concentration, BMI and plasma fibrinogen level). Total cholesterol and LDL concentrations were not included in the analysis because of high statin usage in the cases resulting in the cases having significantly lower values than the controls.

P2Y1 1622A>G polymorphism

After adjusting for age, gender, smoking, diabetes mellitus, hypertension, HDL concentration, BMI and fibrinogen level, and using the co-dominant model, the OR for the risk of premature MI associated with the G allele of the P2Y1 1622A>G polymorphism was 1.08 (95% C.I., 0.71-1.66), p=0.715. The results of dominant and recessive models are shown in Table 29. There was no significant association between the P2Y1 1622A>G polymorphism and risk of MI despite adjustment for other covariates and irrespective of the model used.

Table 29 ***The P2Y1 1622A>G polymorphism and risk of premature MI after adjustment for other covariates***

Adjusted model	Odds ratio (95% CI)	p-value
Co-dominant	1.08 (0.71-1.66)	0.715
Dominant	1.18 (0.72-1.95)	0.508
Recessive	0.68 (0.19-2.46)	0.559

P2Y12 IntB742T>C polymorphism

After adjusting for age, gender, smoking, diabetes mellitus, hypertension, HDL concentration, BMI and fibrinogen level, and using the co-dominant model, the OR for the risk of premature MI associated with the C allele of the P2Y12 IntB742T>C polymorphism was 1.09 (95% C.I., 0.71-1.68), p=0.679. The results of dominant and recessive models are shown in Table 30. Again, there was no significant association between the P2Y12 IntB742T>C polymorphism and risk of MI despite adjustment for other covariates and irrespective of the model used.

Table 30 ***The P2Y12 IntB742T>C polymorphism and risk of premature MI after adjustment for other covariates***

Adjusted model	Odds ratio (95% CI)	p-value
Co-dominant	1.08 (0.71-1.68)	0.679
Dominant	1.16 (0.71-1.90)	0.544
Recessive	0.76 (0.19-3.11)	0.703

4.6.4 Discussion

At the time of writing (December 2005), there has been little published work on genetic variation within the P2Y1 and P2Y12 platelet ADP receptor genes and risk of myocardial infarction. This is largely a consequence of the fact that prior to the work of Fontana *et al* [Fontana *et al*, 2003a], there had not been a systematic interrogation of either the P2Y1 or P2Y12 genes for novel, functional polymorphisms. Thus this study represents the largest investigation of platelet ADP receptor polymorphisms on platelet function and risk of MI to date.

The P2Y1 1622A>G polymorphism was found to have a significant effect on platelet fibrinogen-binding in response to ADP in the PRAMIS controls. At the lowest stimulating concentration of ADP (0.1 μmol/L), subjects carrying the GG genotype had 130% greater fibrinogen-binding as compared to AA homozygotes (p=0.011). This polymorphism accounts for between 4.2 and 5.7% of the variability observed in the platelet response to ADP of the population (see section 4.4.4, Table 20). This compares favourably with the variance explained by other covariates known to affect platelet reactivity (such as age, GPIIb-IIIa expression and smoking status). On a

population basis, the P2Y1 1622 polymorphism therefore exerts a small but significant effect on platelet function.

This study did not find a significant association between this polymorphism and risk of MI, before or after adjustment for other significant risk factors. However, it remains possible that an effect may still exist. As the aetiology of MI is complex and multifactorial, genetic variation within a single gene is unlikely to have a major impact on global risk. It is more likely that multiple gene-gene and gene-environment interactions combine to determine a particular individuals risk. As such, this study may have been underpowered to accurately determine the effect of the polymorphism on risk of MI, even despite the use of cases exhibiting an extreme phenotype. For example, from the 95% confidence intervals in Table 29, and using the most favourable (co-dominant) model, this study has been adequately powered to exclude an association between the P2Y1 1622A>G polymorphism and risk of premature MI that results in a greater than 66% increase in risk with G allele carriage. If, however, one considers the least favourable (recessive) model, this study has only excluded an association that results in a 150% increase in risk, or greater. Thus the relatively small cohort size in this study limits its power to detect increases in risk less than between 66% and 150%, depending on the model used, for the P2Y1 1622 polymorphism.

A haplotype equivalent to that defined by the P2Y12 IntB742T>C polymorphism in this study, has previously been shown to exert an effect on platelet function, measured using platelet aggregation to ADP.[Fontana *et al*, 2003a] Fontana *et al* demonstrated carriage of the minor haplotype, H2, to be significantly associated with greater platelet aggregation when compared to non-carriage (see section 4.4.6). In a follow-up study,[Fontana *et al*, 2003b] the same group also found that the presence of the H2 haplotype was significantly more common in 184 male subjects with peripheral arterial disease, whom were below the age of 70 years, compared to 330 matched controls (OR 1.6, 95% C.I. 1.1 to 2.5, p=0.02). This effect persisted after adjustment for diabetes, smoking, hypertension and hypercholesterolaemia (OR 2.3, 95% C.I. 1.4 to 3.9, p=0.002). There has been no other published data on this haplotype and platelet function, or risk of coronary artery disease.

In the current study, carriage of the P2Y12 IntB742C allele was not associated with either differences in ADP-stimulated fibrinogen-binding to platelets or risk of MI. In the absence of a demonstrable functional effect in the PRAMIS population, it is

perhaps unrealistic to expect to find an association with risk of MI, as any effect of the polymorphism on susceptibility to MI would need to be mediated through an intermediate phenotype, such as an effect on platelet reactivity. With respect to the Fontana case-control study,[Fontana *et al*, 2003b] it is difficult to provide a satisfactory biological explanation for their positive results, as the subjects recruited suffered with prevalent peripheral arterial disease, but had not necessarily experienced a thrombotic event.

4.7 Conclusions

In this Chapter:

- Assays were developed to genotype the common P2Y1 and P2Y12 ADP receptor gene polymorphisms in a cohort of 200 normal individuals and 204 subjects with a history of premature myocardial infarction
- Genotype frequencies within the two cohorts did not differ significantly from those predicted by Hardy-Weinberg equilibrium
- A silent, common A>G polymorphism at position 1622 of the P2Y1 gene was significantly associated with platelet response to ADP in the control cohort, where homozygous carriage of the G allele conferred up to 130% greater platelet reactivity as compared to individuals homozygous for the A allele
- Adjusting for other independent predictors of the platelet ADP response in the control population (resting fibrinogen-binding, age, GPIIb-IIIa expression and smoking status) did not diminish the association between the P2Y1 1622 polymorphism and platelet reactivity
- None of the common P2Y12 gene polymorphisms, including the haplotype previously identified by Fontana *et al*,[Fontana *et al*, 2003a] were found to be significantly associated with platelet responses in either the case or control cohorts. Adjusting for other independent predictors of the platelet response did not impact on this finding
- No association was observed between risk of premature myocardial infarction and any of the platelet P2Y1 or P2Y12 receptor polymorphisms identified

Chapter 5

Confirmation of the P2Y1 1622A>G polymorphism genotype effects on platelet function and signalling

5.1 Introduction

In this chapter the studies undertaken to further analyse the effects of the P2Y1 1622A>G polymorphism on platelet function and signalling are described. Specifically it details: (i) the development and optimisation of the platelet function assays; (ii) the comparison of platelet function and signalling between the P2Y1 1622 AA and GG homozygotes; (iii) assessment of the correlations between the different platelet function tests used.

5.2 Development of platelet function assays

Three different methods were used for testing platelet function in this study – flow cytometric analysis of fibrinogen-binding to the activated GPIIb-IIIa receptor (using a similar method to that used in PRAMIS), platelet aggregation measured by light transmittance and fluorometric analysis of platelet intracellular calcium flux.

5.2.1 Flow cytometric analysis of fibrinogen-binding

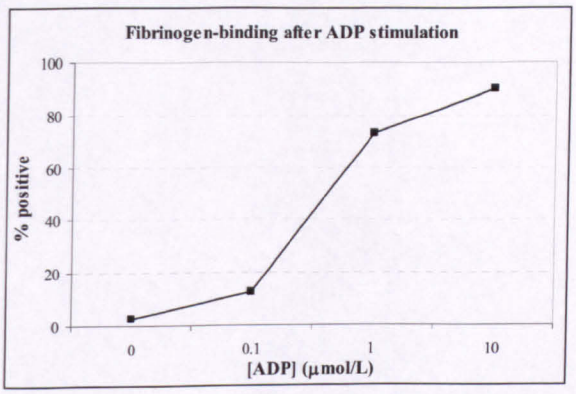
The principles and methods used in whole blood flow cytometry have been covered in detail in Chapters 1 and 2. In this part of the study, flow cytometry was only used to measure fibrinogen-binding to platelets in unstimulated samples, and following stimulation with a range of doses of ADP and a single dose of TRAP. ADP was used as an agonist to attempt to replicate the results of the first part of the study. TRAP was used in combination with two ADP receptor antagonists (MRS2179, a specific P2Y1 receptor antagonist and AR-C69931MX, a specific P2Y12 antagonist), to quantify platelet reactivity following stimulation with a different agonist, with any additive effects of released ADP removed. The purpose behind analysing the isolated TRAP response was to assess whether the platelets in the different genotype groups are specifically sensitive to ADP stimulation, or whether they are generally hyper-responsive to stimulation, irrespective of the agonist used.

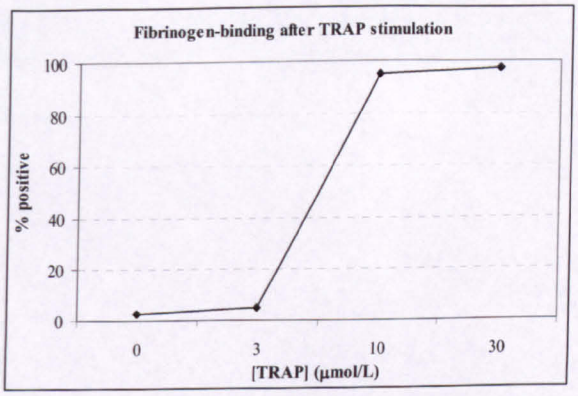
After preparing aliquots of varying concentrations of ADP, TRAP, MRS2179 and AR-C69931MX, dose-ranging studies were performed to determine the optimum concentrations of agonist that would allow differentiation of high responders from low responders in the study population. Figure 27 shows data from a single subject,

and demonstrates the typical dose response curves following stimulation with ADP and TRAP.

Figure 27 ***Example of flow cytometric analysis of platelet function***

Agonist (μmol/L)	Fibrinogen-binding (% positive)
0	2.7
ADP 0.1	12.8
ADP 1	73.2
ADP 10	90
TRAP 3	4.9
TRAP 10	94.9
TRAP 30	97.1





After this initial optimisation, reactions were performed to produce dose inhibition curves for the two ADP receptor antagonists when stimulating platelets with a range of doses of ADP. The rationale behind these experiments was to determine what doses of ADP receptor antagonists were needed to block the effects of ADP released following stimulation of platelets with TRAP. In line with their known effects, MRS2179 (Figure 28) was only able to completely abolish fibrinogen-binding at low concentrations of ADP and high concentrations of MRS2179, whereas AR-C69931MX (Figure 29) was able to completely abrogate the platelet ADP response, even at low doses.

Figure 28 ***ADP-stimulated platelet activation, measured by flow cytometry, in the presence or absence of varying concentrations of MRS2179 (n=1)***

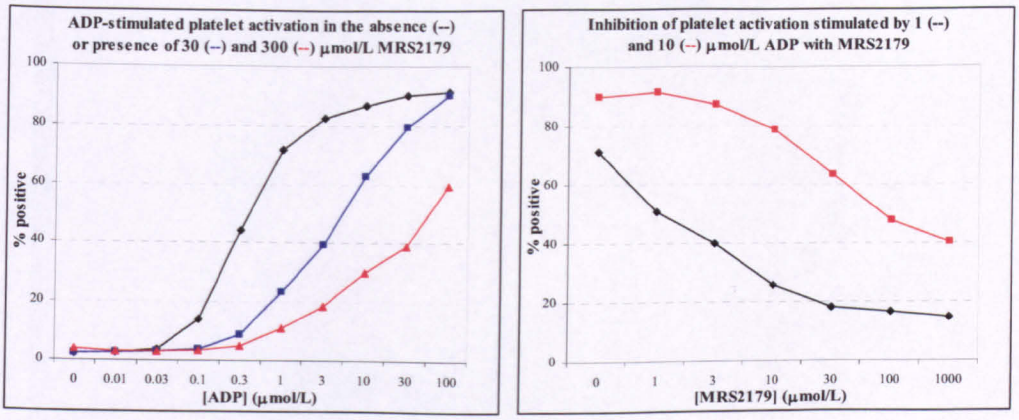
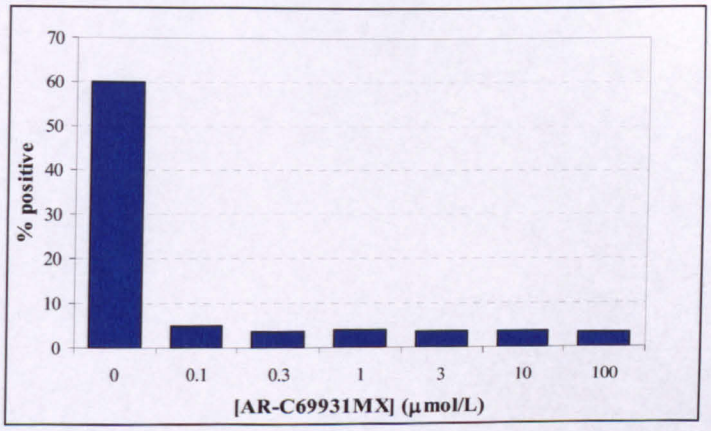
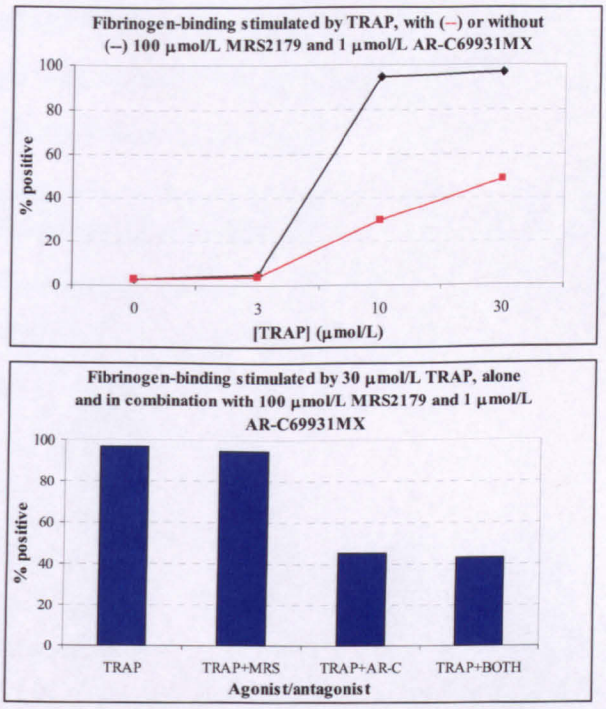


Figure 29 ***Inhibition of platelet activation stimulated by 1 µmol/L ADP with varying concentrations of AR-C69931MX (n=1)***



On the basis of the preliminary experiments with the two ADP receptor blockers, concentrations of each were chosen to eliminate the additive effect of released ADP when stimulating platelets with TRAP. It was felt likely that local concentrations of ADP following dense granule secretion would not exceed 1 µmol/L ADP, and therefore concentrations of 1 µmol/L AR-C69931MX and 100 µmol/L MRS2179 were used. Figure 30 shows the effects of MRS2179 and AR-C69931MX alone, and in combination, on the platelet response to varying concentrations of TRAP in a single subject.

Figure 30 ***Effect of MRS2179 and AR-C69931MX on platelet activation stimulated by TRAP (n=1)***



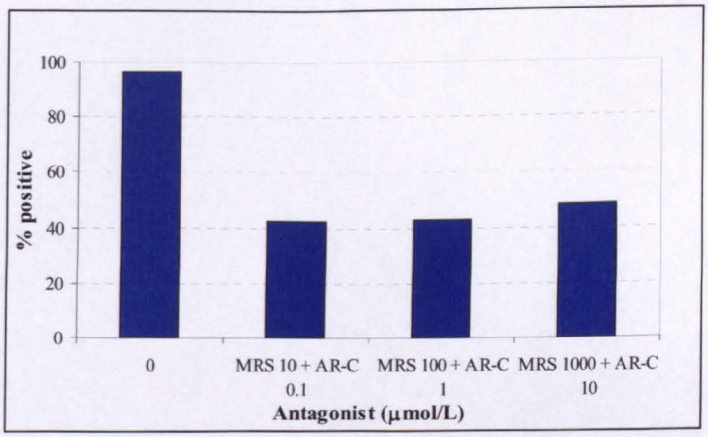
The upper figure shows platelet fibrinogen-binding (% positive) on the y-axis with TRAP dose on the x-axis demonstrating the effects of combined P2Y1 and P2Y12 blockade on TRAP response. The lower figure demonstrates the effects of P2Y1 and P2Y12 receptor blockade in isolation and together on platelet reponses to 30µmol/L TRAP

From these graphs it can be seen that between 50-70% of the fibrinogen-binding stimulated by TRAP is mediated via the action of ADP on the P2Y1 and P2Y12 receptors, dependent on the concentration of TRAP used (Figure 30). Inhibition of P2Y1-mediated activation does not result in a significant reduction in fibrinogen-binding following TRAP stimulation, however ADP acting via P2Y12 plays a significant role in augmenting the TRAP response. This is to be expected as both P2Y1 and PAR1 couple with Gαq, resulting in a degree of redundancy between the two receptors.

The final stage of optimisation of the flow cytometric analysis of the TRAP response was to perform an experiment to establish that complete inhibition of ADP-induced augmentation of the response had been achieved. In order to assess this, whole blood

was stimulated with 30 μmol/L TRAP in the absence, or presence of three different combinations of concentrations of MRS2179 and AR-C69931MX, covering three orders of magnitude on a log-scale (Figure 31), in a single subject. This showed that there is complete inhibition of augmentation of the TRAP response by released ADP with all three dose combinations. On the basis of this, 10 μmol/L MRS2179 and 0.1 μmol/L AR-C69931MX were chosen for use in the study.

Figure 31 ***Platelet fibrinogen-binding stimulated by 30 μmol/L TRAP alone, and in the presence of varying concentrations of MRS2179 and AR-C69931MX (n=1)***



5.2.2 Platelet aggregation assay

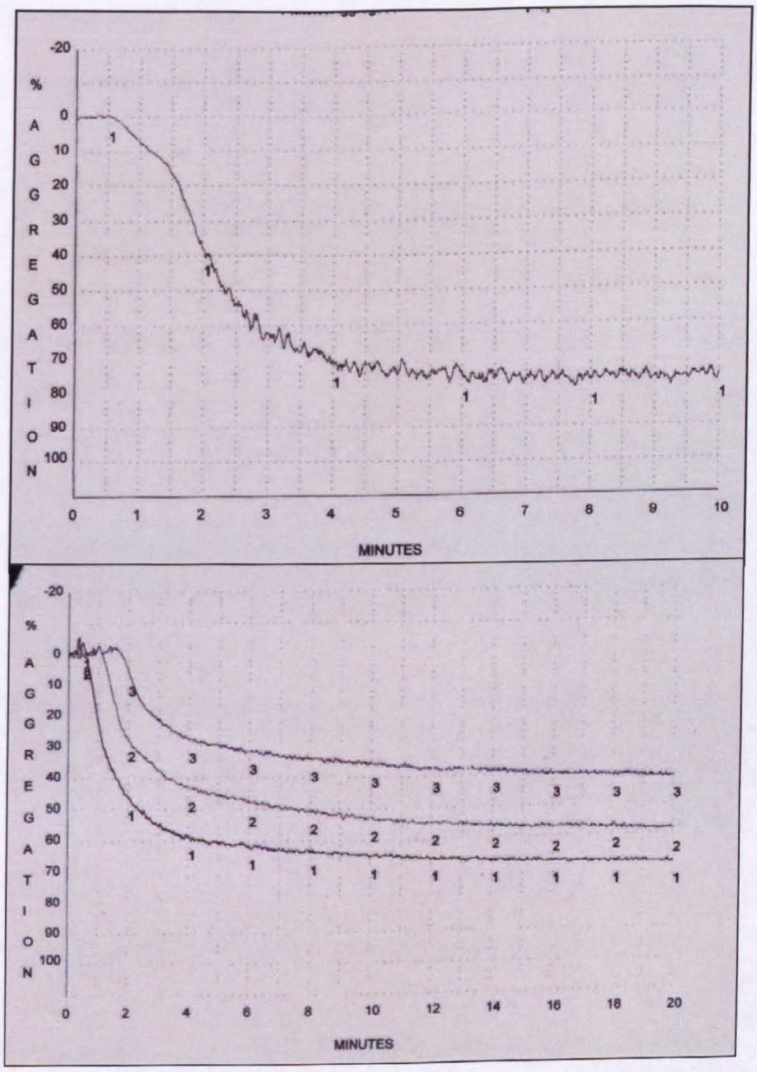
Platelet aggregometry was performed essentially based on the method previously described by Born.[Born GVR, 1962b] The principles and methods used have been described in detail in Chapters 1 and 2.

5.2.2.1 Effects of aspirin

When platelets are stimulated with "weak" agonists such as ADP in the presence of low extracellular calcium, TxA2 is rapidly generated (see Chapter 1, section 1.2.3.1). This augments the primary aggregation response to ADP, and thus could potentially dilute any effect of the P2Y1 1622 polymorphism. In order to minimise this effect, 100 μmol/L aspirin was added to the PRP prior to stimulation with 2MeSADP. Figure 32 demonstrates the effect of aspirin on platelet aggregation induced by 2MesADP. The first graph shows platelet aggregation induced by 40 nmol/L 2MeSADP in the absence of aspirin. The secondary phase of aggregation induced by generated TxA2

can be clearly seen starting at approximately 1 minute following the addition of agonist. In the second graph, platelet aggregation is shown following stimulation with 160 (line 1), 80 (line 2) and 40 (line 3) nmol/L 2MeSADP in the presence of 100 mmol/L aspirin. There is no secondary aggregation visible, just the primary aggregation induced by stimulation through the P2Y1 and P2Y12 ADP receptors alone.

Figure 32 ***Platelet aggregation stimulated by 2MeSADP alone, and with 100 μmol/L aspirin***



The upper figure shows primary and secondary platelet aggregation after stimulation with low dose 2MeSADP in the absence of aspirin. The lower figure shows isolated primary platelet aggregation following stimulation with a range of doses of 2MeSADP and after treatment with aspirin

5.2.2.2 Selection of agonist doses

As discussed in Chapter 2 (section 2.8.1), the rationale behind using 2MeSADP as the agonist for stimulation of platelet aggregation was to provide consistency when comparing aggregation with calcium flux data and because of its stability in aqueous solution. The range of concentrations used in the assay were determined empirically, and were selected so as to maximise the chance of observing a difference between the two genotype groups. The second graph from Figure 32 shows an example of the typical aggregation profile observed with 160, 80 and 40 nmol/L 2MeSADP.

5.2.3 Calcium flux assay

The calcium flux assay used in this study was developed in-house, and was based around previously published methods.[MacKenzie *et al*, 1996; Sage *et al*, 1987] Washed platelets were prepared from PRP by centrifugation and loaded with a calcium-sensitive fluorescent indicator (fluo3-AM). A known quantity of loaded platelets was then maximally stimulated with 2MeSADP at 37°C, in the presence or absence of a maximum inhibiting concentration of AR-C69931MX, used to block any effect of P2Y12 receptor stimulation on calcium flux. A baseline fluorescence reading was taken prior to addition of agonist after which fluorescence was measured for a period of 40 seconds, starting immediately upon addition of agonist. The change in fluorescence was determined by subtracting the baseline reading from those following stimulation, and initial rate of calcium flux and area under the calcium flux curve were also calculated in the post hoc analysis.

5.2.3.1 Optimisation of assay

A brief summary of the experiments performed to optimise the assay is detailed below.

Sample preparation

In order to be able to measure calcium flux, the platelets must first be loaded with the fluorescent indicator. This is undertaken in PRP, however, following successful loading the platelets must then be removed from the loading media, washed and resuspended in indicator free buffer. As a result of this, there is a significant amount of handling of the platelets, thus an inherent risk of inducing platelet activation and desensitisation of the ADP receptors.

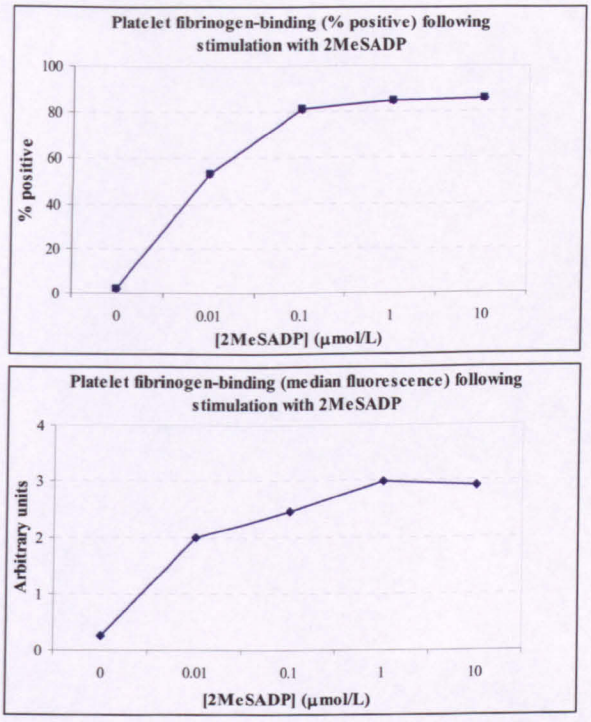
Initially, 6β-prostaglandin I_1, a prostacyclin analogue, was used with an aim to prevent platelet activation during sample preparation. It became readily apparent, however, that inhibition of the platelets by this method significantly altered their responsiveness to 2MeSADP-induced activation. This was in agreement with work recently published by Fox *et al.*[Fox *et al*, 2004] Consequently a method was employed to prevent platelet activation by means of blocking TxA2 generation, which arises as a result of the close cell-cell contact that occurs during sedimentation of the platelets, with aspirin, and using apyrase to hydrolyse any released ADP that may cause platelet activation and receptor desensitisation. In addition, centrifugation was performed at relatively low g, again to minimise the shear stress on the platelets.

Selection of agonist/antagonist concentrations

Due to technical limitations of the sample injection mechanism on the Fluoroskan Ascent fluorometer, it was not possible to perform detailed dose-response curves of 2MeSADP-induced calcium flux. As a surrogate for this, flow cytometric analysis of fibrinogen-binding was performed following stimulation with varying concentrations of 2MeSADP, to determine the dose at which maximal platelet activation occurs (Figure 33).

This experiment showed that 1 μmol/L 2MeSADP was sufficient to induce maximum fibrinogen-binding, both in terms of % positive cells and median fluorescence. This concentration was therefore chosen for the calcium flux assay. Comparison of this data with the aggregation optimisation data reveals that the concentration of 2MeSADP required to induce 50% aggregation stimulates approximately 80% of platelets to bind fibrinogen. The second part of this stage of optimisation was to determine the concentration of AR-C69931MX that would produce maximum inhibition of 1 μmol/L 2MeSADP-induced platelet fibrinogen-binding. A further experiment was therefore performed using a range of concentrations of AR-C69931MX, the results of which are shown in Figure 34. From this it can be seen that an AR-C69931MX concentration of 1 mmol/L was required to maximally inhibit 2MeSADP-induced fibrinogen-binding, and this concentration was selected for use in the final calcium flux assay.

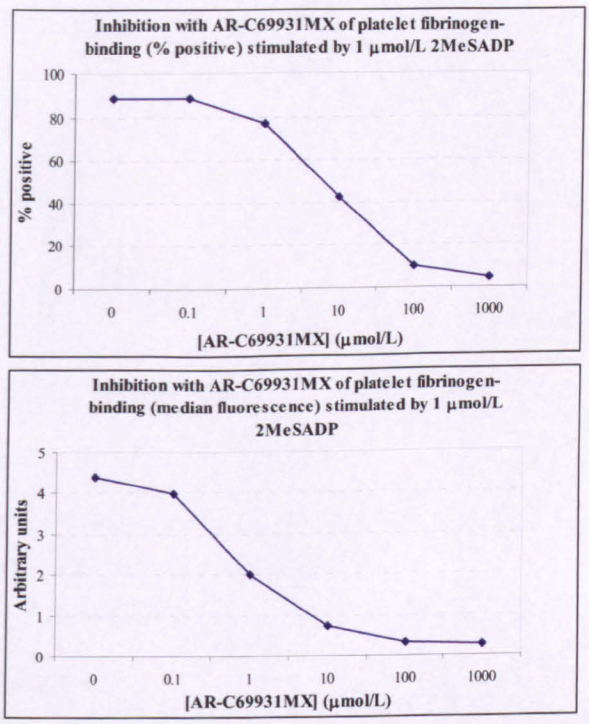
Figure 33 ***Platelet fibrinogen binding following stimulation with 2MeSADP (n=1)***



This figure shows platelet activation as fibrinogen-binding (y-axis, % positive – top, median fluorescence – bottom) following stimulation with varying concentrations of 2MeSADP (x-axis)

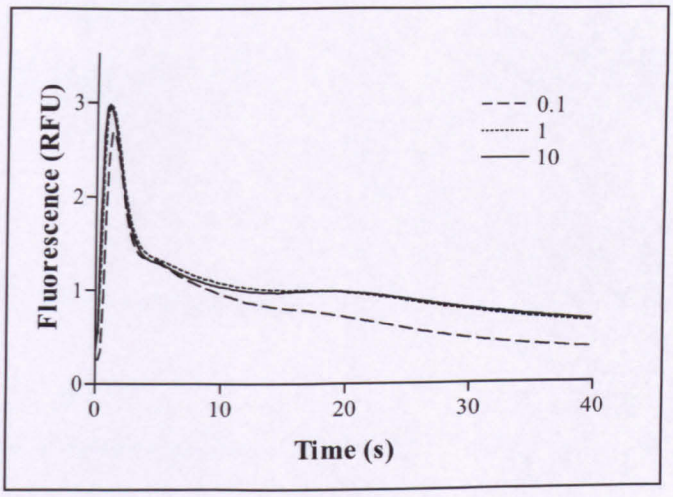
In order to verify that the chosen concentration of 1 µmol/L 2 MeSADP that produced maximal fibrinogen-binding, also produced maximal calcium flux, a single calcium flux experiment was performed stimulating with 0.1, 1 and 10 µmol/L 2MeSADP (Figure 35). This demonstrated that 1 µmol/L 2 MeSADP is sufficient to induce maximal calcium flux.

Figure 34 ***Fibrinogen-binding dose-inhibition curve for AR-C69931MX following stimulation with 2MeSADP (n=1)***



This figure shows fibrinogen-binding (y-axis, % positive – top, median fluorescence – bottom) following stimulation with 1 µmol/L 2MeSADP in the presence of varying concentrations of AR-C69931MX (x-axis)

Figure 35 ***Platelet calcium flux, measured in relative fluorescence units (RFU), following stimulation with 2MeSADP (n=1)***



This figure shows platelet calcium flux (y-axis) against time (x-axis) following stimulation with 3 different concentrations of 2MeSADP (0.1, 1 and 10 μmol/L)

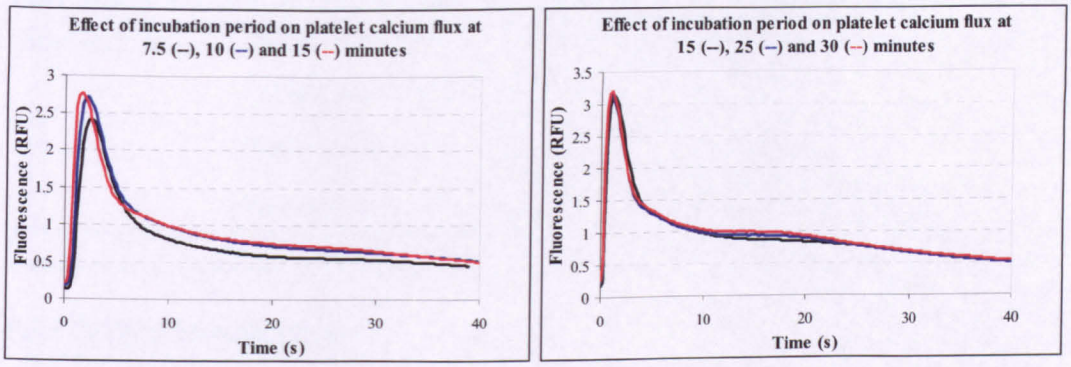
Time course experiment

Because of the inherent delays in sample preparation with the calcium flux method, it was important to determine whether there was any change in response over time. The effects of time delay between resuspension of the loaded, washed platelets and use in the calcium flux assay was assessed in two different individuals, over two overlapping time periods (Figure 36). In the first subject, calcium flux was quantified following incubation at 37°C for 7.5, 10 and 15 minutes after resuspension of the loaded platelets, stimulated with 1 μmol/L 2MeSADP. In the second subject, calcium flux was quantified following 15, 25 and 30 minute incubations. This data demonstrates that incubation for any period of time between 10 and 30 minutes results in stable/reproducible platelet calcium flux. A 7.5 minute incubation appeared to result in lower measured calcium flux, which may be as a result of incomplete de-esterification of the fluo3-AM within the platelets.

Based on the results of these experiments, a final incubation time of 15 minutes was selected for use in the study protocol, which also ensured that the samples had sufficient time to reach 37°C prior to analysis in the calcium flux assay.

Figure 36 ***Effect of variable incubation periods on platelet calcium flux in two individuals***

Subject 1 **Subject 2**



5.3 Subject recruitment and characteristics

5.3.1 Genotyping of GRAPHIC population

Subjects suitable for inclusion in the platelet functional analysis, dependent on their P2Y1 1622 genotype, were identified from the GRAPHIC study. The cohort consisted of 185 family units of two parents and two offspring. These subjects were part of an ongoing population-based family study of cardiovascular phenotypes and were recruited from 3 primary care practices located within Leicestershire, UK. In total, 740 healthy adults were screened for the P2Y1 1622A>G polymorphism using a specifically designed TaqMan assay for allelic discrimination (Table 31). This method was employed rather than using restriction fragment length analysis, for its ease of use and high throughput. The method used in the assay is described in Chapter 2.

Twenty-three subjects were found to be homozygous for the P2Y1 1622 G allele, and twelve of these consented to take part in this study. Nineteen randomly selected age and sex-matched AA homozygotes were also studied.

Table 31 ***Oligonucleotide primers and fluorescent dye-labelled probes used in P2Y1 1622 TaqMan assay***

Polymorphism	Description	Oligonucleotide sequence
P2Y1 1622 A>G	A allele-specific probe	*6FAM*- TACCTGGT**A**ATCATTG
	G allele-specific probe	*VIC*- ACCTGGT**G**ATCATT
	Forward primer	AAAGATCTGGACAACTCTCCTCTGA
	Reverse primer	TCAAGTTCATCGTTTTCATCACATG

5.3.2 Subject characteristics

As the population from which the study subjects were recruited from was a family study, eight of the GG homozygotes recruited were found to be related (four parent/offspring pairs). Consequently the age distribution of the study cohort was bimodal (Figure 37). Due to the larger number of AA homozygotes from which to select matched subjects, it was possible to avoid recruiting related individuals in the AA subset. Because the full cohort consisted of 2 discreet age populations, statistical analysis of genotype-related effects was performed only on the larger, older aged group. This prevented biasing of data due to familial correlations between the parent-sibling pairs as it resulted in separation of the eight related GG subjects.

Figure 37 ***Bimodal age distribution***

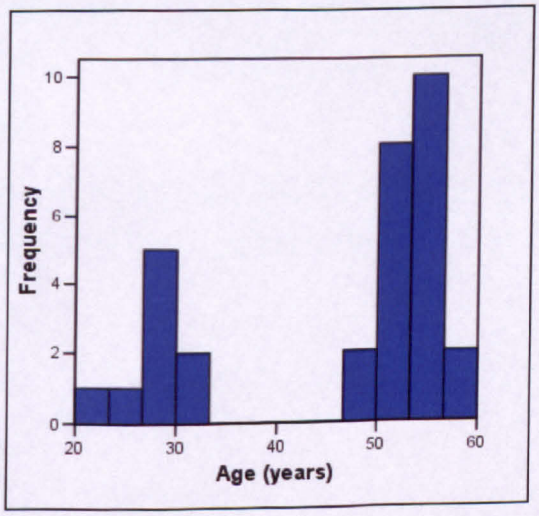


Table 32 shows the demographic characteristics for the older cohort. Continuous variables are given as means (95% confidence intervals). The AA and GG subjects were well matched for all characteristics, with the exception of systolic blood pressure, which bordered on significantly higher in the AA subjects. This difference was likely to have occurred by chance.

Table 32 ***Demographic characteristics of the older group***

	P2Y1 1622 Genotype		p value
	AA (n=14)	GG (n=8)	
Age (yrs)	53.8 (52.1-55.5)	52.8 (50.4-55.1)	0.424
Male/Female (n)	8/6	4/4	0.746
Systolic (mmHg)	130 (122-139)	117 (107-127)	0.051
Diastolic (mmHg)	82 (78-86)	82 (75-90)	0.915
Body mass index (kg/m^2)	27.3 (24.9-29.7)	26.2 (23.9-28.5)	0.534
WCC (x 10^9 /L)	4.9 (4.3-5.5)	5.0 (4.3-5.6)	0.919
Platelet Count (x 10^9 /L)	222 (194-249)	231 (180-283)	0.683
MPV (fl)	9.1 (8.6-9.6)	9.6 (9.0-10.2)	0.209

5.4 Platelet function comparison between P2Y1 1622 AA and GG homozygotes

The platelet function tests were compared between those subjects homozygous for the G allele at position 1622 of the platelet P2Y1 ADP receptor gene, and those homozygous for the A allele, in the older age group.

5.4.1 Flow cytometry

Analysis of platelet reactivity, determined by flow cytometric analysis of fibrinogen-binding to the activated GPIIb-IIIa receptor, partitioned by P2Y1 1622 genotype is shown in Table 33.

Table 33 ***Platelet fibrinogen-binding partitioned by P2Y1 1622 genotype in the older age group***

Agonist (μmol/L)	Mean fibrinogen-binding (% positive)		p-value
	AA (n=14)	GG (n=8)	
Resting	2.8	2.6	0.591
ADP 0.1	15.2	20.9	0.190
ADP 1	68.0	74.6	0.183
ADP 10	85.4	88.8	0.141
TRAP 30	34.1	45.6	0.078

There was no significant difference in platelet fibrinogen-binding following stimulation with either ADP or TRAP, between the AA and GG subjects. Although the GG subject responses were numerically greater than those from the AA subjects, the differences failed to reach statistical significance.

5.4.2 Aggregometry

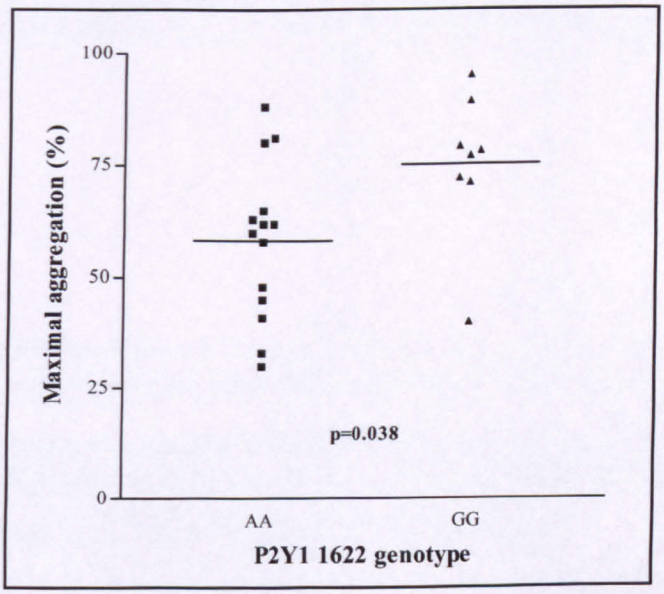
Genotype differences in platelet aggregation following stimulation with a range of doses of 2MeSADP, are shown in Table 34.

Table 34 ***Platelet aggregation partitioned by P2Y1 1622 genotype in the older age group***

Aggregation measure	[2MeSADP] (nmol/L)	Platelet aggregation (%)		p-value
		AA (n=14)	GG (n=8)	
Initial rate	40	30.8	32.9	0.736
	80	52.1	51.0	0.884
	160	66.9	64.9	0.823
Maximal	40	31.2	43.3	0.108
	80	48.6	65.9	0.062
	160	**58.3**	**75.1**	**0.038**

Subjects homozygous for the G allele at position 1622 of the P2Y1 ADP receptor gene were found to have significantly greater maximal platelet aggregation following stimulation with 160 nmol/L 2MeSADP (58.3% vs 75.1%, AA vs GG, p=0.038, see Figure 38), with a trend towards an effect at the lower concentrations of agonist. No effect of genotype was observed on the initial rate of aggregation at any dose of 2MeSADP.

Figure 38 ***Maximal platelet aggregation following stimulation with 160 nmol/L 2MeSADP by P2Y1 1622 genotype***



General linear modelling in the older age group was performed to adjust the genotype effects on maximal platelet aggregation following stimulation with 160 nmol/L 2MeSADP, for MPV. Although this resulted in loss of statistical significance, a similar numerical difference in response was maintained (AA vs GG, 60.4% vs 71.4%, p=0.108).

5.4.3 Calcium flux

The effect of the P2Y1 1622 genotype on platelet calcium flux, in the presence and absence of the specific P2Y12 antagonist AR-C69931MX, is shown in Table 35.

The initial rate of platelet calcium flux was significantly higher in the older GG subjects compared to the AA subjects, irrespective of blockade of the P2Y12 receptor.

Maximal calcium flux (Figure 39) and the area under the calcium flux curve (Figure 40) in the presence of AR-C69931MX were also significantly greater in the GG subjects, with a trend towards an increased maximal response in the absence of the P2Y12 antagonist. Significant differences were also observed in intracellular calcium concentration before stimulation with 2MeSADP.

Table 35 ***Platelet calcium flux partitioned by P2Y1 1622 genotype in the older age group***

Calcium flux measure	AR-C69931MX	Calcium flux (mean responses)		p-value
		AA (n=14)	GG (n=8)	
Baseline	-	**0.90**	**1.10**	**0.005**
	+	**0.82**	**1.00**	**0.002**
Initial rate	-	**2.96**	**3.98**	**0.043**
	+	**1.48**	**2.16**	**0.012**
Maximal	-	2.48	2.89	0.160
	+	**1.77**	**2.18**	**0.041**
Area under curve	+	**21.4**	**29.3**	**0.036**

Figure 39 ***Maximal calcium flux in response to 1 μmol/L 2MeSADP and in the presence of 1 mmol/L AR-C69931MX by P2Y1 1622 genotype***

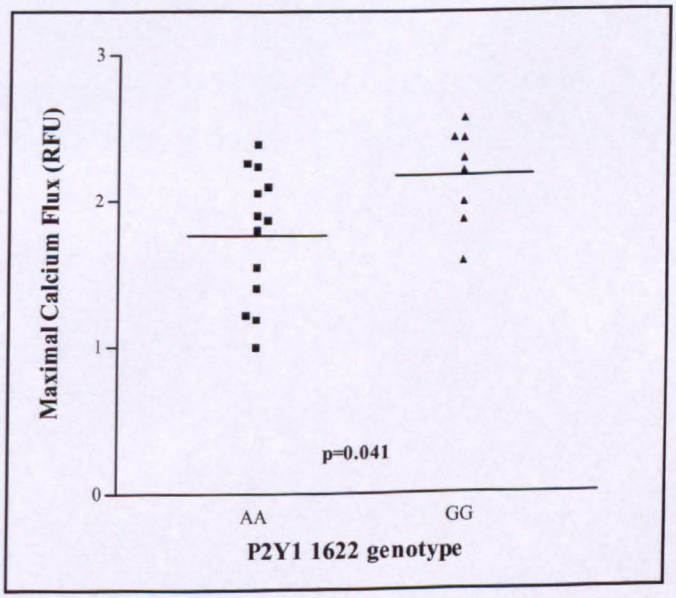
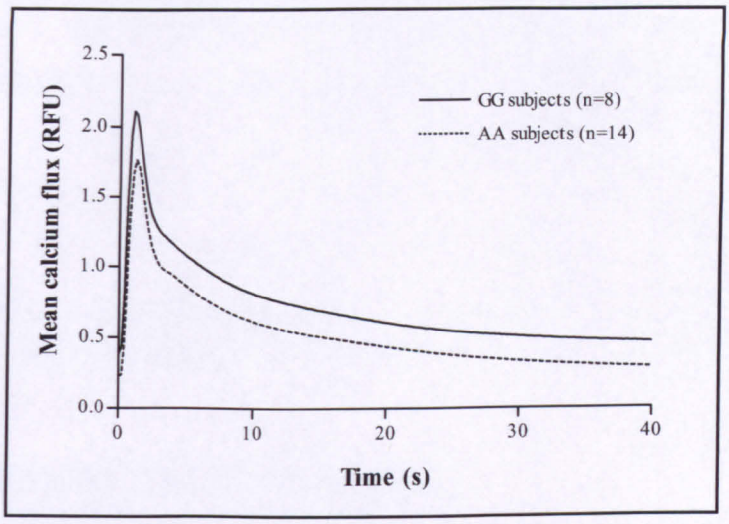


Figure 40 ***Mean calcium flux-time graph following stimulation with 1 μmol/L 2MeSADP and in the presence of 1 mmol/L AR-C69931MX by P2Y1 1622 genotype***



As significant differences were observed in the baseline fluorescence/intracellular calcium concentration between the genotype groups, general linear modelling was used to adjust for this covariate (see Table 36). Adjusting for baseline calcium concentration leads to loss of statistical significance for all measures of platelet calcium flux, however, strong trends towards higher responses persist in the cases of initial rate of flux and the area under the calcium flux curve. Adjusting the area under the calcium flux curve for baseline fluorescence and also MPV, the other independent predictor of area under the calcium flux curve, makes little difference to the magnitude of difference observed (AA vs GG, 21.5 RFU.s vs 29.1 RFU.s, p=0.112).

Table 36 ***Platelet calcium flux adjusted for baseline intracellular calcium concentration in the older age group***

Calcium flux measure	AR-C69931MX	Adjusted calcium flux		p-value
		AA (n=14)	GG (n=8)	
Initial rate	-	2.93	4.01	0.083
	+	1.50	2.14	0.065
Maximal	-	2.58	2.73	0.660
	+	1.87	2.02	0.525
Area under curve	+	21.0	29.8	0.075

5.5 Correlation between platelet functions

Tables 37, 38 and 39 show the correlations between platelet fibrinogen-binding and aggregation (Table 37), calcium flux and fibrinogen-binding (Table 38), and calcium flux and aggregation (Table 39) respectively, the analysis of which was performed in the full cohort, including both younger and older age groups.

Strong, significant correlations were observed between the different doses of agonists used within the platelet fibrinogen-binding and platelet aggregation assays (all p-values <0.001 and all Pearson correlations >0.5, Table 37). Strong correlations were also observed between calcium flux stimulated by 2MeSADP in the presence or absence of the specific P2Y12 antagonist AR-C69931MX (Table 38).

The resting level of bound fibrinogen determined by flow cytometry did not correlate with agonist stimulated fibrinogen-binding (Table 37), however there was a modest significant correlation (p-values >0.001 and Pearson correlations <0.5) between the resting fibrinogen-binding and platelet aggregation measures following stimulation with 40 and 80 nmol/L 2MeSADP. No correlation was observed between resting fibrinogen-binding and platelet aggregation following stimulation with 160 nmol/L 2MeSADP.

More modest, but still significant correlations are observed between platelet fibrinogen-binding and maximal platelet aggregation (but not the initial rate of aggregation, Table 37), and between platelet calcium flux and maximal platelet

aggregation (Table 39). Fibrinogen-binding correlated more strongly to platelet aggregation stimulated by the lower concentrations of 2MeSADP than the higher, whereas the correlation between calcium flux and platelet aggregation showed the opposite trend.

The peak and initial rate of calcium flux (with or without AR-C69931MX) only correlates with maximal aggregation, but not the initial rate of aggregation, whilst the area under the calcium flux curve calculated in the presence of the P2Y12 antagonist correlates with both platelet aggregation measures (Table 39).

A surprising finding from the correlation analysis is that there is no correlation between any measure of calcium flux and platelet fibrinogen-binding in response to ADP (Table 38).

Table 37 ***Correlations between platelet fibrinogen-binding and aggregation***

Correlations

		Resting	ADP 0.1	ADP 1	ADP 10	TRAP 30	Agg slope 40	Agg peak 40	Agg slope 80	Agg peak 80	Agg slope 160	Agg peak 160
Resting	Pearson Corr	1	.301	.267	.197	.223	.376*	.479**	.430*	.417*	.270	.289
	Sig. (2-tailed)	.	.100	.146	.288	.228	.037	.006	.016	.020	.142	.115
	N	31	31	31	31	31	31	31	31	31	31	31
ADP 0.1	Pearson Corr	.301	1	.893**	.720**	.560**	.325	.608**	.201	.485**	.027	.345
	Sig. (2-tailed)	.100	.	.000	.000	.001	.074	.000	.279	.006	.887	.057
	N	31	31	31	31	31	31	31	31	31	31	31
ADP 1	Pearson Corr	.267	.893**	1	.927**	.682**	.394*	.625**	.293	.562**	.139	.474**
	Sig. (2-tailed)	.146	.000	.	.000	.000	.028	.000	.110	.001	.456	.007
	N	31	31	31	31	31	31	31	31	31	31	31
ADP 10	Pearson Corr	.197	.720**	.927**	1	.614**	.304	.502**	.267	.485**	.124	.477**
	Sig. (2-tailed)	.288	.000	.000	.	.000	.096	.004	.146	.006	.507	.007
	N	31	31	31	31	31	31	31	31	31	31	31
TRAP 30	Pearson Corr	.223	.560**	.682**	.614**	1	.341	.495**	.130	.432*	.041	.337
	Sig. (2-tailed)	.228	.001	.000	.000	.	.060	.005	.486	.015	.825	.063
	N	31	31	31	31	31	31	31	31	31	31	31
Agg slope 40	Pearson Corr	.376*	.325	.394*	.304	.341	1	.852**	.921**	.729**	.869**	.672**
	Sig. (2-tailed)	.037	.074	.028	.096	.060	.	.000	.000	.000	.000	.000
	N	31	31	31	31	31	31	31	31	31	31	31
Agg peak 40	Pearson Corr	.479**	.608**	.625**	.502**	.495**	.852**	1	.746**	.889**	.612**	.747**
	Sig. (2-tailed)	.006	.000	.000	.004	.005	.000	.	.000	.000	.000	.000
	N	31	31	31	31	31	31	31	31	31	31	31
Agg slope 80	Pearson Corr	.430*	.201	.293	.267	.130	.921**	.746**	1	.688**	.921**	.685**
	Sig. (2-tailed)	.016	.279	.110	.146	.486	.000	.000	.	.000	.000	.000
	N	31	31	31	31	31	31	31	31	31	31	31
Agg peak 80	Pearson Corr	.417*	.485**	.562**	.485**	.432*	.729**	.889**	.688**	1	.544**	.903**
	Sig. (2-tailed)	.020	.006	.001	.006	.015	.000	.000	.000	.	.002	.000
	N	31	31	31	31	31	31	31	31	31	31	31
Agg slope 160	Pearson Corr	.270	.027	.139	.124	.041	.869**	.612**	.921**	.544**	1	.607**
	Sig. (2-tailed)	.142	.887	.456	.507	.825	.000	.000	.000	.002	.	.000
	N	31	31	31	31	31	31	31	31	31	31	31
Agg peak 160	Pearson Corr	.289	.345	.474**	.477**	.337	.672**	.747**	.685**	.903**	.607**	1
	Sig. (2-tailed)	.115	.057	.007	.007	.063	.000	.000	.000	.000	.000	.
	N	31	31	31	31	31	31	31	31	31	31	31

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

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

Table 38 ***Correlations between platelet calcium flux and fibrinogen-binding***

Correlations

		Baseline, P2Y1/12	Ca flux slope, P2Y1/12	Ca flux peak, P2Y1/12	Baseline, P2Y1	Ca flux slope, P2Y1	Ca flux peak, P2Y1	Ca flux AUC, P2Y1	Resting	ADP 0.1	ADP 1	ADP 10	TRAP 30
Baseline, P2Y1/12	Pearson Corr	1	.332	.541**	.976**	.312	.528**	.361	.030	.289	.228	.227	.079
	Sig. (2-tailed)	.	.068	.002	.000	.093	.003	.050	.873	.115	.218	.220	.672
	N	31	31	31	30	30	30	30	31	31	31	31	31
Ca flux slope, P2Y1/12	Pearson Corr	.332	1	.825**	.452*	.916**	.845**	.856**	-.173	-.106	.033	.109	.134
	Sig. (2-tailed)	.068	.	.000	.012	.000	.000	.000	.352	.569	.860	.559	.472
	N	31	31	31	30	30	30	30	31	31	31	31	31
Ca flux peak, P2Y1/12	Pearson Corr	.541**	.825**	1	.585**	.762**	.953**	.794**	.000	.070	.163	.179	.193
	Sig. (2-tailed)	.002	.000	.	.001	.000	.000	.000	.998	.708	.380	.334	.298
	N	31	31	31	30	30	30	30	31	31	31	31	31
Baseline, P2Y1	Pearson Corr	.976**	.452*	.585**	1	.365*	.550**	.461*	-.047	.236	.213	.230	.059
	Sig. (2-tailed)	.000	.012	.001	.	.047	.002	.010	.804	.209	.258	.221	.758
	N	30	30	30	30	30	30	30	30	30	30	30	30
Ca flux slope, P2Y1	Pearson Corr	.312	.916**	.762**	.365*	1	.864**	.784**	-.299	-.070	.041	.113	.202
	Sig. (2-tailed)	.093	.000	.000	.047	.	.000	.000	.108	.713	.828	.552	.284
	N	30	30	30	30	30	30	30	30	30	30	30	30
Ca flux peak, P2Y1	Pearson Corr	.528**	.845**	.953**	.550**	.864**	1	.774**	-.142	-.011	.066	.092	.155
	Sig. (2-tailed)	.003	.000	.000	.002	.000	.	.000	.454	.955	.729	.628	.415
	N	30	30	30	30	30	30	30	30	30	30	30	30
Ca flux AUC, P2Y1	Pearson Corr	.361	.856**	.794**	.461*	.784**	.774**	1	-.162	.063	.193	.180	.317
	Sig. (2-tailed)	.050	.000	.000	.010	.000	.000	.	.391	.740	.308	.342	.088
	N	30	30	30	30	30	30	30	30	30	30	30	30
Resting	Pearson Corr	.030	-.173	.000	-.047	-.299	-.142	-.162	1	.301	.267	.197	.223
	Sig. (2-tailed)	.873	.352	.998	.804	.108	.454	.391	.	.100	.146	.288	.228
	N	31	31	31	30	30	30	30	31	31	31	31	31
ADP 0.1	Pearson Corr	.289	-.106	.070	.236	-.070	-.011	.063	.301	1	.893**	.720**	.560**
	Sig. (2-tailed)	.115	.569	.708	.209	.713	.955	.740	.100	.	.000	.000	.001
	N	31	31	31	30	30	30	30	31	31	31	31	31
ADP 1	Pearson Corr	.228	.033	.163	.213	.041	.066	.193	.267	.893**	1	.927**	.682**
	Sig. (2-tailed)	.218	.860	.380	.258	.828	.729	.308	.146	.000	.	.000	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31
ADP 10	Pearson Corr	.227	.109	.179	.230	.113	.092	.180	.197	.720**	.927**	1	.614**
	Sig. (2-tailed)	.220	.559	.334	.221	.552	.628	.342	.288	.000	.000	.	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31
TRAP 30	Pearson Corr	.079	.134	.193	.059	.202	.155	.317	.223	.560**	.682**	.614**	1
	Sig. (2-tailed)	.672	.472	.298	.758	.284	.415	.088	.228	.001	.000	.000	.
	N	31	31	31	30	30	30	30	31	31	31	31	31

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

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

Table 39 *Correlations between platelet calcium flux and aggregation*

Correlations

		Baseline, P2Y1/12	Ca flux slope, P2Y1/12	Ca flux peak, P2Y1/12	Baseline, P2Y1	Ca flux slope, P2Y1	Ca flux peak, P2Y1	Ca flux AUC, P2Y1	Agg slope 40	Agg peak 40	Agg slope 80	Agg peak 80	Agg slope 160	Agg peak 160
Baseline, P2Y1/12	Pearson Corr	1	.332	.541**	.976**	.312	.528**	.361	.083	.219	.100	.395*	-.050	.468**
	Sig. (2-tailed)	.	.068	.002	.000	.093	.003	.050	.657	.237	.592	.028	.789	.008
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Ca flux slope, P2Y1/12	Pearson Corr	.332	1	.825**	.452*	.916**	.845**	.856**	.304	.225	.281	.297	.352	.486**
	Sig. (2-tailed)	.068	.	.000	.012	.000	.000	.000	.096	.223	.125	.105	.052	.006
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Ca flux peak, P2Y1/12	Pearson Corr	.541**	.825**	1	.585**	.762**	.953**	.794**	.379*	.345	.337	.399*	.289	.517**
	Sig. (2-tailed)	.002	.000	.	.001	.000	.000	.000	.036	.057	.064	.026	.115	.003
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Baseline, P2Y1	Pearson Corr	.976**	.452*	.585**	1	.365*	.550**	.461*	.096	.221	.111	.400*	-.018	.502**
	Sig. (2-tailed)	.000	.012	.001	.	.047	.002	.010	.615	.240	.558	.029	.925	.005
	N	30	30	30	30	30	30	30	30	30	30	30	30	30
Ca flux slope, P2Y1	Pearson Corr	.312	.916**	.762**	.365*	1	.864**	.784**	.267	.159	.169	.176	.267	.376*
	Sig. (2-tailed)	.093	.000	.000	.047	.	.000	.000	.154	.402	.371	.353	.154	.041
	N	30	30	30	30	30	30	30	30	30	30	30	30	30
Ca flux peak, P2Y1	Pearson Corr	.528**	.845**	.953**	.550**	.864**	1	.774**	.287	.218	.215	.271	.201	.411*
	Sig. (2-tailed)	.003	.000	.000	.002	.000	.	.000	.124	.248	.253	.148	.288	.024
	N	30	30	30	30	30	30	30	30	30	30	30	30	30
Ca flux AUC, P2Y1	Pearson Corr	.361	.856**	.794**	.461*	.784**	.774**	1	.449*	.369*	.307	.371*	.398*	.504**
	Sig. (2-tailed)	.050	.000	.000	.010	.000	.000	.	.013	.045	.099	.043	.029	.004
	N	30	30	30	30	30	30	30	30	30	30	30	30	30
Agg slope 40	Pearson Corr	.083	.304	.379*	.096	.267	.287	.449*	1	.852**	.921**	.729**	.869**	.672**
	Sig. (2-tailed)	.657	.096	.036	.615	.154	.124	.013	.	.000	.000	.000	.000	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Agg peak 40	Pearson Corr	.219	.225	.345	.221	.159	.218	.369*	.852**	1	.746**	.889**	.612**	.747**
	Sig. (2-tailed)	.237	.223	.057	.240	.402	.248	.045	.000	.	.000	.000	.000	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Agg slope 80	Pearson Corr	.100	.281	.337	.111	.169	.215	.307	.921**	.746**	1	.688**	.921**	.685**
	Sig. (2-tailed)	.592	.125	.064	.558	.371	.253	.099	.000	.000	.	.000	.000	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Agg peak 80	Pearson Corr	.395*	.297	.399*	.400*	.176	.271	.371*	.729**	.889**	.688**	1	.544**	.903**
	Sig. (2-tailed)	.028	.105	.026	.029	.353	.148	.043	.000	.000	.000	.	.002	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Agg slope 160	Pearson Corr	-.050	.352	.289	-.018	.267	.201	.398*	.869**	.612**	.921**	.544**	1	.607**
	Sig. (2-tailed)	.789	.052	.115	.925	.154	.288	.029	.000	.000	.000	.002	.	.000
	N	31	31	31	30	30	30	30	31	31	31	31	31	31
Agg peak 160	Pearson Corr	.468**	.486**	.517**	.502**	.376*	.411*	.504**	.672**	.747**	.685**	.903**	.607**	1
	Sig. (2-tailed)	.008	.006	.003	.005	.041	.024	.004	.000	.000	.000	.000	.000	.
	N	31	31	31	30	30	30	30	31	31	31	31	31	31

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

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

5.6 Discussion

The P2Y1 1622 polymorphism was found to have significant effects on platelet aggregation and calcium flux, where GG homozygotes were seen to have increased responses for both of these measures compared to AA homozygotes. This confirms the findings following analysis of fibrinogen-binding data from PRAMIS (Chapter 4), and provides support for the hypothesis that the mechanism by which the polymorphism exerts its effect is through increased receptor expression with carriage of the G allele resulting in increased receptor-mediated signalling and ultimately platelet activation.

Although numerically greater in the GG homozygotes, the difference in platelet fibrinogen-binding did not reach statistical significance, and overall the differences in fibrinogen-binding between the genotype groups were smaller than anticipated from the PRAMIS data. An explanation for this discrepancy is not immediately obvious, but may reflect the presence of a hitherto unidentified confounding factor that only affected the flow cytometric assay in the second part of the study. Alternatively this may have occurred by chance as a result of the small cohort size.

Another interesting finding was the presence of significantly higher baseline fluo3 fluorescence measurements in the P2Y1 1622 GG subjects compared to the AA subjects. Adjusting the calcium flux responses for this variable abolished the significant association, and to a large extent the numerical difference, observed in maximal calcium flux between the genotype groups (Tables 35 and 36). This loss of association with adjustment was to be anticipated, as a strong, significant correlation was seen between baseline fluorescence and maximal calcium flux in the preliminary analyses. A possible explanation for this difference in baseline fluorescence relates to the preparation of samples in the calcium flux assay. The preparation of fluo3-loaded platelets involves significant handling and manipulation of the samples, and it is possible that this process induces a minor degree of platelet activation. Since the platelets of the GG homozygotes were found to be more reactive than those of the AA homozygotes, these would be more affected by the handling process, which then becomes measurable as an increase in baseline fluorescence, i.e. an increase in basal cytoplasmic calcium concentration. This hypothesis is supported by the fact that even despite the presence of high concentrations of apyrase to breakdown any ADP released during preparation, the addition of the specific P2Y12 receptor antagonist AR-

C69931MX results in a significant reduction in measured baseline fluorescence (baseline fluorescence with vs without AR-C69931MX using paired t-test, 0.89 RFU vs 0.98 RFU, $p<0.001$). This indicates that released ADP still present in the washed samples could be responsible for the observed difference in baseline fluorescence between the genotype groups.

Strong correlations were observed within the three different tests of platelet function when comparing responses to different doses of agonists. This is, perhaps, to be expected, as when comparing responses only to different concentrations of agonists, essentially the same pathways are being stimulated, but to differing degrees.

An unexpected finding was the lack of a significant correlation between fibrinogen-binding and calcium flux, especially given there were significant correlations between calcium flux and aggregation, and fibrinogen-binding and aggregation. A possible explanation may be the different agonists used in the flow cytometry and platelet aggregation assays, or the blockade of TxA2 generation in the calcium flux assay, but given fibrinogen-binding and aggregation correlate, this seems unlikely. Other explanations include the presence of an undefined confounding factor that affected the flow cytometric assay in isolation, or that calcium flux has a greater influence on platelet aggregation, as a result of the close platelet-platelet contact that occurs during aggregation, that is prevented by dilution in the flow cytometric assay.

With hindsight, given the results of the platelet function tests above, other useful additional experiments that could have been performed to provide a more detailed analysis of the genotype/phenotype relationship include performing flow cytometry with 2MeSADP and platelet aggregation with ADP, to allow additional correlations to be assessed, and the calcium flux experiments with TRAP + ADP receptor antagonists. Measurement of calcium flux in response to TRAP stimulation in the presence of P2Y1 and P2Y12 receptor antagonists would help to ensure that the increase in calcium flux observed was specific to stimulation through the P2Y1 receptor.

5.7 Conclusions

In this Chapter I have shown that:

- Platelet aggregation stimulated with 2MeSADP was significantly greater in P2Y1 1622 GG homozygotes compared to AA homozygotes

- Platelet calcium flux following stimulation of the P2Y1 receptor in isolation, with 2MeSADP, was significantly greater in GG homozygotes compared to AA homozygotes
- There were significant correlations between platelet calcium flux and aggregation, and between platelet aggregation and fibrinogen-binding to the activated GPIIb-IIIa receptor following stimulation through the platelet P2Y receptors

Chapter 6

Exploration of the mechanism of effect of the P2Y1 1622A>G polymorphism

6.1 Introduction

The data presented in Chapter 5 lends further support to the hypothesis that the mechanism by which the P2Y1 1622 polymorphism exerts its effect is through differences in P2Y1 receptor numbers between genotype groups. This is the likely explanation for its effect as the polymorphism does not affect the amino acid composition of the receptor protein, yet the polymorphism is associated with significant differences in platelet aggregation, platelet fibrinogen-binding (which is the prerequisite to aggregation) and P2Y1 receptor signalling. The association observed with calcium flux is of particular importance because this is a direct consequence of P2Y1 receptor stimulation, and in the presence of P2Y12 receptor blockade, is not likely to suffer from significant "interference" due to interaction with other cell-signalling pathways.

In order to determine if the P2Y1 1622A>G polymorphism mediates its functional effects through alteration of P2Y1 receptor expression, attempts were made, using various techniques, to identify any differences in receptor expression between P2Y1 1622 AA and GG subjects.

6.2 Radioligand binding assay

Radioligand binding assays work on the principle that in a simple ligand-receptor interaction, such as between ADP and the P2Y1 receptor, a single receptor molecule can bind a single ligand molecule in a reversible fashion. If a saturating concentration of a radiolabelled ligand is incubated with cells or membranes bearing its specific receptor, all receptors will be occupied with the labelled ligand when the interaction reaches equilibrium. If the liquid containing the cells that bear the ligand-receptor complex are then filtered through fine glass fibre filters, these cells will become trapped within the filter. After washing to remove any unbound radioligand, the amount of radioligand trapped within the filter can be quantified by measuring its specific radioactivity using scintillation counting. Assuming there is one molecule of radioligand bound to one receptor molecule, the amount of radioligand bound to the filter equals the number of receptors present in the original sample.

In reality, there is always a degree of non-specific binding of the ligand to the cell sample preparation and to the filter. However, as the amount of non-specific binding

with increasing ligand concentration does not follow saturation kinetics, an estimation of non-specific binding can be made by displacing specifically bound labelled ligand using either a different, specific receptor ligand (such as MRS2179 in the case of the P2Y1 receptor), or by adding an excess of the unlabelled ligand (non-specific binding of labelled ligand will not be displaced, following the assumption that there is an infinite number of non-specific binding sites).

6.2.1 Assay development

Experiments were performed to set up the radioligand binding experiments on two occasions. Fractional activity of the radioligand needs to be calculated on each day of use, because radioactive decay occurs within the sample. As the radioligand is provided with a specific activity (1000 Ci/mmol) on a specific activity date, it is possible, using the known half-life of the radioisotope used, to calculate the specific activity of the radioligand sample on any day after this. On the first occasion, fractional activity of the radioligand was calculated to be 0.57 using standard fractional activity tables, and on the second 0.561.

Detailed methods and rationale for the assay are given in Chapter 2 (section 2.10.2). Briefly, 50 μg of platelet protein was incubated with 240 nmol/L [^{35}S]dATPαS for 20 minutes at 25°C. Varying concentrations of MRS2179, 2MeSADP and AR-C69931MX were added to the reactions to displace specific P2Y receptor binding. The reaction was terminated by dilution with ice cold buffer and aliquots were filtered over glass fibre filters, followed by washing with further large volumes of ice cold buffer. The platelet protein used for each experiment was from the same subject/sample to allow comparisons to be made.

Two experiments were performed on the first day. The purpose of these experiments was to determine if specific binding of the radioligand to P2Y1 could be demonstrated, through displacement of the radioligand with 1 mmol/L MRS2179 and 1 μmol/L 2MeSADP. Two aliquots were analysed per sample by scintillation counting over a period of ten minutes, with the mean counts per minute (CPM) recorded. Counting efficiency was calculated to be 88.8% (calculated from actual/expected counts per 100μl standard, see Chapter 2, section 2.10.2). Data from the day 1 experiments are shown in Table 40.

Table 40 ***Day 1 radioligand binding experiments***

Experiment	Displacing ligand (μmol/L)	Mean counts per minute	Mean disintegrations per minute
1	-	59506	67011
	MRS2179 1000	57274	64497
	-	56778	63939
2	MRS2179 1000	46667	52552
	2MeSADP 1	19877	22384

From the data in Table 40 the amount of radioligand binding per mg of platelet protein that is displaceable by MRS2179 can be calculated. This works out to be 0.1 and 0.45 pmol/mg of platelet protein for experiments 1 and 2 respectively. The displaceable binding in respect of 2MeSADP was 1.64 pmol/mg of platelet protein. These initial results were somewhat disappointing for a number of reasons. First, the experiments using MRS2179 to displace specific P2Y1 binding yielded markedly different results, casting doubt over the reproducibility of the assay. Second, additional binding was displaced with 2MeSADP on top of that displaced by MRS2179. In view of the fact that the ligand is specific to P2Y1, this may mean that non-specific binding was being displaced in addition to specific binding. Third, overall there appeared to be a much larger degree of non-specific binding compared to specific binding.

Before abandoning the assay, further experiments were performed using the same sample but on a different day, and using different doses of MRS2179 and AR-C69931MX (in an attempt to identify any specific binding to the P2Y12 receptor) in isolation and together. The results are shown in Table 41.

Counting efficiency was determined to be 84.3% for these experiments, and this value was used to calculate the mean disintegrations per minute (DPM) from CPM. There did not appear to be any correlation between the displacing ligand used and changes in CPM/DPM. Consequently it was not felt to be appropriate to continue trying to develop the radioligand assay with [^{35}S]dATPαS.

Table 41 ***Day 2 radioligand binding experiments***

Experiment	Displacing ligand (μmol/L)	Mean counts per minute	Mean disintegrations per minute
3	-	40764	48355
	MRS2179 1000	31615	37502
	AR-C69931MX 100	32063	38034
	Both	34178	40543
4	-	37108	44018
	MRS2179 1	27425	32532
	AR-C69931MX 1	46536	55202
	Both	40979	48610

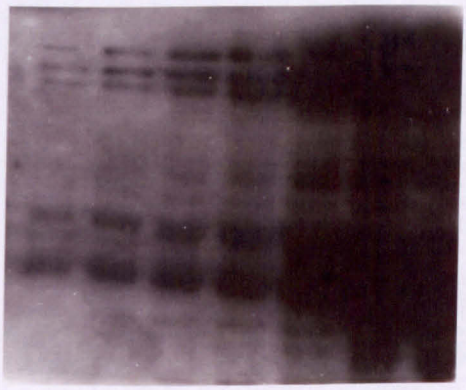
6.3 Western blot assay

The detailed method for the Western blot assay is given in Chapter 2 (section 2.10.3). There is very limited information in the literature on the detection of P2Y1 receptor protein using Western blotting techniques. Two companies were identified that marketed a P2Y1 receptor antibody that was advertised as suitable for Western blotting applications. As both antibodies had been raised against the same epitope on the P2Y1 receptor, a rabbit anti-rat polyclonal antibody supplied by United States Biological (Massachusetts, USA) was chosen. The peptide epitope used to raise the antibody has the following amino acid sequence – RALI YKDLD NSPLR RKS, which corresponds to residues 242-258 of rat or human P2Y1. As this was a C-terminal peptide, and therefore intracellular, it was unsuitable for determination of receptor numbers by flow cytometry. Control antigen was provided by the company to confirm specific binding. The antibody was used at a dilution of 1:400, which is the manufacturer's recommended dilution for use on human platelets.

An initial experiment was performed to determine the optimum amount of platelet protein to use in the final assay. Platelet protein was added to the reaction in the form of freeze-thawed whole platelet suspensions (see Chapter 2, section 2.10.1 for method used to quantify platelet protein in the freeze-thawed washed platelet preparations). Quantities from 1 to 100 μg per lane were used. Figure 41 shows the resulting

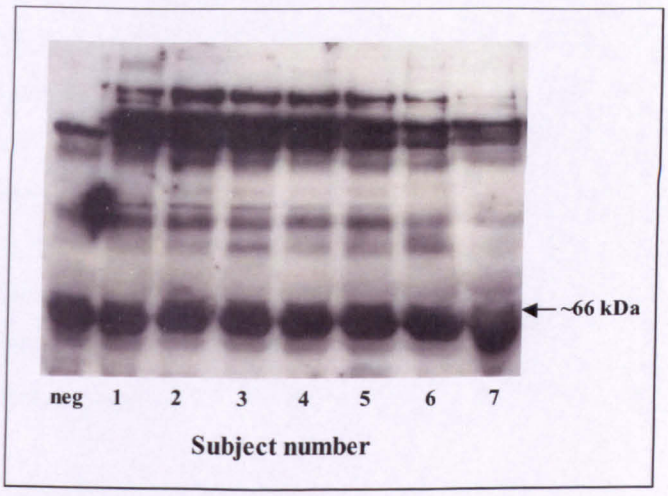
autoradiograph. From this it can already be seen that there were multiple protein bands present indicating significant non-specific binding of the P2Y1 receptor antibody.

Figure 41 ***Western blotting of 1 to 100 μg of platelet protein using a P2Y1 receptor polyclonal antibody***



Using data from this initial experiment, *50 μg of* platelet protein was used in the final assay. The assay was then performed on the study samples with one duplicate sample per gel to allow the use of the control peptide, permitting identification of specific binding of the antibody (negative control, labelled lane "neg" in Figure 42). The control peptide works by binding to the P2Y1 receptor antibody and preventing it from hybridising with the blot membrane. An example autoradiograph is shown in Figure 42. The P2Y1 receptor has an approximate molecular weight of 66 kDa. It was not possible to identify any specific binding at this molecular weight (see Figure 42). Of note, is the presence of a large amount of non-specific binding of the P2Y1 receptor antibody. This finding was consistent following repeated experiments. Consequently, it was not felt that this assay could be developed any further with the P2Y1 antibodies that were commercially available. The use of flow cytometry to determine receptor numbers was also deemed unsuitable because of the lack of commercially available antibody to an extracellular domain and because of the low number of P2Y1 receptors per platelet.

Figure 42 ***Typical Western blot using 50 µg/lane of study samples and a P2Y1 receptor antibody +/- control peptide***



6.4 Relative P2Y1 mRNA expression

Messenger RNA was extracted from 3 randomly selected P2Y1 1622 GG subjects and 3 age and sex matched AA subjects, in a pilot experiment to determine whether differences in P2Y1 mRNA, and hence receptor, expression may account for the differences seen in platelet function and signalling. The detailed method for platelet preparation, mRNA extraction, first strand cDNA synthesis and the principle technique employed in relative quantification of cDNA is given in Chapter 2. The primers specific to the P2Y1 gene that were used for these experiments consisted of the forward primer from primer pair P2Y1-5 and the reverse primer from pair P2Y1-4, giving a product size of 260 base pairs (see Chapter 2). 150 nmol/L of each primer was used in the otherwise standard PCR master mixture. The PCR program used for amplification of P2Y1 cDNA is given below.

PCR Program:

HOLD: 95°C for 3 min.

CYCLE: 95°C for 1 min, 58°C for 1 min, 72°C for 1 min x 36 cycles.

HOLD: 72°C for 2 min.

HOLD: 10°C until tubes were removed.

Multiple optimisations of the quantities of cDNA that were added to each reaction, for each subject, were performed, to ensure that equivalent adjusted volumes of cDNA were used when amplifying P2Y1. In brief, an initial experiment was performed where 2 µl of cDNA from each subject, prepared from platelet mRNA, was used to prime the PCR reaction in addition to GAPDH primers. Spot densitometry was used to analyse yield following completion and resolving on agarose gels, and on the basis of this, adjustments were made to the volumes of cDNA added to the reaction mixtures for each subject. Further PCR reactions were performed to verify that GAPDH-specific product yield was similar for all subjects. Additional experiments were performed, using increasing volumes of cDNA template, to ascertain that PCR amplification was in the linear phase. This was undertaken to allow valid relative quantification of P2Y1 mRNA.

In the 3 paired GG and AA subjects tested, P2Y1 expression relative to GAPDH was consistently higher in 2 out of 3 GG homozygotes compared to the AA homozygotes (see typical agarose gels in Figure 43 and data from 3 repeat experiments in Table 42). P2Y1 transcript expression in GG subjects was found to be approximately 2 times that observed in AA subjects in pairs 1 and 2, when measured by spot densitometry of DNA bands following agarose gel electrophoresis. P2Y1 transcript expression was slightly lower in the GG subject compared to the AA subject in the third pair.

Figure 43 ***Typical agarose gels demonstrating amplification of GAPDH and P2Y1 cDNA after reaction optimisation***

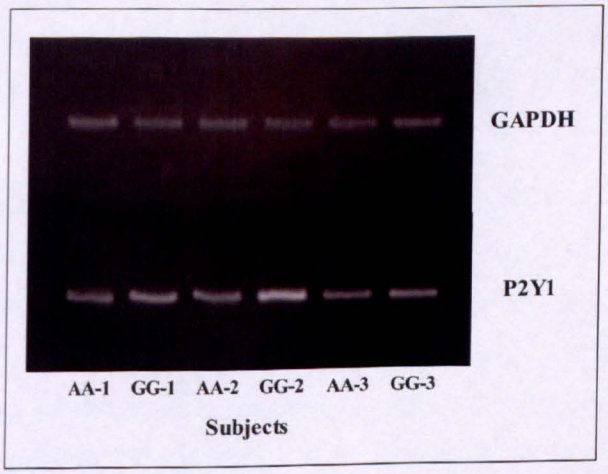


Table 42 ***Relative quantification of P2Y1 transcript expression, expressed as a proportion of GAPDH expression in 3 P2Y1 1622 GG and 3 AA subjects***

Subject	P2Y1 transcript expression relative to GAPDH			
	Exp 1	Exp 2	Exp 3	Mean
AA-1	1.64	0.87	1.08	1.20
GG-1	2.96	1.27	2.26	2.16
AA-2	2.03	0.50	1.78	1.44
GG-2	4.61	1.65	2.17	2.81
AA-3	2.02	0.90	1.61	1.51
GG-3	2.01	1.08	0.97	1.35

6.5 Sequencing of P2Y1 gene promoter region

Substitution of an A to a G at position 1622 of the P2Y1 ADP receptor gene does not result in an alteration in the amino acid sequence of the P2Y1 receptor protein. From this observation it can be inferred that any effect of the polymorphism is likely to be through differences in receptor expression, associated with the base substitution. This theory is lent further support by the finding of increased P2Y1 mRNA levels found in a small number of GG subjects compared to AA subjects. There are two possible mechanisms by which the P2Y1 1622 polymorphism may affect expression. First, the 1622 polymorphism may be in linkage with other polymorphisms upstream of the gene in its promoter region, and that these may affect the genes transcriptional regulation. Second, the change from an adenosine to guanidine nucleotide at position 1622 may affect the stability of the transcribed mRNA through changes in its secondary structure, thereby having a downstream effect on receptor expression.

To investigate the first possible mechanism further, 13 subjects were selected at random from the three P2Y1 1622 genotype groups found in the PRAMIS cohort (4 GG, 5 AG and 4 AA subjects), and underwent additional sequencing of the minimal P2Y1 gene promoter region. A further primer pair was required to allow sequencing of this region, which was up to 1000 base pairs upstream of the P2Y1 transcription start site (Table 43). Primer pair P2Y1-1 from the earlier sequencing experiments (Chapter 2, section 2.5.2) was also used to amplify the region from –388 to the transcription start site. PCR

amplification and sequencing was performed using the standard methods described in Chapter 2 (section 2.5.2), with each primer at a concentration of 100 nmol/L in the reaction mixture and using an annealing temperature of 58°C during thermal cycling.

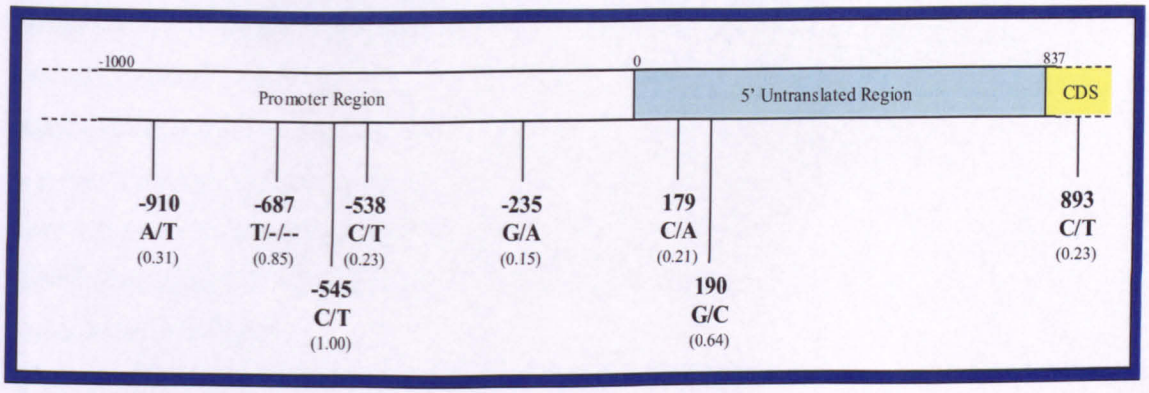
Table 43 ***Oligonucleotide primers used for amplifying and sequencing of the P2Y1 gene promoter***

Primer pair	Position and sequence	Product size (bp)
P2Y1-6	F (-1009) 5'- TTCATTTAGTTTCCAATTAAACTTGCTCA-3' (-981) R (-388) 5'-AATGAGGGTTGGGAGAGGAGTG-3' (-409)	622
P2Y1-1	F (-536) 5'- TTTTGGAAATTCTCACGTTGATGTT-3' (-512) R (473) 5'-GAGTTTCCCTTGACCTCGCTG-3' (453)	1009

F – forward primer, R – reverse primer. Nucleotide positions are given in relation to the transcription start site of the P2Y1 gene

In total, 5 polymorphisms were identified in the P2Y1 gene promoter that exhibited varying degrees of linkage with the P2Y1 1622 A>G polymorphism. These are -910A>T, -687T/TTindel, -545C>T, -538C>T and -235G>A. A schematic diagram representing the 5' region of the P2Y1 gene, its promoter region and including the promoter polymorphisms, is shown in Figure 44. The numbers in parentheses are estimations of the degree of co-inheritance of the less common allele for each promoter polymorphism, with the P2Y1 1622 G allele, assuming that in the case of compound heterozygosity for the promoter polymorphism and 1622 polymorphism, the linkage phase of the less common allele for the promoter polymorphism is with the P2Y1 1622 G allele. For example, with regard to the -235G>A substitution, the -235 A allele is carried with 1622 G allele in 15% of cases and is therefore partially linked, whereas with regard to the -545C>T substitution, the -545 T allele is always carried with the 1622 G allele and is thus in complete linkage disequilibrium.

Figure 44 ***Schematic diagram representing the 5'UTR and promoter region of the P2Y1 gene showing sequence variation***



6.6 Discussion

In this chapter, details of the attempts made to quantify platelet P2Y1 receptor expression have been presented. Regrettably the two main assays used to quantify platelet P2Y1 receptor protein, namely using radioligand binding of [^{35}S]dATPαS or Western blotting, were both unsuccessful. With regard to the radioligand-binding assay, its failure likely relates to the very high level of non-specific binding of [^{35}S]dATPαS to the platelets and also the glass fibre filters. Normally one would expect nonspecific binding to be a fraction of the specific binding, allowing confidence in the assay. However in this study, the majority of radiolabelled ligand bound to the glass fibre filters was not displaceable with ADP receptor specific ligands, such as 2MeSADP, MRS2179 and AR-C69931MX. Freeze-thawed platelets were used in the assay, which may have also affected the accuracy of the assay due to degradation of receptor protein. Although the use of fresh platelets would have been preferable, this was not done, to simplify the collection of samples into a single visit. It would also have been necessary to perform the assay in a single batch to prevent the introduction of a random time variable, given there is continuous radioactive decay of the radioligand. Thus if using fresh platelets, all subjects would have to have reattended on the same day to give a sample, making it logistically difficult. Although Oras *et al* [Oras *et al*, 2002] were able to confidently use this radioligand to quantify P2Y1 receptor expression, other groups have also found it to be unsuitable due to its high non-specific binding.[Schachter *et al*,

1997] In respect of the Western blotting assay, failure can again be attributed to high non-specific binding of the only P2Y1 receptor antibody that was commercially available at the time of the study. A search of the literature has shown that a single group has successfully quantified P2Y1 expression using Western blotting, however this was accomplished with an in-house developed antibody.[Moore *et al*, 2000] Attempts were made to acquire this antibody to use in our assay, but unfortunately none was available for our use. In retrospect, specificity of the Western blot assay may have been improved if platelet membranes had been used, rather than whole platelets, as this would have removed the majority of cytoplasmic proteins that are likely to contribute to the non-specific antibody binding.

Amplification and relative quantification of platelet P2Y1 receptor mRNA was successfully achieved in a small subset of the study cohort. Despite the limitations of relative quantification as compared to techniques that provide absolute quantification using fluorogenic 5' nuclease-based assays (such as TaqMan), these results still provide useful clues as to the mechanism by which the P2Y1 1622 polymorphism exerts its effect, namely via increased mRNA, and hence receptor, expression. On average P2Y1 mRNA was found to be 1.5 fold greater in subjects homozygous for the P2Y1 1622 G allele as compared to those homozygous for the A allele. This result was not statistically significant due to the small numbers tested, but is hypothesis generating. Evidence exists in the literature that increased expression of P2Y1 receptors on platelets can cause platelet hyper-reactivity.[Hechler *et al*, 2003] Hechler *et al* created transgenic mice that over-expressed the P2Y1 receptor, and found that these mice had significantly greater aggregation in response to ADP, showed a significantly shorter bleeding time and were more sensitive to *in vivo* induced platelet aggregation following injection of a mixture of collagen and epinephrine, when compared to wild-type mice.

Working on the assumption that the P2Y1 1622 polymorphism affects platelet reactivity through differences in P2Y1 transcript expression, the promoter region of the P2Y1 gene was interrogated for single nucleotide polymorphisms that were in linkage disequilibrium with the 1622 polymorphism. This comprehensive screening of the region 1000 base pairs upstream of the P2Y1 gene transcription start point led to the identification of five polymorphisms, all of which bore a degree of linkage to the 1622 polymorphism. An interesting hypothesis to explain this mechanism is that one or more of these polymorphisms may affect the transcriptional regulation of the P2Y1 gene,

through modulation of transcription factor binding. This hypothesis could be tested in additional studies using expression systems to analyse the effects of the individual polymorphisms on transcription and further evidence linking these polymorphisms to platelet function could be sought through genotyping the PRAMIS cohort and analysing haplotype data.

6.7 Conclusions

In this chapter, attempts were made to provide support to the hypothesis that the increase in platelet reactivity conferred by carriage of the P2Y1 1622 G allele was as a result of differences in P2Y1 receptor numbers using radioligand-binding and Western blotting of the P2Y1 receptor. Unfortunately these attempts were unsuccessful.

Although by no means conclusive, the finding of increased P2Y1 transcript expression in a small pilot study using a subset of the homozygous subjects would lend support to this theory. Furthermore, a number of polymorphisms have been identified in the promoter region of the P2Y1 gene, and these provide targets for further molecular research.

Chapter 7

General discussion and perspective

7.1 Introduction

The genetic aetiology of myocardial infarction is complex and yet to be fully understood. Multiple interacting genetic and environmental factors determine the risk of such an event in any individual, and include established risk factors such as smoking, hypertension, disorders of lipid and glucose metabolism, and a strong family history. Many genes are likely to be implicated, including those that predispose to other conventional risk factors such as hypertension, diabetes and dyslipidaemia. Intuitively, genetic influences will be more important in determining premature disease.

The development of a myocardial infarction usually depends on the presence of an abnormal, atherosclerotic coronary artery, which has developed chronically over a number of years, in addition to the abrupt onset of thrombosis following plaque disruption. The main influences on the thrombotic process are haemostatic, fibrinolytic and platelet factors. As the atherosclerotic burdon in younger people is generally lower, premature myocardial infarction is more likely to be influenced by the thrombotic response, and in particular, platelets.

Defining genetic factors that influence thrombotic risk will help to further our knowledge and understanding of atherothrombotic disease processes. This knowledge may, in time, lead to the development of novel therapeutic and preventative strategies, and thus, they are the subject of intense molecular research. This study has identified and assessed the contribution of a number of potential genetic-mediated haemostatic risk factors, in the form of platelet ADP receptor polymorphisms.

7.2 Summary of main findings

This study utilised twenty subjects with extreme phenotypes (ten high and low responders to ADP each) from the well-characterised PRAMIS cohort to identify novel sequence variation within the P2Y1 and P2Y12 ADP receptor genes. In total, sixteen polymorphisms were identified, five in the P2Y1 gene and eleven in the P2Y12 gene, from which five were selected for further investigation in the full PRAMIS population.

The detailed analysis of the effects of P2Y1 and P2Y12 genotypes on platelet function demonstrated that, in the control cohort of 200 healthy adult subjects, homozygous carriage of the P2Y1 1622G allele conferred a significant, 130% increase in platelet

reactivity compared to non-carriers. This effect was not seen in the case cohort, which may have been secondary to the use of aspirin in this group, or the disease itself. None of the P2Y12 gene polymorphisms had an effect on platelet function. None of the polymorphisms identified in either the P2Y1 or P2Y12 gene were associated with a significantly increased risk of premature myocardial infarction.

The effects of the P2Y1 1622A>G polymorphism on platelet aggregation and platelet calcium flux were also determined separately, in a small, highly selected group of AA and GG homozygotes. This work confirmed that carriage of the P2Y1 1622G allele was also significantly associated with an increase in platelet aggregation response and calcium flux. The effect on calcium flux was most pronounced when any modifying effect of ADP stimulation through the P2Y12 receptor was blocked, using a specific antagonist.

Given the finding of increased receptor-mediated signalling with homozygous carriage of the P2Y1 1622G allele and that the base substitution is synonymous, the likely mechanism through which it exerts its effect is via effecting an increase in P2Y1 receptor numbers. Radioligand binding, and western blot assays were attempted to quantify this, but were unsuccessful. Relative quantification of P2Y1 mRNA in a small pilot study, demonstrated 1.5 to 2 fold greater expression in the GG subjects compared with AA subjects, in 2 out of 3 AA/GG subject pairs. Furthermore, a number of polymorphisms have been identified upstream of the P2Y1 gene that may affect its transcriptional regulation, providing targets for future research.

7.3 Future studies

Overall the work completed in this study satisfactorily addressed the aims set out in Chapter 1. However, there are two aspects of the work where the findings were not conclusive, permitting scope for further investigation.

First, given that the effect of any common polymorphism on risk of myocardial infarction is likely to small, it is possible that the case control study undertaken in this thesis was underpowered to detect such a small increase in relative risk. Consequently it would be prudent to perform further MI case-control studies using larger cohorts to attempt to characterise further the effect of the P2Y1 1622A>G polymorphism on risk. In addition, studies could be undertaken in more selected cohorts, where the effects of

differences in platelet reactivity on outcome is likely to be greater. For example, such groups could include subjects that have suffered sub-acute stent thrombosis following percutaneous coronary intervention, or thrombotic re-occlusion of a coronary artery following thrombolysis for acute myocardial infarction.

Second, in the absence of a suitable, specific P2Y1 receptor antibody that could be used in Western blotting, it was not possible to elucidate, with certainty, the mechanism by which the P2Y1 1622 polymorphism exerts its effect. The results of the platelet signalling and mRNA work indicate that the likely mechanism is through an increase in P2Y1 receptor numbers mediated via an increase in the amount of P2Y1 transcript available for translation. Although the P2Y1 1622 polymorphism is silent, an increase in stability of the mRNA message as a consequence of carriage of the P2Y1 1622 G allele could result in an increase in transcript availability for translation. This hypothesis could be tested in further studies by transfecting an appropriate cell line with P2Y1 receptor gene constructs carrying the P2Y1 1622 A and G alleles. Absolute quantification of P2Y1 transcripts could then be performed, after development of a sutiable TaqMan fluorogenic 5'-nuclease assay, to address this hypothesis.

Alternatively, the P2Y1 1622 polymorphism may be linked with one or more polymorphisms upstream of the gene that affect its transcriptional regulation. Up-regulation of transcription, resulting in increased receptor numbers could also explain the mechanism of action of the polymorphism. A number of promoter region polymorphisms linked to the P2Y1 1622 polymorphism have been identified in this work, and thus future studies could also be directed at investigating how these promoter polymorphisms affect transcriptional regulation by cloning promoter fragments containing different haplotypes of the upstream polymorphisms into a luciferase expression plasmid. Transfection of these plasmids into an appropriate cell line would allow reporter activity to be measured and comparisons of relative promoter activity to be made.

7.4 Implications of results

The critical role of platelets in thrombosis and in the pathophysiology of acute myocardial infarction is incontrovertible. Despite this, debate still continues as to whether increased platelet reactivity, *per se*, predisposes to the development of

atherothrombotic disease states such as acute MI, largely due to conflicting results from studies addressing this question. However, given the proven benefits of various antiplatelet therapies, which work by reducing platelet reactivity, in reducing morbidity and mortality suffered as a consequence of atherothrombotic disease,[Antithrombotics Trialists' Collaboration, 2002] it remains logical to think that those patients with higher intrinsic platelet reactivity would be at greater risk. This is lent support by data from a number of studies that have measured platelet reactivity and related this to complications post thrombolysis and percutaneous coronary intervention (PCI, such as coronary angioplasty and stenting).

Nordt *et al* [Nordt *et al*, 1998] assessed the relationship between platelet aggregation and risk of re-occlusion of the infarct-related coronary artery following thrombolysis in 31 subjects with acute MI. They found that re-occlusion (defined as re-infarction or angiographically proven re-occlusion) was significantly associated with higher post-thrombolysis platelet aggregation to 2 μmol/L ADP, such that those subjects with re-occlusion demonstrated greater platelet aggregation at both 2 and 12 hours post-thrombolysis ($p<0.001$ and $p<0.01$ respectively). In addition, they identified platelet aggregation slope at 2 hours to be the best predictor of both early (5 to 14 days) and late (within 1 year) re-occlusion.

Kabbani *et al* [Kabbani *et al*, 2001b] studied 112 subjects undergoing PCI for angina (stable or unstable syndromes) and MI. They determined platelet reactivity by flow cytometric analysis of fibrinogen-binding to the activated GPIIb-IIIa receptor, a method identical to that used in this work, following stimulation with 0.2 μmol/L ADP prior to the procedure. Subjects were divided into two groups using median fibrinogen-binding as the cut-off point. At 90 days, a composite end-point of MI, urgent revascularisation or repeat revascularisation, was significantly greater in the high responders as compared to the low responders (26.8% vs 7.1%, $p=0.01$).

There is also growing interest in the field of "drug-resistance" to antiplatelet therapy, such as clopidogrel. Clopidogrel is used to prevent recurrent events[Yusuf *et al*, 2001a] and re-occlusion post MI,[Sabatine *et al*, 2005] and subacute stent thrombosis (SAT) following PCI.[Gurbel *et al*, 1999] Clopidogrel is a non-competitive antagonist of the P2Y12 receptor, and is a potent inhibitor of platelet activation and aggregation.

Gurbel *et al* [Gurbel *et al*, 2003] have analysed the association between variability in platelet reactivity before treatment with clopidogrel, and subsequent response to clopidogrel therapy in 96 subjects undergoing elective coronary stenting. They found that clopidogrel resistance, defined as <10% absolute difference between pre- and post-treatment platelet aggregation to 5 μmol/L ADP, was present in 31% and 15% of patients at days 5 and 30 respectively. Subjects with a pre-treatment platelet aggregation to 5 μmol/L ADP of >70%, remained the most reactive after 24 hours of treatment ($p<0.0001$).

Gurbel went on to study the effects of clopidogrel on platelet reactivity in 20 subjects with SAT compared with 100 age-matched subjects without SAT.[Gurbel *et al*, 2005] Platelet reactivity was determined by a number of methods including platelet aggregation to ADP and flow cytometric measurement of GPIIb-IIIa activation using PAC-1. All subjects were taking clopidogrel at the time of testing. They found that platelet reactivity was significantly greater by all measuring methods in those subjects with SAT compared to those without. Overall 60% of patients with SAT had platelet aggregation higher than the 75th percentile of the non-SAT group.

Given the data described in the studies above, and as carriage of the P2Y1 1622G allele confers significantly greater platelet reactivity to ADP, measured by platelet aggregation or fibrinogen-binding, this raises the interesting possibility that the P2Y1 1622A>G polymorphism may be implicated not only in the pathogenesis of myocardial infarction, but also in the development of complications following the treatment of AMI either by thrombolysis or PCI. As discussed earlier in this chapter, potential further clinical studies would include determining whether this polymorphism predisposes to the development of complications, such as subacute stent thrombosis, post-PCI.

The mechanism by which resistance to clopidogrel occurs has yet to be fully understood. It remains plausible that polymorphisms that increase platelet reactivity, such as the P2Y1 1622A>G polymorphism, may be implicated in the development of this. Thus studying the effects of clopidogrel on platelet function and signalling in subjects with differing P2Y1 1622 genotypes may help to elucidate part of this mechanism.

Finally, if further studies were to find that the P2Y1 1622A>G polymorphism significantly increase the risk of developing an acute MI, or thrombotic complications

post thrombolysis and PCI, potential future applications of this data may include the use of genetic testing to permit identification of subjects that would benefit from more aggressive therapy at the time of a myocardial infarction, or at the time of percutaneous coronary intervention.

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