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Mechanisms of Variability in Response to Antiplatelet Drugs

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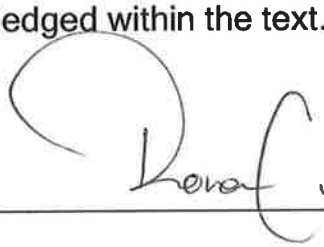
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a Medical Doctorate (MD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed 

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Summary

A variety of antiplatelet drugs decrease the risk of thrombotic events in at risk patients. However, more recent evidence suggests that the benefit derived from antiplatelet drugs is not consistent and that certain patients respond unfavourably to therapy. In four separate studies, we examined different components of individual variability in antiplatelet drug response. A key hypothesis was that the PI^{A2} polymorphism of the platelet fibrinogen (GPIIb/IIIa) receptor modulated the response to aspirin and GPIIb/IIIa antagonists.

In a study of 199 patients with coronary artery disease, 30 (15%) were aspirin resistant when measured by the Platelet Function Analyser (PFA)-100. Non-responsiveness was related to enhanced reactivity to epinephrine (median 38% vs 23%, $p < 0.05$) and increased inducible platelet activation (median 66% vs 56%, $p < 0.05$). There was no association between the PI^{A2} genotype and aspirin response.

In another study of the GPIIb/IIIa antagonist tirofiban in patients ($n = 21$) presenting with an acute coronary syndrome we found evidence for inadequate inhibition of platelet aggregation in the majority (66%) of patients at standard dose. There was evidence for significant interindividual variation in the antithrombotic response to tirofiban ($p < 0.05$), however there was poor agreement between the different assays used. No predictors of variability were detected. Consistent with a partial

agonist effect, there was enhanced inducible platelet activation during tirofiban treatment (61% versus 37%, p = 0.004).

In a third study we examined the role of GPIIIa promoter polymorphisms on platelet fibrinogen receptor (GPIIb/IIIa) expression in 207 patients with a history of an acute coronary syndrome. The promoter polymorphisms were in tight linkage disequilibrium with the PI^{A2} polymorphism of GPIIIa, but did not affect GPIIb/IIIa receptor expression density.

Finally, to determine the effect of PI^{A2} on the antithrombotic response to GPIIb/IIIa antagonists, we studied an enriched group of individuals with the PI^{A2} genotype (n = 20). We confirmed a partial agonist effect with GPIIb/IIIa antagonists *in vitro* manifest as increased platelet P-selectin expression and thromboxane generation. Corrected p-selectin expression was significantly increased in PI^{A2} homozygotes compared to wildtype controls after incubation with orbofiban (24.8% vs 10.1%, p = 0.02).

In conclusion, we detected variability in the response to aspirin and GPIIb/IIIa antagonists. Aspirin resistance was linked to increased reactivity to epinephrine and increased platelet activation. Variation in the expression of the GPIIb/IIIa receptor was not related to the PI^{A2} genotype or its associated promoter polymorphisms. However PI^{A2} appears to enhance partial agonism in the presence of small molecule GPIIb/IIIa antagonists, suggesting an effect of this polymorphism on outside-in signalling by the GPIIb/IIIa receptor.

~ Chapter 1 ~

General Introduction: Variability in the Response to Antiplatelet Drugs

Antiplatelet drugs are vital in the treatment of patients at high risk for atherothrombotic events. In the Antithrombotic Trialists' Collaboration, a review of 287 studies of antiplatelet therapy in 212,000 patients, the combined outcome of any serious vascular event, including death, myocardial infarction and stroke, was reduced by about 25% compared to control [1]. Aspirin was the most widely studied antiplatelet drug and was effective at doses as low as 75 mg daily. The thienopyridines, clopidogrel and ticlopidine, appeared to be slightly more effective antiplatelet agents, decreasing vascular events by a further 10 to 12% compared to aspirin. The subsequently published CURE study showed that addition of clopidogrel to aspirin therapy in patient with acute coronary syndromes (ACS) excluding ST elevation reduced the risk of death from cardiovascular causes, MI, and stroke by 20%. The COMMIT and CLARITY studies demonstrated that addition of clopidogrel to aspirin in patients presenting with ST elevation MI was associated with decrease in the risk of major adverse cardiac events compared to treatment with aspirin alone [2,3]. However in the CHARISMA study, addition of clopidogrel to aspirin in lower risk patients with either clinically evident cardiovascular disease or multiple risk factors did not reduce the primary combined outcome of cardiovascular death, MI, or stroke [4]. Addition of another antiplatelet drug,

dipyridamole, to aspirin may provide a small additional benefit in patients with a history of stroke [5]. In patients undergoing coronary stenting, short term addition of an intravenous glycoprotein (GP) IIb/IIIa antagonist to aspirin and a thienopyridine reduces the risk of major adverse cardiac events at 30 days [6]. Newer and more potent oral $P2Y_{12}$ ADP receptor antagonists have shown superior efficacy to clopidogrel in recently published, randomised controlled trials of patients presenting with acute coronary syndromes (ACS) undergoing percutaneous intervention (PCI). In the TRITON-TIMI 38 trial, treatment with prasugrel was associated with a significant 19% relative risk reduction in cardiovascular death, nonfatal MI, or nonfatal stroke compared to clopidogrel at the cost of a small increase in non-coronary artery bypass grafting (CABG) related major bleeding [7]. Ticagrelor is a novel ADP antagonist which reversibly inhibits the $P2Y_{12}$ receptor and has a more rapid onset of action than clopidogrel [8]. In the PLATO study, treatment with ticagrelor was associated with a significant 16% relative risk reduction in death from vascular causes, MI, or stroke compared to clopidogrel, again at the cost of a small increase in non-CABG related major bleeding [9]. Ticagrelor is not yet available for clinical use.

Despite the benefits of antiplatelet drugs in large randomized controlled trials, there is increasing evidence that there is individual variability in the response to these agents that may limit the benefit in certain patients [10]. These concerns have arisen in particular with aspirin, but also have relevance to the thienopyridines and

GPIIb/IIIa anatagonists [11,12]. We sought to investigate some of the factors that may lead to this interindividual variation in antiplatelet drug response.

Aspirin Resistance

Pharmacology of Aspirin

Aspirin was the earliest, and remains the principal antiplatelet agent in clinical use. Originally developed for its analgesic and antipyretic properties in the late 1800s, it was not until the mid-twentieth century that Gibson and Craven independently first described the antithrombotic properties of aspirin [13,14]. However their work went largely unnoticed and it was many years later before large scale clinical trials confirmed the anti-clotting effects of aspirin leading to FDA approval for aspirin in the secondary treatment of stroke in 1980 and myocardial infarction (MI) in 1985. Sir John Vane first described the mechanism of action of aspirin in 1971[15]. Aspirin irreversibly inhibits cyclooxygenase (COX)-1 due to acetylation of serine reside 530, thereby preventing formation of prostaglandin endoperoxides and thromboxane (TX) A_2 (Figure 1.1). Prostaglandin (PG) I_2 causes vasodilation and inhibits platelet aggregation whereas TXA_2 is a potent vasoconstrictor and promotes platelet aggregation. Although aspirin prevents production of both of these compounds, two factors lead to greater inhibition of TXA_2 than PGI_2. Firstly, platelets are the primary source of TXA_2 and the vascular endothelium is the primary source of PGI_2. Secondly, the anucleate platelet is unable to regenerate COX-1 that is irreversibly acetylated by aspirin, whereas the endothelium retains its

capacity to generate new COX-1 and recovers normal function shortly after exposure to aspirin [16]. Therefore inhibition of platelet production of TXA_2 by aspirin is the key to its antithrombotic effect. In the 1990's a second isoform of cyclooxygenase was discovered called COX-2 [17]. COX-2 is not routinely expressed in normal functioning cells, but is induced in the inflammatory state and also by shear stress [18]. Earlier studies found no evidence of COX-2 expression in mature platelets [19], however more recent reports have found evidence for platelet COX-2, likely related to immature circulating platelets [20,21]. In addition, although aspirin irreversibly inhibits both COX-1 and COX-2, it is a relatively weak COX-2 inhibitor. Its inhibitory effect on COX-1 is approximately 170 times greater than on COX-2 [22]. For these reasons, COX-2 is unlikely to be an important factor in the antithrombotic effect of aspirin.

Evidence for Aspirin Resistance

Aspirin has been conclusively shown to reduce the risk of further thromboembolic events in patients with a history of cardiovascular, cerebrovascular and peripheral vascular disease [1]. However 10 to 20% of patients suffer recurrent vascular events over long term follow up despite treatment with aspirin [1]. Treatment failures with aspirin are not surprising. Aspirin is a relatively weak antiplatelet agent because it antagonises only one of several pathways that lead to platelet activation and aggregation. The occurrence of clinical failures during aspirin therapy and the observation of variability in the biological response to aspirin using an assortment

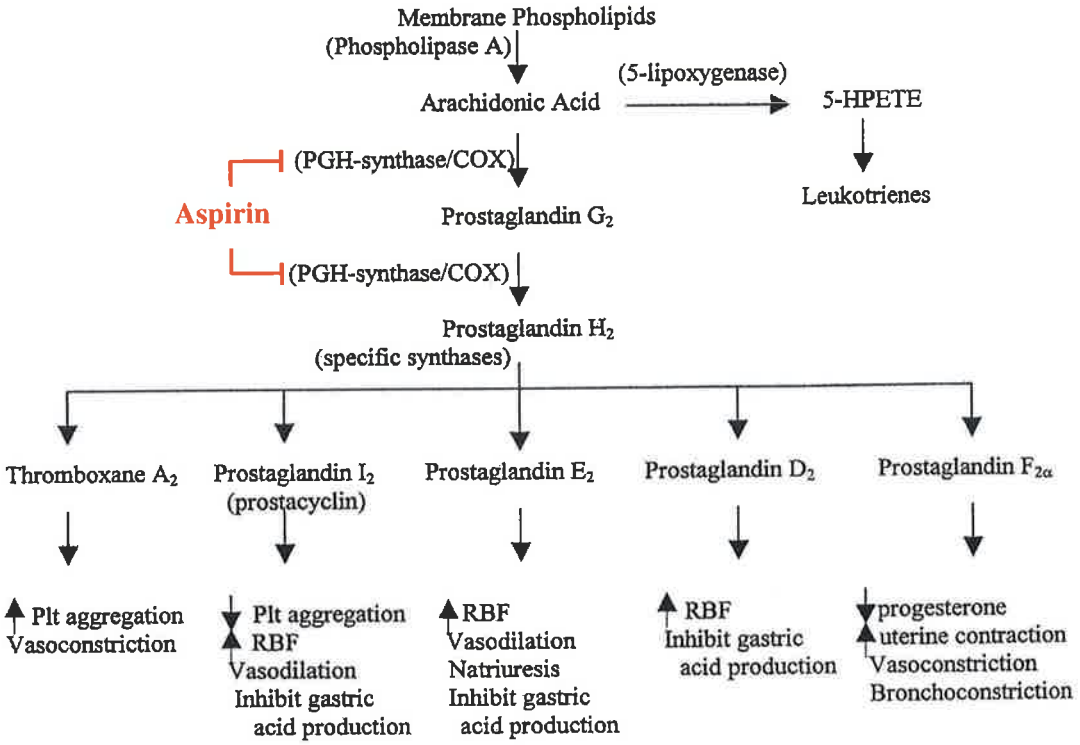

Figure 1.1. The eicosanoid pathway. Aspirin inhibits the conversion of arachidonic acid to Prostaglandin H_2 by Cyclooxygenase(COX)-1.

of platelet function assays has led to the concept of "aspirin resistance" [23]. However, "aspirin resistance" remains poorly defined and is not universally accepted [24,25]. As mentioned above, clinical "aspirin resistance" occurs in 10 to 20% of patients over long term follow up. Although no medication is 100% effective, it seems that certain patients are at particularly high risk of recurrent events on aspirin. Patients presenting with acute coronary syndromes (ACS) despite treatment with aspirin have up to a 60% higher risk of death or a recurrent cardiac

event at approximately 1 month [26,27]. Functional "aspirin resistance", detected as enhanced platelet activity despite treatment with aspirin, has been reported in 0% to 57% of patients [28]. Discrepancy in its reported frequency in part reflects the wide variety of tests used and arbitrary cut off values imposed [29]. A major confounding factor in assessment for aspirin resistance is patient non-compliance, which has been poorly evaluated in many studies of aspirin resistance. Non-compliance to aspirin has been described in 2 to 16% of patients in studies of aspirin resistance [30,31]. Non-adherence or withdrawal of aspirin therapy in itself portends a worse prognosis and is associated with a 3-fold increased risk of major adverse cardiovascular events (MACE) in moderate to high risk patients [32].

The most scientifically precise method of testing the antithrombotic efficacy of aspirin is to directly measure its inhibition of platelet cyclooxygenase thromboxane generation (Figure 1.2). TXA_2 is rapidly metabolised in vivo to the stable metabolite TXB_2 and subsequently excreted in the urine as 11-dehydro-TXB_2. ELISA or radioimmunoassay can readily measure TXB_2 *ex-vivo* in the serum of clotted blood samples. Levels of urinary 11-dehydro-TXB_2 represent systemic production of TXA_2 and have also been used to determine the efficacy of aspirin. However, although urinary 11-dehydro-TXB_2 is largely platelet derived, it may also reflect TXA_2 generation by other tissues[33,34].

A single loading dose of 100 mg of aspirin causes almost complete suppression (95%) of platelet thromboxane production [35]. Lower daily doses (0.45 mg/kg) are needed to maintain the same level of inhibition of thromboxane generation [35].

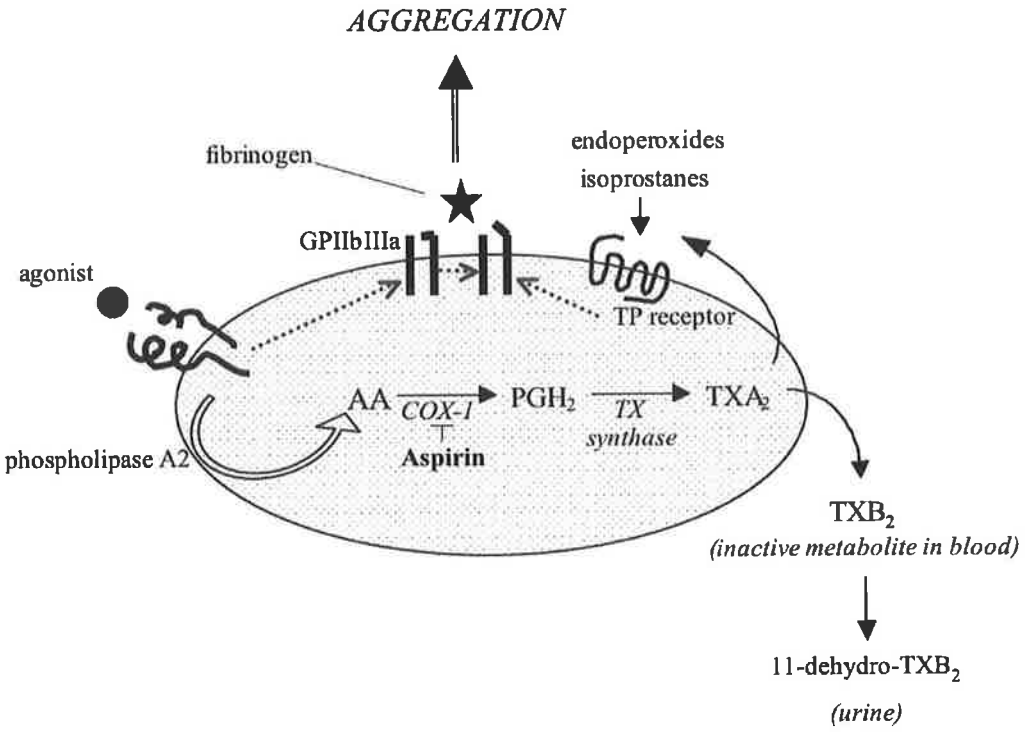

Figure 1.2. The central role of the cylooxygenase pathway illustrated in the platelet. AA = Arachidonic Acid. PGH_2 = Prostaglandin H_2. TX = Thromboxane.

However, enteric coated aspirin has lower bioavailability than dispersible preparations and at a dose of 75 mg appears to be associated with incomplete suppression of thromboxane synthesis in a large proportion of individuals [36,37]. In one cross-over study of 71 healthy volunteers, 13% of patients failed to inhibit thromboxane generation more than 95% with enteric coated aspirin compared to 0% with dispersible aspirin [36]. Using the more stringent criteria of > 99% inhibition of thromboxane generation, 54% of individuals on enteric coated aspirin were

deemed treatment failures compared to 8% on dispersible aspirin. In a population of patients with coronary artery disease, we have similarly shown that 44% of patients had incomplete suppression of thromboxane generation defined by a serum TXB_2 > 2.2 ng/ml [37]. Patients with a serum TXB_2 > 2.2 ng/ml were more likely to have platelet aggregation in response to stimulation with arachidonic acid. In addition, they were younger, heavier, and were more likely to have had a previous MI. Inadequate suppression of TXA_2 generation by aspirin may also occur due to concomitant therapy with non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen [38,39]. There is also evidence that genetic variation in COX-1 may contribute to differences in suppression of thromboxane generation by aspirin. We have shown that a common (12% of the study population) single nucleotide polymorphism (SNP) that results in an A to G substitution at position -842 in the promoter region of COX-1 is associated with aspirin resistance defined by increased aggregation to arachidonic acid (AA)[40]. Further studies are needed to validate this result and elucidate the possible role of other SNPs in aspirin resistance.

Aspirin insensitive eicosanoid biosynthesis has also been postulated as a mechanism for aspirin resistance [41]. Possible mechanisms include COX-2 expression in immature platelets; aspirin insensitive isoforms of COX-1; generation of isoprostanes such as isoprostane(iP)$F_{2\alpha}$-III (formerly known as 8-iso-prostaglandin(PG)$F_{2\alpha}$); and vasoactive cysteinyl leukotrienes. A recent study showed a relationship between higher levels of immature or reticulated platelets,

platelet COX-2 expression and platelet aggregation and activation prior to and after treatment with aspirin [20]. Levels of 8-iso-$PGF_{2\alpha}$, a bioactive product of arachidonic acid peroxidation, are increased in unstable angina and correlate with 11-dehydro-TXB_2 excretion ($r = 0.721$, $p < 0.0001$) [42]. 8-iso-$PGF_{2\alpha}$ activates the thromboxane (TP) receptor resulting in platelet aggregation [43,44]. However any association between isoprostanes and aspirin resistance remains hypothetical at this time. Most studies of aspirin resistance have used COX-independent assays such as the bleeding time test, platelet aggregometry, platelet adhesion, flow cytometric analysis of platelet activation and automated assays such as the Platelet Function Analyzer (PFA)-100 [45]. The wide variety of tests employed is further complicated by the variety of platelet agonists and doses used in the many studies of aspirin resistance. It is not surprising therefore that the reported prevalence of aspirin resistance varies widely and that there is poor agreement between the different assays in predicting failure to respond to aspirin [28,29,46,47]. Incomplete inhibition of platelet cyclooxygenase by aspirin may contribute to some of the treatment failures observed with functional assays of platelet adhesion and aggregation. Some studies have demonstrated that higher doses of aspirin can reverse therapy failures, suggesting inadequate dosing of aspirin in certain individuals [46,48,49]. However these and other studies show that despite higher doses of aspirin and evidence of complete suppression of platelet COX-1, some patients have higher levels of platelet reactivity, presumably related to up-regulation of non-COX-1 pathways [31]. In fact healthy individuals not on antiplatelet therapy demonstrate a

wide range of platelet reactivity. A recent study of platelet aggregation in 359 healthy individuals showed considerable interindividual variability in the aggregation response to the agonists adenosine diphosphate (ADP), epinephrine, collagen, collagen-related peptide, and ristocetin, especially at concentrations lower than those typically used in clinical laboratories [50]. Increased sensitivity to low dose epinephrine (0.4 μM) was detected in 14% of individuals and was particularly reproducible on multiple testing (intra-class correlation coefficient (ICC) = 0.81). Subjects who exhibited hyperreactivity to one agonist tended to demonstrate a similar response to others, suggesting that hyperreactivity is a global characteristic of platelets. Similar variation and consistency in response to different platelet agonists has been observed in healthy volunteers in a study of platelet activation using flow cytometric analysis of platelet p-selection (CD-62) and activated GPIIb/IIIa expression [51]. Increased baseline platelet activation in some individuals is not altered by the *ex vivo* addition of aspirin [31]. However, an outstanding concern with functional assays of platelet function in aspirinated patients is their poor reproducibility over time, the lack of correlation between different assays, and the absence of conclusive evidence of an ability to predict increased clinical risk or improve therapeutic strategies [47,52].

Incomplete inhibition of cyclooxygenase by aspirin has been linked to a higher risk of clinical events [53,54]. Gum et al found that aspirin resistance, defined as mean aggregation of ≥ 70% with 10 μM ADP and ≥ 20% with 0.5 mg/ml of arachidonic acid, was associated with an approximate 3-fold increased risk of death, MI, or

CVA at 2 years compared to aspirin responsive patients (24% vs 10%, p = 0.03) [54]. Eikelboom et al, in a nested case control study of the HOPE trial, showed that patients with urinary 11-dehydro-TXB_2 levels in the upper quartile had a 1.8-fold increased risk of cardiovascular death, MI, or stroke at 5 years compared to those in the lowest quartile (95% CI, 1.2 to 2.7; p = 0.009) [53]. In addition, a number of small studies have shown an association between aspirin resistance detected with automated assays of platelet function such as the Ultegra RPFA and PFA-100 and major adverse cardiac events [55-59]. However agreed definitions of aspirin resistance, large trials to confirm its prognostic value, and proof that changes in treatment can alter outcomes are needed before aspirin resistance can be considered clinically relevant.

Variability in the Response to GPIIb/IIIa Antagonists

Pharmacology of GPIIb/IIIa Antagonists

Glycoprotein IIb/IIIa antagonists are specific and potent inhibitors of platelet aggregation. The intravenous compounds are an important and effective therapy in the setting of coronary angioplasty and stenting, where mechanical injury to the vessel wall is a strong stimulus to thrombus formation. However, their efficacy in the treatment of patients with acute coronary syndromes, who do not undergo percutaneous coronary intervention (PCI), is not so certain. Abciximab, eptifibatide, and tirofiban, the three commercially available intravenous agents, have been extensively evaluated in several large randomized controlled trials of patients

presenting with ACS and/or undergoing PCI. A fourth agent, lamifiban, has been studied in the setting of ACS without routine PCI.

Abciximab, a chimeric monoclonal antibody, was the first of these agents to be developed. It has been extensively evaluated in the setting of PCI. Subsequent intravenous and oral compounds are synthetic peptide and peptidomimetic compounds modelled on the Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) recognition sequences of the fibrinogen α and γ chains respectively [60]. These "small molecule" GPIIb/IIIa antagonists are short acting and include the intravenous compounds eptifibatide, tirofiban and lamifiban and the oral compounds orbofiban, sibrafiban, lefradafiban, and xemilofiban.

Eptifibatide is a cyclic heptapeptide based on the Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) sequence located at the carboxy terminus of the fibrinogen γ-chain. Tirofiban and lamifiban are non-peptide derivatives of tyrosine based on the Arg-Gly-Asp (RGD) sequence found in fibrinogen and other natural ligands of the GPIIb/IIIa receptor. Tirofiban and lamifiban bind specifically to GPIIb/IIIa, whereas abciximab binds in addition to other receptors, such as the vitronectin receptor, $\alpha_v\beta_3$ [61], and the neutrophil associated integrin, Mac-1 ($\alpha_M\beta_2$) [62]. Eptifibatide binds both $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) and $\alpha_v\beta_3$, but has an approximately 300- to 400-fold higher affinity for $\alpha_{IIb}\beta_3$ [63]. Nevertheless at therapeutic concentrations of eptifibatide, some inhibition of $\alpha_v\beta_3$ may occur. Inhibitors of the vitronectin receptor inhibit proliferation of smooth muscle cells in models of vascular injury [64]. Inhibition of the leukocyte adhesion receptor Mac-1 improves microvascular reflow and myocardial salvage in

animal models of acute myocardial infarction (AMI) [65]. However the clinical significance of these differences in agent specificity remain unclear.

The GPIIb/IIIa antagonists also vary in their affinity for the GPIIb/IIIa receptor. Abciximab binds tightly to and dissociates slowly from GPIIb/IIIa. Its effects can be measured for several days following discontinuation of the drug and receptor occupancy at low levels persists for up to 14 days [66]. Eptifibatide and tirofiban have low affinity for GPIIb/IIIa and in addition they have short half-lives of elimination (t_β). Once either of these agents is discontinued platelet function rapidly returns to normal and there is little or no measurable antiplatelet effect after 4 hrs [67].

Clinical Trials of GPIIb/IIIa Antagonists

A. Percutaneous Coronary Intervention

There have been more than ten large randomised trials of GPIIb/IIIa antagonists in PCI, involving a total of more than 25,000 patients (Table 1.1). Abciximab has been comprehensively studied in PCI and is associated with a 35 to 56% reduction in major adverse cardiac events (MACE) at 30 days [68-71]. Eptifibatide and tirofiban have been associated with more modest reductions of 15 to 35% at 30 days [72-74]. In TARGET (Do Tirofiban And Reopro Give similar Efficacy Trial), the only head-to-head comparison of the GPIIb/IIIa antagonists, abciximab was associated with a lower rate of death, MI and revascularisation than tirofiban at 30 days (6.0% versus (14.3% versus 14.8%, p=0.591) [76]. 7.6%, p=0.038) [75]. However, this difference no longer existed at 6-month follow up.

Table 1.1. GPIIb/IIIa antagonist therapy in PCI. [a] Event rate is for death, MI and urgent revascularisation at 30 days.

Study (year)	Subjects (number)	Treatment Arms	Event rate[a]	P value
EPIC [68] (1994)	2,099	Placebo	12.8%	
		Abciximab bolus only	11.5%	0.43
		Abciximab bolus + infusion	8.3%	0.008
IMPACT II [72] (1997)	4,010	Placebo	11.4%	
		Eptifibatide (low dose)	9.2%	0.063
		Eptifibatide (high dose)	9.9%	0.22
RESTORE [73] (1997)	2,139	Placebo	12.2%	
		Tirofiban	10.3%	0.16
CAPTURE [69] (1997)	1,265	Placebo	15.9%	
		Abciximab	11.3%	0.012
EPILOG [70] (1997)	2,792	Placebo + standard heparin	11.7%	
		Abciximab + low dose heparin	5.2%	<0.001
		Abciximab + standard heparin	5.4%	<0.001
EPISTENT [71] (1998)	2,399	Stent + placebo	10.8%	
		PTCA+ abciximab	6.9%	0.007
		Stent + abciximab	5.3%	<0.001
ESPRIT [74] (2000)	2,064	Placebo	10.5%	
		Eptifibatide (double bolus)	6.8%	0.0034
TARGET [75] (2001)	5,308	Tirofiban	7.6%	
		Abciximab	6.0%	0.038
ISAR-REACT [77]	2,159	Placebo	4.0%	
		Abciximab	4.0%	0.91
ISAR-REACT 2 [78]	2,022	Overall		
		Placebo	11.9%	
		Abciximab	8.9%	0.03
		Troponin +ve		
		Placebo	18.3%	
		Abciximab	13.1%	0.02
		Troponin -ve		
		Placebo	4.6%	
		Abciximab	4.6%	0.98

The majority of events prevented by GPIIb/IIIa antagonists in the PCI studies were MIs, many of which were characterized by small rises in cardiac markers. Although the individual trials were not powered to detect a mortality benefit with GPIIb/IIIa antagonist therapy, subsequent meta-analyses have demonstrated a sustained 20 to 30% reduction in the risk of death with these agents in PCI [79,80]. Although the early studies showed that all patient subgroups benefit from GPIIb/IIIa antagonist therapy in PCI, high-risk patients defined by an elevation in troponin T or I benefited to a greater extent [69]. However, subsequent refinements in the dosing of the thienopyridines prior to PCI led many to question the additional benefit of a GPIIb/IIIa antagonist in low risk patients. The recently completed ISAR REACT (elective PCI) and ISAR-REACT 2 (ACS patients) studies confirmed that in patients undergoing PCI after pre-treatment with clopidogrel 600 mg and aspirin 325 to 500 mg at least 2 hours prior to the procedure, the benefit of abciximab therapy was confined to troponin positive patients [77,78]. Another study by the same group, entitled ISAR-SWEET confirmed that abciximab reduces the risk of restenosis in diabetic patients, seen initially in a secondary analysis of the EPISTENT study [71,81].

B. Acute Myocardial Infarction

The role of GPIIb/IIIa antagonists in PCI for ST-segment-elevation MI is less certain. Several small, randomized trials have shown that administration of abciximab in this setting improves outcome [82-85]. In the ADMIRAL (Abciximab before Direct Angioplasty and Stenting in Myocardial Infarction Regarding Acute and Long term follow up) and ISAR (Intracoronary Stenting and Antithrombotic

Regimen)-2 trials, there was an impressive 59% and 52% reduction respectively in the composite of death, re-infarction, or target vessel revascularisation at 30 days in the stenting plus abciximab group compared to the stenting plus placebo group [84,85]. However in the more recent and larger CADILLAC (Controlled Abciximab and Device Investigation to Lower Late Angioplasty Complications) study, the composite of death, reinfarction, disabling stroke, and ischemia-driven revascularisation of the target vessel at 6 months was no different in the stenting plus abciximab and stenting alone group (10.2% versus 11.5%, p=NS) [86]. Treatment with abciximab was not randomized in the CADILLAC study, which was designed to assess angioplasty versus stenting, with or without abciximab in ST-segment-elevation MI using a 2 x 2 factorial design. But unlike CADILLAC, neither ADMIRAL nor ISAR-2 used a loading dose of a thienopyridine. Another confounding factor may be the timing of GPIIb/IIIa antagonist administration. In the ADMIRAL study, reduction in clinical events was more pronounced in those patients who received abciximab early, in the ambulance or emergency room (ER), compared to those who received it later in the cardiac care unit or catheterisation laboratory [85]. Similarly the recent TIGER-PA (TIrofiban Given in the Emergency Room before Primary Angioplasty) trial showed that early administration of tirofiban in the ER improved angiographic outcomes compared to later administration in the catheterisation laboratory just prior to PCI [87].

Despite promising preliminary results, addition of GPIIb/IIIa antagonist therapy to thrombolytic therapy for acute ST-segment-elevation MI does not appear to accrue

any clinical benefit. Although combined therapy results in improved rates of TIMI-3 or normal blood flow of the infarct related vessel [88,89], this has not translated into an improvement in 30 day or 1 year mortality [90,91].

C. Acute Coronary Syndromes

There is doubt regarding the benefit of GPIIb/IIIa antagonists in the medical treatment of patients who present with unstable angina or non-ST-segment-elevation MI, despite results from six large clinical trials (Table 1.2). Whereas treatment with eptifibatide and tirofiban has been associated with modest reductions in recurrent cardiac events in this population, abciximab showed no benefit over placebo in the recent GUSTO IV-ACS (Global Use of Strategies to Open Occluded Coronary Arteries IV in Acute Coronary Syndromes) trial [92].

In the PURSUIT (Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy) trial 10,948 high risk ACS patients were randomised to high or low dose eptifibatide or placebo [97]. There was a small but significant reduction in the primary endpoint of death or MI at 30 days in the higher dose eptifibatide group (14.2% vs 15.7%, p=0.04). The PRISM (Platelet Receptor Inhibition in Ischaemic Syndrome Management) study randomized 3,232 patients to either heparin or tirofiban [95]. Although death, MI, or refractory ischemia were reduced at 48 hours in the tirofiban group (3.8% vs 5.6%, p=0.01) this benefit was not evident at 30 days. In the PRISM-PLUS (Platelet Receptor Inhibition in Ischaemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms) trial 1,570 high risk patients were randomised to treatment with

Table 1.2. GPIIb/IIIa antagonists in acute coronary syndromes. [a] Event rate is for death or MI at 30 days.

Study (year)	Subjects (number)	Treatment Arms	Event Rate[a]	P value
PARAGON A [93] (1998)	2,282	Placebo + heparin Lamifiban (low dose) Lamifiban (high dose)	11.7% 10.6% 12.0%	 0.67
PARAGON B [94] (2002)	5,225	Placebo + heparin Lamifiban	11.5% 10.6%	 0.32
PRISM [95] (1998)	3,232	Heparin Tirofiban	5.8% 7.1%	0.11
PRISM-PLUS [96] (1998)	1,915	Heparin Tirofiban Tirofiban + heparin	8.7% Stopped 11.9%	0.03
PURSUIT [97] (1998)	10,948	Placebo Epifibatide bolus + infusion	15.7% 14.2%	0.04
GUSTO IV ACS [92] (2001)	7,800	Placebo Abciximab (24hr) Abciximab (48hr)	8.0% 8.2% 9.1%	 NS 0.19

tirofiban plus heparin or heparin alone[96]. The primary endpoint of death, MI or refractory ischemia at 7 days was significantly reduced in the tirofiban group (12.9% vs 17.9%, risk ratio 0.68, 95% CI = 0.53 - 0.88, p = 0.004). The rates of the composite end point in the tirofiban group were also lower than those in the heparin only group at 30 days (18.5% vs 22.3%, P = 0.03) and at 6 months (27.7% vs 32.1%, P = 0.02). The benefit was consistent in the various subgroups of patients and in those treated medically as well as those treated with angioplasty (30.5% of patients between 49 to 96 hours after randomisation). In the CAPTURE (Chimeric

c7E3 Antiplatelet therapy in Unstable angina Refractory to standard treatment) trial patients with refractory unstable angina were randomised to abciximab or placebo for 18 to 24 hours prior to PCI. There was a reduced incidence of MI with abciximab treatment before PCI (0.6% vs 2.1%, p = 0.029) as well as during PCI (2.6% vs 5.5% p = 0.009). The GUSTO IV-ACS study was expected to confirm the benefit of abciximab in ACS patients who do not undergo PCI [92]. In this trial 7,800 high risk ACS patients were randomly assigned to placebo, abciximab bolus and 24 hr infusion, or abciximab bolus and 48 hr infusion in addition to standard treatment. The primary endpoint of death or MI at 30 days occurred in 8.0% patients on placebo, 8.2% on abciximab for 24 hrs, and 9.1% on abciximab for 48 hrs (p = NS). This finding was similar across all subgroups, and in particular, no benefit was seen in patients with raised cardiac troponin T or I concentrations at enrolment. Similarly, the GPIIb/IIIa antagonist, lamifiban had no effect on major cardiac events at 30 days in ACS patients in the dose-finding PARAGON (Platelet IIb/IIIa Antagonism for the Reduction of Acute coronary events in a Global Organisation Network) -A trial [93] and the subsequent PARAGON-B trial [94]. Meta-analysis of the above studies suggests that there is a modest benefit with GPIIb/IIIa antagonist therapy in unstable angina and non-ST-segment-elevation MI [98]. Overall the combined 30-day incidence of death or MI was 10.8% with GPIIb/IIIa blockade versus 11.8% with placebo (p = 0.015). However, GUSTO-IV ACS has cast a shadow on the benefit GPIIb/IIIa antagonists in patients not undergoing intervention.

How can we explain the differences in benefit between the PCI and ACS trials of the GPIIb/IIIa antagonists? One explanation is that mechanical injury to the coronary artery during PCI is more thrombogenic than the spontaneously ruptured plaque of an ACS. If this is the case then a less potent antiplatelet regimen, such as the combination of aspirin and clopidogrel may be sufficient in ACS patients who do not undergo PCI. In the CURE study prolonged oral therapy (mean 9 months) with clopidogrel was associated with a 21% relative risk reduction in cardiovascular death, MI and stroke at 30 days (relative risk, 0.79; 95% CI 0.67 to 0.92) [99]. Although not directly comparable, the reduction in ischaemic endpoints observed in CURE compares favourably with that observed in PRISM, PRISM-PLUS and PURSUIT.

The timing of initiation of the GPIIb/IIIa antagonist relative to the injury of the vessel wall is also important. In the PCI trials, GPIIb/IIIa antagonists were commenced prior to coronary angioplasty and vessel wall injury. In the ACS studies, GPIIb/IIIa antagonists were commenced up to 24 hours after the onset of chest pain. In the PURSUIT trial earlier treatment with eptifibatide was consistently associated with a greater reduction in death or MI at 30 days. For example, those treated within 24 hrs of symptom onset had a significant 2.2% absolute reduction in death or MI ($p = 0.003$), whereas those treated after 24 hrs derived no benefit [100].

Duration of treatment has been empiric in the clinical trials of GPIIb/IIIa antagonists in ACS and based on the greatest risk period for ischaemic complications being within the first 48 to 96 hrs. In the PURSUIT trial eptifibatide was administered for

72 hrs and in the PRISM and PRISM-PLUS studies tirofiban was administered for 48 to 96 hrs (mean 71 hrs). Extending treatment with a GPIIb/IIIa antagonist beyond the first few days may be important, particularly in patients who have a residual coronary stenosis that may act as a stimulus for platelet activation, adhesion and thrombus formation. Indeed the significant reduction in ischaemic events associated with clopidogrel therapy in the CURE study may be in no small part due to its prolonged administration [99]. However, only with the development of a safe and effective oral agent will prolonged therapy with GPIIb/IIIa antagonists be possible [101].

Reasons for Variability in Response to GPIIb/IIIa Antagonists

The pharmacology of GPIIb/IIIa antagonist therapy is more complex than was initially appreciated. The disappointing results with GPIIb/IIIa antagonists in a number of large clinical trials has led to further investigation of their pharmacology. It would appear that the first step to success with these agents is getting the dose right. Other issues such as interindividual variation, partial agonism, inhibition of inflammation are also being investigated.

A. Inadequate Dosing

It is likely that dosing explains the differences in outcome in the clinical trials with the GPIIb/IIIa antagonists in PCI rather than any differences in property between the agents. Occupancy of ≥ 50% of GPIIb/IIIa receptors by drug is needed before any significant effect is seen on ADP induced platelet aggregation. This is

consistent with the observation that patients heterozygous for Glanzmann's thrombasthenia who express only 50% of the usual number of GPIIb/IIIa receptors do not show an increased bleeding tendency [102]. Early studies of the GPIIb/IIIa antagonists in animals suggested that > 80% receptor occupancy, with nearly complete inhibition of platelet aggregation, was necessary to prevent platelet-induced thrombosis in models of coronary stenosis [103,104]. Subsequent clinical trials of the intravenous GPIIb/IIIa antagonists were therefore designed with dosing regimens to achieve this level of platelet inhibition. The IMPACT (Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis) -II and subsequent ESPRIT (Enhanced Suppression of the Platelet IIb/IIIa Receptor with Integrilin Therapy) trial inadvertently demonstrated the importance of achieving high levels of receptor blockade [72,74]. Pharmacodynamic studies used to calculate the dose of eptifibatide in IMPACT-II substantially overestimated its antiplatelet effect due to calcium chelation by the anticoagulant sodium citrate. When samples were collected instead in the thrombin anticoagulant, PPACK, the IMPACT-II dose of eptifibatide only achieved levels of receptor occupancy of around 50% [105]. In the later ESPRIT trial a dose of eptifibatide 3 to 4 times higher than that used in IMPACT-II almost doubled the clinical benefit of treatment, with a significant 35% risk reduction compared to a non-significant 19% risk reduction in the earlier trial. The platelet inhibitory effect of tirofiban, but not abciximab, is also overestimated when blood is collected in sodium citrate [106]. Several recently published studies have suggested that tirofiban is under-dosed during the first 6 hours of infusion [106-

[109]. This may explain, in particular, the modest and statistically non-significant 16% reduction in ischaemic endpoints with tirofiban at 30 days seen in the RESTORE (Randomized Efficacy Study of Tirofiban for Outcomes and Restenosis) study [73]. In contrast, tirofiban treatment in the PRISM-PLUS (Platelet Receptor Inhibition in Ischaemic Syndrome Management in Patients Limited by Unstable Angina) study was associated with a statistically significant 28% reduction in ischaemic endpoints at 7 days [96]. In PRISM-PLUS coronary angioplasty was not undertaken early in the infusion; investigators were encouraged to delay angiography/PCI until 48 to 96 hrs after randomisation. It is possible to achieve higher levels of platelet inhibition by increasing the dose of tirofiban [109]. Although a higher level of platelet inhibition is likely to translate into an enhanced clinical benefit, this has yet to be confirmed in a clinical trial. The PURSUIT dosing of eptifibatide for ACS is similarly associated with a small fall in the inhibition of platelet aggregation at 1 and 4 hrs [110]. Therefore administration of a second 180 μg/kg bolus of eptifibatide, as used in the later ESPRIT trial, should be considered if a patient proceeds to intervention within this time frame [111].

Abciximab is associated with high levels of platelet inhibition in the first hour of treatment, possibly explaining the particular benefit observed with this agent in the setting of PCI. However, by 12 hrs there is a fall off in receptor occupancy and inhibition of platelet aggregation [66,106]. In addition, because of the long half-life of abciximab, a gradual decline in the level of inhibition of platelet aggregation is measurable for up to 5 days and low levels of receptor occupancy are detectable

for up to 14 days after discontinuation of the infusion [66]. This slow dissociation property of abciximab is particularly relevant in the setting of partial agonism (see below) and may in part explain its failure to reduce ischaemic events in patients with ACS who do not undergo PCI.

B. Interindividual Variation

Apart from the differences in platelet inhibition observed with current dosing regimens of the GPIIb/IIIa antagonists, there is evidence of interindividual variation in response to these drugs. In GOLD, a prospective study of 503 patients undergoing PCI, platelet inhibition was measured using a rapid point of care test, the Ultegra Rapid Platelet Function Assay (RPFA) [112] at 10 mins, 1 hr, 8 hrs and 24 hrs after GPIIb/IIIa administration [12]. One quarter of all patients did not achieve ≥ 95% platelet inhibition at the 10 minute time point and this group had a significantly higher incidence of death, MI or target vessel revascularisation (14.4% vs 6.4%, p = 0.006). In addition patients with < 70% inhibition of platelet function at 8 hrs after the start of therapy had an event rate of 25% in contrast to 8.1% for those with levels ≥ 70%. The majority of patients, 84%, received abciximab, 9% received tirofiban and 7% eptifibatide. Although numbers were small for tirofiban and eptifibatide, their results were consistent with those of abciximab. Several clinical, procedural and haematologic factors were assessed for their relationship with platelet inhibition by GPIIb/IIIa antagonist therapy but only the presence of angiographic thrombus was associated with lower levels of platelet inhibition at all time points. It is reasonable to assume that titrating therapy to a specific level of

inhibition in certain patients will reduce their risk of ischaemic events, although this requires further investigation. There are a number of point-of-care devices, which like the RPFA, will in the future allow a simple and convenient method of monitoring GPIIb/IIIa antagonist therapy [113,114].

C. Partial Agonism

It is now recognized that platelets play a significant and central role in vascular homeostasis that extends beyond the traditional view of them as passive participants in thrombus formation. When activated, platelets release an array of prothrombotic and vasomotor factors, mitogens, and inflammatory mediators (Figure 1.3). Platelet activation also induces a conformational change in the GPIIb/IIIa receptor, so called inside-out signalling, which allows it to bind its natural ligands, fibrinogen and von Willebrand Factor (vWF) [115]. In turn binding of fibrinogen and vWF to the GPIIb/IIIa receptor results in outside-in signalling and further platelet activation [116]. It would appear that engagement of GPIIb/IIIa antagonists with the receptor also results in outside-in signalling and GPIIb/IIIa antagonist-induced platelet activation [117]. Enhanced expression of the platelet activation marker CD63 was observed during treatment with orbofiban in the OPUS-TIMI 16 trial [118]. This partial agonism would appear to be particularly important at lower doses of GPIIb/IIIa antagonists, where platelet activation occurs without significant inhibition of platelet aggregation. For example doses of abciximab, eptifibatide and tirofiban that incompletely inhibit platelet aggregation *in vitro* enhance sCD40L generation, although higher doses reduce its levels [119].

Although the intravenous agents generally achieve high levels of inhibition of platelet aggregation, the slow dissociation of abciximab from the GPIIb/IIIa receptor on discontinuation of the drug provides a period of several days where potential GPIIb/IIIa antagonist-induced platelet activation can act unopposed [66]. In the absence of PCI this window for GPIIb/IIIa antagonist-induced platelet activation with abciximab may be particularly important. PCI restores normal coronary blood flow, thereby reducing shear stress and consequent platelet activation and aggregation. This combination of a residual prothrombotic coronary stenosis and unopposed GPIIb/IIIa antagonist-induced platelet activation may account for the failure of abciximab to reduce events in GUSTO-IV ACS. This is also likely to be particularly relevant to the failure of the oral GPIIb/IIIa antagonist trials as these agents were dosed to achieve lower levels of platelet inhibition than their intravenous counterparts. The results of these trials were universally negative irrespective of whether in an ACS or PCI population. The higher mortality observed in the trials of the oral agents maybe related to longer periods of unopposed GPIIb/IIIa antagonist-induced platelet activation [101].

D. Inflammation

Platelets are integral to the process of vascular inflammation. Platelets directly contribute to inflammation by release of newly synthesised and pre-stored inflammatory mediators. In addition, activated platelets bind leukocytes through Mac-1 and p-selectin to form platelet-leukocyte aggregates in circulating blood [120].

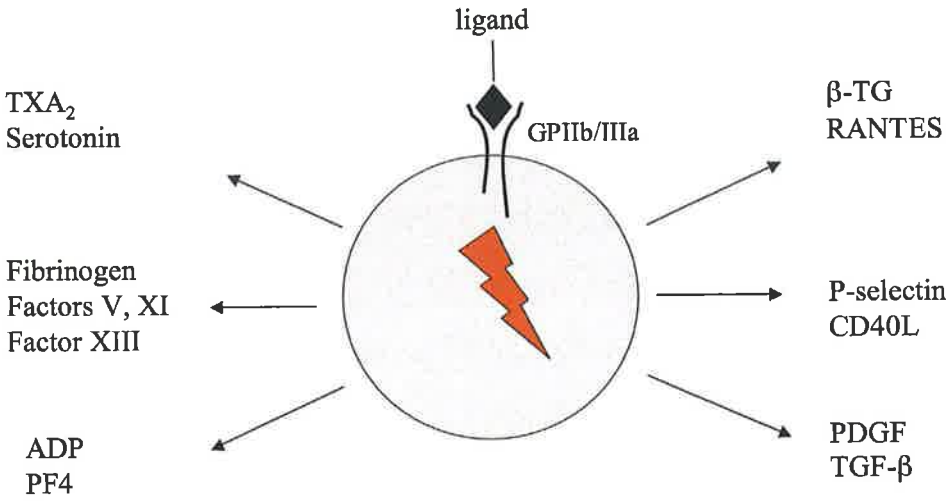

Figure 1.3. "Outside-in" signalling following ligand binding to the GPIIb/IIIa receptor results in platelet activation and release of a large number of humoral factors.

Elevated levels of inflammatory markers such as interleukin (IL)-1, interleukin (IL)-6 and C-reactive protein (CRP) have been linked to increased cardiac event rates and increased mortality in cardiovascular patients. There is also a rise in inflammatory markers following PCI, which has been associated with an increased risk of restenosis [121,122]. The source of this elevation may be the mechanically ruptured plaque and/or areas of myonecrosis generated by platelet micro-emboli. It has been speculated that abciximab, because of its cross-reactivity with the Mac-1

and vitronectin receptor, may have a specific advantage in suppressing inflammation. Evidence so far suggests that both abciximab and eptifibatide, but not tirofiban appear to significantly attenuate the rise of CRP post PCI [123-125]. The rise in inflammatory markers post PCI correlate with a rise in troponin-T [125], suggesting that the bulk of the inflammatory response may be related to myonecrosis rather than ruptured plaque. This suggests that the anti-inflammatory effect of GPIIb/IIIa antagonists in PCI is secondary to their antithrombotic activity rather than differential properties of the agents themselves. The observed failure of tirofiban to influence CRP post PCI may therefore reflect deficiencies in its current dosing schedule. Further support for this is provided by the observation that none of the current antiplatelet agents (aspirin, clopidogrel, GPIIb/IIIa antagonists) prevent platelet induced endothelial cell activation [126].

Pharmacogenetics

Age, concurrent medications, and renal and liver dysfunction are well-characterised factors that can affect the efficacy or toxicity of drug treatment. Inherited factors are also emerging as determinants of the pharmacokinetics and pharmacodynamics of many medications. Pharmacogenetics is that field of science concerned with this hereditary component of drug response. Variations in genes that encode proteins involved in drug metabolism can be important determinants of a patient's response to therapy. Genetic variations in many drug metabolizing enzymes and their phenotypes have been extensively characterised [127,128]. However, genetic variation

in drug transporters and drug targets, as well as genetic heterogeneity underlying the disease being treated can also modulate response to medications [129]. Initially, identification of inherited differences in drug metabolism was based on observations in patients who suffered adverse effects despite being treated with standard doses of a drug [130]. However the focus of research in pharmacogenetics, triggered in part by the human genome project, has shifted to identification of single nucleotide polymorphisms (SNPs) in target genes. Drug trials or disease cohorts provide an opportunity to determine whether such SNPs modify drug response. For example, a variant of the β_2 adrenergic receptor enhances the response to beta-agonist therapy in asthmatics, as the receptor fails to desensitise [131]. Variants in genes that may not be targets for drugs but are involved in the disease may also influence drug response. For example, recent evidence suggests that the angiotensin converting enzyme (ACE) DD genotype modifies the response to β-blocker therapy in heart failure [132]. Of particular importance in platelet biology and pharmacology are its surface receptors. Some of these receptors, including GPIIb/IIIa are highly polymorphic [133]. It is possible that genetic variation in these receptors is a determinant of response to antiplatelet agents. Our primary research interest is in the pharmacogenetics of the PI^{A2} polymorphism of GPIIIa, which has been extensively studied in relation to its effects on platelet function. The role of other polymorphisms in platelet surface proteins has not been as extensively evaluated.

The PI^{A2} Polymorphism

PI^{A2} and Coronary Artery Disease

The GPIIb/IIIa receptor ($\alpha_2\beta_3$) is critical to platelet aggregation and thrombus formation (Figure 1.4). Following platelet activation by agonists such as collagen, ADP, thrombin, thromboxane A_2 and epinephrine the GPIIb/IIIa receptor undergoes a conformational change that allows it to bind fibrinogen and form platelet aggregates [134]. The Platelet Antigen (PI^A) polymorphism results in a leucine (PI^{A1}) to proline (PI^{A2}) substitution at position 33 in the extracellular portion of the IIIa subunit of GPIIb/IIIa [135]. It is responsible for most cases of post transfusion purpura or neonatal alloimmune thrombocytopenic purpura in the Caucasian population, the amino acid substitution causing a conformational change in IIIa and exposure of novel epitopes [133]. The PI^{A2} polymorphism is common in non-Asians with about 20% heterozygous and 2% homozygous for the mutation [136].

Given the importance of the GPIIb/IIIa receptor in platelet aggregation it was postulated that the PI^{A2} polymorphism might also affect thrombotic risk. Weiss et al. in a small case control study showed that the PI^{A2} polymorphism was associated with a 2.8-fold (95% CI 1.2-6.4) increased risk of MI or angina. In patients less than 60 years of age, the association appeared stronger with a relative risk (RR) of 6.2 (95% CI 1.8-22.4)[137]. More than 30 case-control studies have been published since and most have not confirmed the PI^{A2} genotype as a risk factor for coronary thrombosis. There have been two meta-analyses of these studies, the most recent of which suggests a weak association between the PI^{A2} polymorphism and

cardiovascular disease (RR 1.10, 95% CI 1.03 - 1.18) [138,139]. This is not surprising, as any single variant is unlikely to contribute in a major way to the risk of coronary artery thrombosis. Studies of the PI^{A2} polymorphism and coronary thrombosis continue to be published, with one recent study demonstrating an association of PI^{A2} with younger onset of MI by 5.2 yrs ($p = 0.006$) in 264 patients [140]. Confounding related to genetic heterogeneity in different populations or environmental factors may cause conflicting results in genetic association studies

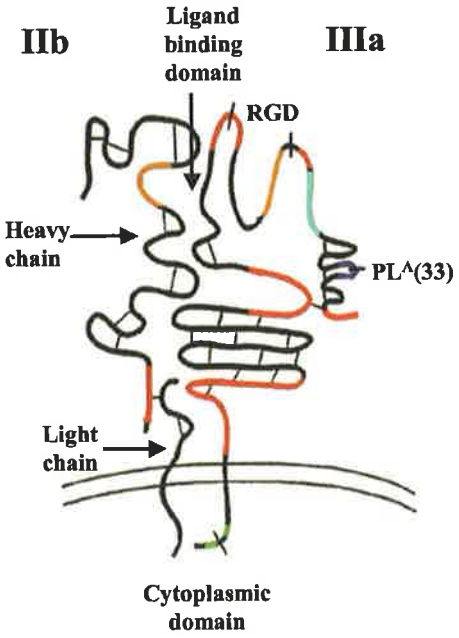

Figure 1.4. The GPIIb/IIIa receptor and PI^{A} polymorphism. The ligand binding site (domain) and RGD binding site are indicated.

of complex diseases. Indeed, there is evidence of an interactions between PI^{A2} and the 4G allele of the plasminogen activator inhibitor (PAI) gene and smoking[136].

PI^{A2} and Platelet Function

Attempts to define the effect of the PI^{A2} polymorphism on platelet function have yielded apparently conflicting results. Although one study has shown an increase in fibrinogen binding in response to ADP [141], others have failed to show an increased affinity of the PI^{A2} receptor for soluble fibrinogen [142-144]. Vijayan et al. have shown increased binding by two cell lines (Chinese Hamster Ovary (CHO) and Human Embryonal Kidney (HEK) 293 cells) expressing the PI^{A2} isoform of GPIIb/IIIa to immobilised but not soluble fibrinogen [143]. However, Bennett et al. did not reproduce this finding in a B lymphocyte cell line [144].

PI^{A2} need not affect affinity of GPIIb/IIIa for its ligand to influence thrombosis. The variant may instead affect thrombus formation by modifying "outside-in" signalling by the receptor. Outside-in signalling describes the process whereby, after ligand binding to GPIIb/IIIa, information is transmitted into the platelet that is required for aggregation [145]. Vijayan et al. have shown increased peripheral F actin content, cell spreading and fibrin clot retraction and a modest increase in $pp125^{FAK}$ phosphorylation, events characteristic of outside-in signalling, in two cell lines expressing PI^{A2} after binding to immobilised fibrinogen [143]. Platelet α-granule release is another consequence of ligand binding to the GPIIb/IIIa receptor. Michelson et al. have shown that $PI^{A1/A2}$ and $PI^{A2/A2}$ platelets express greater

amounts of an α-granule constituent, p-selectin, in a gene-dose manner both at rest and following stimulation with a range of concentrations of ADP [146].

In vitro measurement of platelet aggregation by turbidometric assay is the standard assay in determining the efficacy of antiplatelet drugs. However it detects only larger platelet aggregates and may be relatively insensitive for detecting platelet hyperfunction [147]. Studies assessing the effect of PI^{A2} on platelet aggregation have been most inconsistent. The largest of these, a cohort of 1,422 participants in the Framingham Offspring study, concluded that PI^{A2} was associated with increased platelet reactivity as evidenced by a lower aggregation threshold concentration to epinephrine and ADP [148]. In addition, Andrioli et al. found increased sensitivity of PI^{A2} platelets to thromboxane A_2 [149]. Although one study has reproduced the finding of increased sensitivity to epinephrine in those homozygous for PI^{A2} [146], others have yielded apparently conflicting results. Lasne et al. showed reduced sensitivity to ADP and TRAP in 8 and 14 PI^{A2} carriers respectively [150], and Bennett et al. failed to show any effect of PI^{A2} on TRAP induced aggregation over a range of concentrations of the agonist [144]. Again, confounding related to genetic heterogeneity or environmental factors may explain the conflicting results of PI^{A2} on platelet function [151,152].

PI^{A2} and Aspirin

There is evidence to suggest that PI^{A2} may modulate response to therapy with aspirin. In one study of healthy volunteers a shorter baseline bleeding time and

greater platelet thrombin generation was reported in 26 PI^{A2} carriers versus 54 $PI^{A1/A1}$ controls [153]. Following ingestion of aspirin 300 mg, the bleeding time prolonged by a mean of 46.5 seconds in carriers versus 112.5 seconds in non-carriers, exaggerating a difference found at baseline. Bleeding time was shortened in seven of the 26 carriers versus one of the 54 controls ($p = 0.001$). In addition, this study detected lower levels of prothrombin fragment 1.2 (F1.2), a marker of thrombin generation, at baseline in PI^{A2} carriers versus controls ($p = 0.02$). Platelets are an important contributor to thrombin generation [154]. After treatment with aspirin F1.2 levels showed a mild tendency to increase in carriers and decrease in controls, attenuating the baseline differences between the groups. More recently this group has further characterised the effect of PI^{A2} on thrombin generation in the bleeding-time blood of healthy volunteers before and after aspirin ingestion. By measuring prothrombin and a number of thrombin-mediated events, such as thrombin-antithrombin III production, thrombin β-chain formation, fibrinogen consumption and factor Va generation they showed evidence of enhanced and more rapid thrombin generation in PI^{A2} carriers. Aspirin ingestion resulted in a significant reduction in the velocity of all these processes in $PI^{A1/A1}$ subjects but not in PI^{A2} carriers [155]. Consistent with the effect of the PI^{A2} polymorphism on bleeding time, Cooke et al. observed a decrease in the inhibition of collagen aggregation by aspirin in 30 PI^{A2} carriers versus 30 wildtype controls [156].

Paradoxically, other studies have suggested greater sensitivity to aspirin in those with the PI^{A2} polymorphism when measured by platelet aggregation *in vitro*. Cooke et al. have shown increased inhibition of epinephrine induced platelet aggregation with aspirin in a study of eleven $PI^{A2/A1}$ subjects and fifteen matched $PI^{A1/A1}$ controls [157]. Aggregation in the absence of aspirin was similar in both groups but there was a 10-fold reduction of the IC_{50} for aspirin with $PI^{A2/A1}$ platelets ($p = 0.005$). A subsequent study from the same group confirmed increased inhibition of epinephrine induced platelet aggregation by aspirin in platelets from $PI^{A2/A1}$ subjects, but there was a trend towards an opposite effect in platelets from $PI^{A2/A2}$ subjects [146]. In an analysis of 17 $PI^{A1/A1}$, 13 $PI^{A1/A2}$ and 14 $PI^{A2/A2}$ subjects the IC_{50} for aspirin was lower in heterozygous versus wildtype subjects (7.4 μmol/L vs. 13.1 μmol/L, $p = 0.06$). However the IC_{50} in $PI^{A2/A2}$ subjects was slightly higher (14.0 μmol/L) than in wildtype and significantly higher than in patients heterozygous for PI^{A2} ($p = 0.015$). Another study from Andrioli et al. looked at the effect of acetylsalicylic acid on platelet aggregation in washed platelets after stimulation with arachidonic acid [149]. PI^{A2} homozygotes ($n = 2$) and heteroygotes ($n = 12$) were combined and compared to $PI^{A1/A1}$ controls ($n = 49$). PI^{A2} carriers were significantly more sensitive to inhibition by aspirin, with an IC_{50} of 2.7 ± 0.6 μmol/l and 23.4 ± 3.3 μmol/l for PI^{A2} and $PI^{A1/A1}$ platelets respectively ($p < 0.005$).

These apparently conflicting results using different assays of platelet function make it difficult to determine what effect aspirin may have on platelet inhibition in those with the PI^{A2} polymorphism. The bleeding time test suffers from poor reproducibility

and is sensitive to a host of factors not related to platelet function [158]. *In vitro* platelet aggregation is more sensitive and specific in detecting changes in platelet function and for example will detect changes in platelet reactivity in response to GPIIb/IIIa antagonists at much lower drug concentrations [159]. Prothrombin binds to both activated and resting GPIIb/IIIa. It has been suggested that the reduced number of occupied GPIIb/IIIa receptors resulting from enhanced inhibition of platelet aggregation by aspirin may allow greater prothrombin binding to the platelet surface and subsequent thrombin generation [160]. This theory could explain the contradictory outcome from bleeding time tests and *in vitro* aggregation studies in PI^{A2} carriers on aspirin. However, only a prospective study with clinical endpoints will determine whether PI^{A2} has an effect on the response to aspirin.

PI^{A2} and Glycoprotein IIb/IIIa Antagonists

Although GPIIb/IIIa antagonists are effective antiplatelet agents in the setting of PCI and ACS when administered as intravenous infusions, attempts to provide prolonged GPIIb/IIIa blockade with oral antagonists has met with failure and may even increase the risk of thrombotic events [101].

The PI^{A2} polymorphism does not appear to alter the affinity of the receptor for small molecule GPIIb/IIIa antagonists. The K_i for RGDS has been shown to be similar in $PI^{A1/A1}$ and $PI^{A1/A2}$ platelets [144]. Similarly a peptidomimetic GPIIb/IIIa antagonist RWJ 53308, modeled on the KQAGD sequence of the carboxyl terminus of the fibrinogen γ chain, shows similar binding to the receptor, regardless of PI^{A2} allotype.

In addition, RWJ 53308 showed similar inhibition of TRAP stimulated platelet aggregation at various concentrations of the antagonist in $PI^{A1/A1}$, $PI^{A1/A2}$ and $PI^{A2/A2}$ platelets [144]. However, in a separate study of 23 PI^{A2} carriers (20 $PI^{A1/A2}$, 3 $PI^{A2/A2}$) and 24 $PI^{A1/A1}$ controls another GPIIb/IIIa antagonist modeled on KGD, eptifibatide, appeared to be less effective in inhibiting platelet aggregation in those with the polymorphism [161]. Eptifibatide inhibited both ADP (60 and 100 nM) and collagen (40 nM) induced aggregation less strongly in PI^{A2} carriers with IC_{50} values of 115 nM versus 67nM ($p < 0.05$) and 141 nM versus 110 nM ($p < 0.05$) respectively. Similarly in a study of abciximab in 66 $PI^{A1/A1}$ and 21 $PI^{A1/A2}$ patients undergoing PCI there was less inhibition of platelet aggregation in PI^{A2} carriers as assessed by the Ultegra RPFA device and optical aggregometry to ADP (20 μM) [162]. In contrast, another study determined that abciximab was more effective in inhibiting ADP induced platelet aggregation in PI^{A2} carriers [146]. In an analysis of 20 $PI^{A1/A1}$, 20 $PI^{A1/A2}$ and 15 $PI^{A2/A2}$ patients, there was a trend towards an enhanced inhibitory response to abciximab at therapeutic concentrations in $PI^{A1/A2}$ compared to $PI^{A1/A1}$ and $PI^{A2/A2}$ patients ($p = 0.099$). The inhibitory effect was similar in $PI^{A1/A1}$ and $PI^{A2/A2}$ subjects.

Two sub-studies of oral GPIIb/IIIa antagonist trials suggest that PI^{A2} may interfere with the response to these agents. In a substudy of SYMPHONY (TIMI 12) 98 patients on sibrafiban were analysed for an effect of PI^{A} genotype on platelet aggregability and events [163]. Although there was no significant difference in platelet

inhibition as measured by ADP-induced aggregation, recurrent MI occurred more commonly in the PI^{A2} group (7.4% versus 0%, p = 0.02).

In OPUS, a clinical trial of the oral GPIIb/IIIa antagonist orbofiban, 10,288 patients from 29 countries were recruited within 72 hours of an acute coronary syndrome and randomised to one of three groups: (1) orbofiban 50mg twice daily for 30 days followed by 30 mg twice daily, (2) orbofiban 50 mg twice daily or (3) placebo [164]. A subgroup of 1,014 patients, the majority of whom were from the USA and Canada donated a genetic sample at a mean of 157 days. These patients were genotyped for the PI^{A2} polymorphism and its effect on response to orbofiban was examined [165]. The rate of MI was significantly higher among all PI^{A2} carriers (RR = 2.71, 95% CI 1.37 - 5.38, p = 0.004). When PI^{A2} carriers were broken down by treatment, the relative risk of a recurrent MI in those on orbofiban and placebo were 4.27 and 1.04 respectively (p < 0.001). Of those randomised to treatment with orbofiban, PI^{A2} carriers had a more than fourfold increased risk of MI versus non carriers (2.46 vs 0.59, p = 0.08). In addition, while there was an expected, dose-dependent excess of risk of bleeding events in the non-carriers on treatment, PI^{A2} carriers showed no increased bleeding risk on treatment. The relative risk of a bleeding event on orbofiban was 1.87 (95% CI 1.29 - 2.71) in PI^{A2} non-carriers versus 0.87 (95% CI 0.46 -1.64) in PI^{A2} carriers (p = 0.05). The results of this substudy indicate a reduced response to orbofiban or even negative interaction between drug and PI^{A2} genotype. A possible explanation for these results is that PI^{A2} carriers may have a greater propensity to partial agonism when treated with GPIIb/IIIa antagonists [118].

In the setting of coronary artery disease where there is enhanced platelet activation, GPIIb/IIIa antagonists may trigger 'outside-in' signalling and so cause further platelet activation [166]. If plasma drug levels are too low to prevent fibrinogen binding (which occurs with the oral administration of these agents), this enhanced platelet activation may ultimately lead to thrombus formation and increased clinical events.

PI^{A2} and Other Antiplatelet Drugs

The thienopyridines, ticlopidine and clopidogrel inhibit platelet activation by ADP by acting as antagonists of the $P2Y_{12}$ purinergic receptor [167]. Clopidogrel is at least as effective as aspirin in reducing recurrent vascular events in high-risk patients [168]. The CURE study demonstrated that combination therapy with aspirin and clopidogrel was superior to aspirin alone in patients with unstable angina or non-ST-elevation MI [99]. The COMMIT and CLARITY studies confirmed the benefits of combination therapy in ST-elevation MI also [2,3]. However the CHARISMA study demonstrated no benefit of adding clopidogrel to aspirin therapy in lower risk patients [4]. Variability in the antithrombotic response to clopidogrel has been observed in a number of smaller studies. In a study of 150 patients undergoing PCI, clopidogrel resistance, defined as a less than 10% difference between baseline and post-treatment aggregation to 5 μM ADP, was present in 31% and 15% of patients at 5 and 30 days, respectively [169]. Clopidogrel resistance appears to be related to aspirin resistance, possibly reflecting higher baseline platelet

reactivity in certain patients that is not responsive to drug therapy. Lev et al. found that in a population of coronary artery disease patients undergoing PCI almost 50% of the aspirin resistant patients were also clopidogrel resistant. Elevation of CK-MB after stenting occurred more frequently in aspirin-resistant versus aspirin-sensitive patients (38.9% vs. 18.3%; p = 0.04) and in clopidogrel-resistant versus clopidogrel-sensitive patients (32.4% vs. 17.3%; p = 0.06)[170]. In another study of patients undergoing PCI, clopidogrel resistance was linked to a higher risk of ischaemic events [171]. More recent studies have pointed to the importance of genetic variation in CYP2C19, one of the hepatic enzymes responsible for conversion of clopidogrel to its active metabolite, in determining response to clopidogrel. In the EXCELSIOR, TRITON-TIMI 38 trials and a large French registry, patients with reduced-function CYP2C19 alleles demonstrated decreased platelet inhibition to clopidogrel as well as a significant increases in the risk of death, MI, stroke and stent thrombosis [172-174]. Increasing the dose of clopidogrel results in greater platelet inhibition in patients with CYP2C19 loss of function polymorphic variants [175]. One small prospective study of 162 patients undergoing PCI has shown improved outcome with adjustment of clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation (VASP) index [176]. However the role of platelet function or genetic testing to guide clopidogrel therapy remains unproven and is not endorsed by the most recent AHA/ACC advisory [177]. The antiplatelet efficacy of clopidogrel is also potentially affected by other drugs which are metabolized by CYP3A4. Initially a concern with statins, a post hoc

analysis of the CHARISMA study showed no evidence for a clinically important interaction between clopidogrel and the CYP3A4 metabolised statins, atorvastatin, lovastatin, and simvastatin [178]. More recent attention has focused on a possible interaction with proton pump inhibitors (PPIs) leading to the FDA and European Medicines Agency (EMEA) recommending avoidance of PPIs in patients treated with clopidogrel in March and July 2009 respectively. Although laboratory and observational studies have suggested reduced antiplatelet efficacy and clinical benefit when clopidogrel is co-prescribed with PPIs and omeprazole in particular, data from a post hoc analysis of the TRITON-TIMI 38 study and the COGENT randomized controlled trial indicate that cotherapy of PPIs, including omeprazole, with clopidogrel appears to be safe [179,180]. Prasugrel may be an alternative treatment in patients where there is concern for reduced response to clopidogrel. The newer thienopyridine, prasugrel, appears to be more predictable in its efficacy. Studies so far show greater and more consistent inhibition of platelet function [181,182]. In addition prasugrel does not appear to be affected by genetic variation in CYP2C19 [183].

Although the majority of studies have not shown an effect of the PI^{A2} polymorphism on the antiplatelet effect of clopidogrel [156,184,185], one study showed less platelet inhibition by clopidogrel in PI^{A2} carriers manifested as a higher degree of GPIIb/IIIa activation and p-selectin expression [186], and yet another suggested more platelet inhibition with clopidogrel in PI^{A2} carriers when measured by the bleeding time test, PFA-100 and thrombin generation at the site of microvascular injury [187]. These

discrepant results are likely due to small sample sizes, genetic heterogeneity, or confounding environmental factors.

Other genetic variants have been tested for effects on the antiplatelet response to clopidogrel. Three variants in cytochrome (CY) P450-3A, which is responsible for conversion of clopidogrel to its active metabolite, have been implicated in modulating the antithrombotic effect of clopidogrel [188-190], one of which was associated with a higher incidence of atherothrombotic events 6 months after PCI in 348 patients [190]. Genetic variation in the thienopyridine drug target, the $P2Y_{12}$ receptor, has not been found to affect the response to clopidogrel [184,191,192]. The 807T polymorphism of the collagen receptor GPIa associated with decreased response to collagen but not ADP induced aggregation during clopidogrel therapy [193].

Other antiplatelet drugs that are in use or undergoing development have not been investigated for pharmacogenetic interactions. The most clinically relevant example is dipyridamole, an antiplatelet agent that has been shown to be particularly effective in combination with aspirin in the secondary prevention of stroke [194]. Its mechanism of action includes inhibition of phosphodiesterase and inhibition of adenosine reuptake in platelets [195]. *In vitro*, dipyridamole reduces platelet adhesion, aggregation and platelet factor IV availability with plasma concentrations of greater than 3.5 μmol/l [196]. The plasma concentration of dipyridamole may vary as much as ten fold between individuals [197], but no predictors of this variation have

been described and no pharmacogenetic studies targeting dipyridamole have been conducted.

Linkage Disequilibrium and Haplotype Analysis

During meiosis there is some recombination or crossing over of DNA when the chromosomal pairs separate, so that alleles in the same chromosome can go to different cells. Alleles or genes that lie close to each other are less likely to have recombination between them and are therefore more likely to be inherited together. The association of two or more loci on a chromosome with limited recombination between is defined as linkage. Linkage disequilibrium (LD) describes a situation in which a combination of alleles occurs more frequently in a population than would be expected based on the individual allele frequencies. For example if alleles A and B have frequencies of 10% and 20% in a population respectively and their combination, AB, has a frequency of 5% (compared to an expected frequency of 2% (0.1 x 0.2)), then they are in linkage disequilibrium. If a new genetic mutation arises in a chromosome, it becomes associated with the other alleles or polymorphisms present in that chromosome. Although these associations will be diluted by meiosis over successive generations, the association will remain stronger than predicted by the prevalence of these polymorphisms in the general population. The degree of LD is affected primarily by population dynamics and non-random mating. It is particularly common and extends over greater chromosomal distances in homogenous or founder populations [198,199]. LD is exploited for genome

wide mapping of disease genes [200] and is particularly powerful in homogenous populations as illustrated by the identification of the gene responsible for diastrophic dysplasia in a Finnish population [201,202]. However, the non-random association between different alleles that occurs with linkage disequilibrium is a possible confounder in association studies of single nucleotide polymorphisms with complex diseases or traits. Detection of an association between an SNP and a disease may be erroneous due to linkage between that SNP to another variant. For example, a synonymous SNP (i.e., no change in the encoded amino acid) in exon 26 (C3435T) of the human multidrug-resistance gene has been associated with variable expression of P-glycoprotein in the duodenum and higher bioavailability of digoxin [203,204]. However, the C3435T SNP is in linkage disequilibrium with a non-synonymous SNP (i.e., one causing an amino acid change) in exon 21 (G2677T) [205]. Because of the association between these two SNPs, it is unclear whether the C3435T polymorphism is truly of functional importance or is simply linked with the causative polymorphism in exon 21. To further complicate matters, the G2677T SNP has been associated with enhanced P-glycoprotein function in vitro and lower plasma fexofenadine concentrations in humans, effects opposite to those reported with digoxin [205].

The phenomenon of linkage disequilibrium results in groups of alleles or haplotypes that tend to be maintained over successive generations and may segregate with a disease gene.

Although the number of SNPs in the human genome is vast (> 2 million) the number of existing haplotypes is far smaller because of LD. This diminishes the potential variation in the human genome and will allow smaller sample sizes than predicted by the number of SNPs [206]. In addition, haplotype association studies limit the confounding effects of linkage disequilibrium and heterogenous populations. The HapMap project (www.hapmap.org) is an international collaboration to define common haplotypes in the human genome that will allow more accurate and almost fully capture the genetic variation in a population. An example of the benefits of haplotype analysis is seen in the case of the β_2 adrenergic receptor, coded by the ADRB2 gene. 13 different SNPs have been identified in ADRB2, suggesting 8192 (2^{13}) potential haplotypes. However in a study of 77 patients, only 12 of the 8192 possible ADRB2 haplotypes were actually observed. The bronchodilator response to inhaled β-agonist therapy in patients with asthma revealed a stronger association between bronchodilator response and haplotype than between bronchodilator response and any SNP alone [207].

Conclusion

There is interindividual variation in the response to antiplatelet drugs, with evidence for environmental and inherited causes. Of particular interest to us is the PI^{A2} variant of GPIIIa, which appears to modulate the response to aspirin and GPIIb/IIIa antagonists, although studies report conflicting findings. Contradictory results may reflect the small size of the populations tested, as well as genetic heterogeneity,

and confounding environmental factors. A potential mechanism for a modifying role of the PI^{A2} polymorphism is the reported association between it and enhanced partial agonism to GPIIb/IIIa antagonists [118]. It is also possible that PI^{A2} is in linkage with functional polymorphisms close to the GPIIIa gene and is simply a marker of another variant. Indeed, the PI^{A2} polymorphism is in linkage disequilibrium with promoter polymorphisms of GPIIIa, which have been reported to determine the expression of GPIIb/IIIa on the platelet surface [208]. In the future individually tailored therapy with antiplatelet therapy may be a reality. But first larger studies are necessary to explore this issue and to define the role of environmental and genetic factors.

~ Chapter 2 ~

COX-Dependent and Independent Determinants of Aspirin Response in Patients with Stable Cardiovascular Disease

Introduction

Despite the advent of newer agents, aspirin remains the mainstay of antiplatelet therapy. It reduces the risk of recurrent cardiovascular events by approximately 25% [1]. However, as already discussed in Chapter 1, as many as 20% of patients suffer recurrent vascular events over long term follow up despite treatment with aspirin [1]. Interindividual variation in response to aspirin may explain some treatment failures and has led to the concept of "aspirin resistance" [23]. However, "aspirin resistance" is both poorly defined and controversial [24,25]. Assessment of interindividual variability in response to aspirin may involve either cyclooxygenase (COX)-dependent or cyclooxygenase (COX)-independent assays. Pharmacokinetic tests are not useful in evaluation of aspirin metabolism, due to the fact that its primary site of action is in the enterohepatic circulation, where it is rapidly metabolised to the inactive product salicylate. COX-dependent tests of aspirin measure inhibition of platelet cyclooxygenase-1, the enzyme that converts arachidonic acid to the platelet agonist thromboxane A_2. TXA_2 is rapidly metabolised in the blood to the stable metabolite TXB_2 and subsequently excreted in the urine as 11-dehydro-TXB_2. Levels of urinary 11-dehydro-TXB_2 represent

systemic production of TXA_2, which is largely platelet derived, but may also reflect TXA_2 generated by other tissues [33,34]. Persistent production of platelet thromboxane may be due to inadequate dosing or concomitant therapy with NSAIDs such as ibuprofen [38,39]. Incomplete inhibition of platelet COX activity has been linked to higher risk of clinical events [53,54]. Although incomplete inhibition of cyclooxygenase by aspirin may be the best way to define "aspirin resistance", there has also been much interest in identifying patients with high platelet reactivity despite complete inhibition of COX-1. COX- independent assays of "aspirin resistance" include platelet aggregometry, flow cytometric measurement of platelet activation markers and automated assays such as the PFA-100 [45]. COX-independent mechanisms of non-response to aspirin may represent enhanced signalling through alternative pathways of platelet activation that are not dependent on TXA_2 or generation of products such as isoprostanes that mimic thromboxane but are generated independently of COX [43,44].

In this study we examined variability in the platelet response to aspirin using the PFA-100 in patients with coronary artery disease. A key hypothesis was that phenotypic or genetic variation in the platelet adhesive receptors (GPIIb/IIIa, GPIb, GPIa/IIa), including the PI^{A2} variant of GPIIIa, contributed to aspirin resistance. In contrast to many previously published studies, the analyses were designed to discriminate between COX-dependent and COX-independent response to aspirin after exclusion of non-compliant patients.

Methods

Study Population

The protocol was approved by the institutional ethics committees and all of the subjects gave written informed consent. Men and women age 21 years and older were recruited consecutively while attending cardiology outpatient clinics in two hospitals in Dublin, Ireland between March and September 2002. Patients were suitable for inclusion in the study if they had a documented history of an acute coronary syndrome or stable cardiovascular disease confirmed by coronary angiography or noninvasive testing and were currently being treated with aspirin 75 - 300 mg once daily. Those with a recent history (within 6 weeks) of an acute coronary syndrome, coronary intervention or major surgery were ineligible. Patients with a personal or family history of bleeding disorders, a platelet count outside the normal range (< 150,000/μl or > 450,000/μl), haemoglobin < 8 g/dl, or current treatment (within 2 weeks) with another antiplatelet drug, anticoagulant or NSAID were excluded. 208 patients met the inclusion criteria and consented to take part in the study. 8 patients were excluded from the analysis because the wrong anticoagulant was inadvertently used for PFA-100 blood collection (3.2% vs 3.8% citrate). One patient was excluded because they were found to be on < 75 mg aspirin per day after study enrollment. Therefore complete data was available for 199 patients. 50 patients from the original cohort attended for further blood testing.

Study Design

The study design is outlined in figure 2.1. Blood was drawn from participants at two different time points. On recruitment (phase I) we analysed the interindividual variation to aspirin using the PFA-100 and serum TXB_2 and identified a group of 30 aspirin non-responders based on the PFA-100 results. In phase II, we analysed platelet function in more detail in 25 of 30 aspirin non-responders who agreed to further testing and 25 age and sex matched aspirin responder controls. All patients were recalled within 3 - 13 months of initial sampling. In addition to repeat PFA-100 and serum TXB_2 measurements, we measured platelet aggregation and flow cytometry for expression of platelet activation markers and adhesive receptors. A urine sample was collected for measurement of urinary thromboxane metabolites and the isoprostane (iP) $F_{2\alpha}$-III.

Serum Thromboxane B_2

Whole blood was allowed to clot in a non-siliconised glass tube at 37°C for 1 hr. Serum was separated by centrifugation at 1000 g for 10 minutes and stored at 80°C. Serum TXB_2 levels were later measured by enzyme immunoassay (R&D Systems, Abingdon, U.K.).

Platelet Function Analyser (PFA) -100

The PFA-100 is an automated device in which the process of platelet adhesion and platelet aggregation is simulated *in vitro* [114]. Blood was collected by vacutainer into

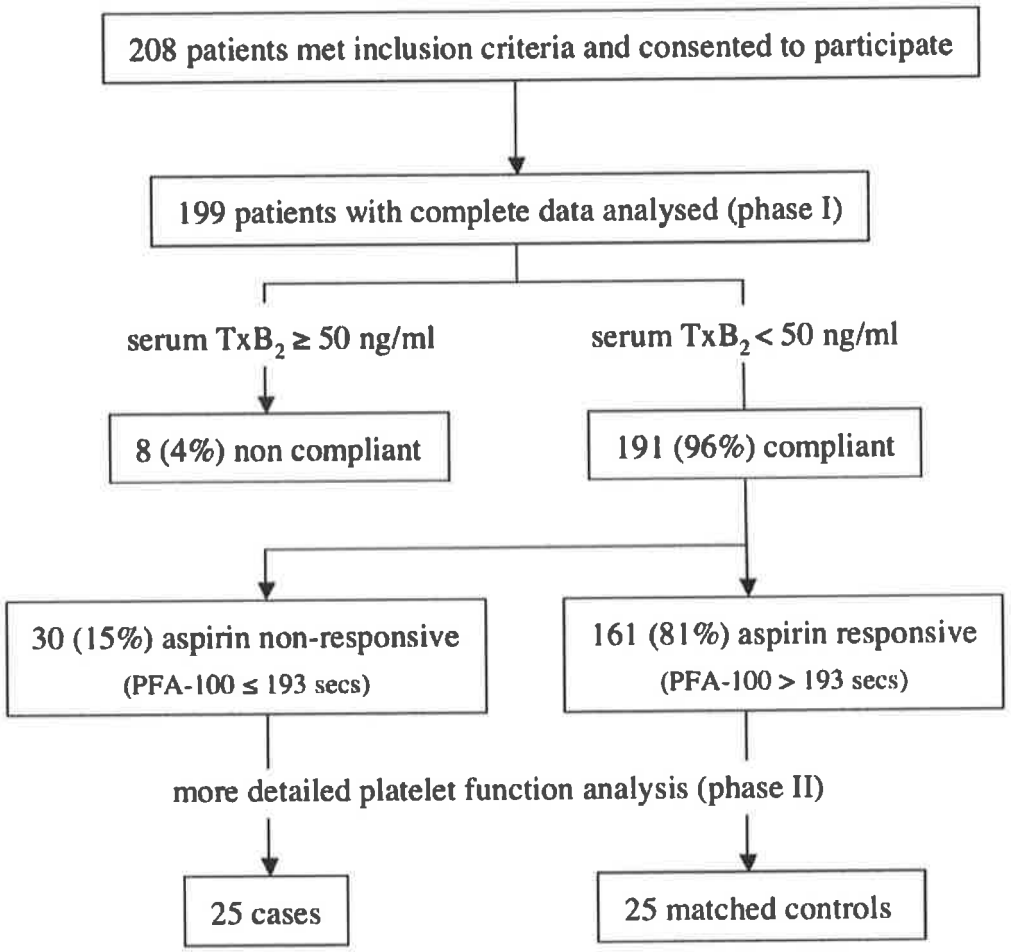

Figure 2.1 Study design and summary of results for phase 1.

sodium citrate 3.8%. Blood was gently mixed by inversion before testing. All samples were analysed in duplicate. 800 µl of anticoagulated whole blood was then dispensed and drawn at high shear through a capillary at the end of which is a membrane with a central aperture (Fig 2.2).

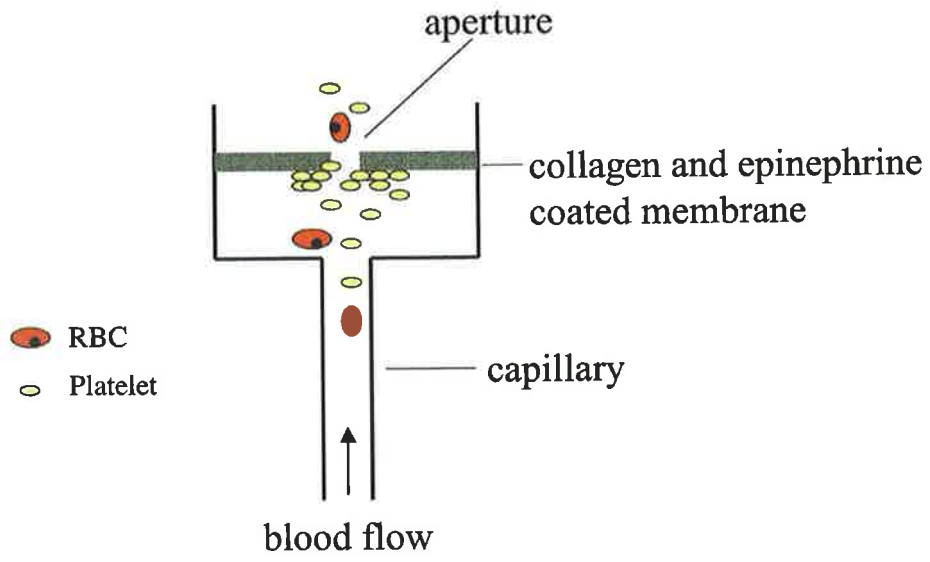

Figure 2.2. Cartoon of the PFA-100 cartridge, which simulates in vitro the process of platelet aggregation and adhesion in whole blood at the site of vascular injury. The membrane is coated with collagen and additionally infused with either ADP or epinephrine. In this study we used the membrane coated with collagen and epinephrine (CEPI cartridge). The time to occlusion ("Closure Time") of the membrane aperture by a platelet plug is automatically measured and reflects baseline platelet function or exposure to platelet inhibiting agents. The maximum closure time reading is ≥ 300 seconds.

Platelet Light Transmission Aggregometry

Platelet aggregation studies were performed within 2 hours of blood collection in 3.2% citrate. Whole blood was centrifuged at 150 g for 10 minutes and platelet rich plasma (PRP) was collected. The remaining blood was spun at 2,500 g for 5

minutes to obtain platelet poor plasma (PPP). Platelet aggregation was determined following addition of arachidonic acid (1.6 mM) and threshold concentrations of fibrillar collagen (0.5 μg/ml) and epinephrine (5 μM) to PRP at 37°C by light transmission (Biodata PAP-4, Biodata Corporation, Horsham, PA, USA). Aggregation was not adjusted for the platelet count of each sample due to the rapidity of analysis.

Marker of Platelet Activation

Platelet activation was quantified by measuring surface expression of the platelet activation marker CD62p (P-selectin). 5 μl whole blood was incubated with 20 μl PE labeled CD62p or isotype antibody (Becton Dickinson) for 20 minutes at room temperature. A similar assay was performed following addition of TRAP (20 μM) to whole blood. Samples were diluted with 2 ml of Biocytex buffer after incubation and then analysed by flow cytometry at 488 nm excitation.

Quantitative Receptor Expression

GPIIb/IIIa, GPIb and GPIa/IIa receptor numbers were analysed using a quantitative assay (Platelet GP screen kit, Biocytex, Marseille, France). 20 μl whole blood, after 1 in 4 dilution in Biocytex buffer, was incubated with 20 μl anti-GPIIIa (p18) or anti-GPIb (SZ2) or anti-GPIa antibody (10 μg/ml) at room temperature for 20 minutes. Antibody binding was determined using fluorescein isothyocyanate (FITC) labeled $F(ab)_2$ fragments of human Ig absorbed, sheep anti-mouse IgG (H+L) antibodies.

Samples were diluted with 2 ml of Biocytex buffer after 10 minutes incubation and then analysed by flow cytometry at 488 nm excitation.

Flow Cytometry

Platelet populations were gated according to their forward and side light scatter. Histograms were generated using 10,000 counts and geometric mean fluorescence or percentage of platelets positive for CD62p was calculated using the CELLQUEST software of the FACScalibur system (Becton Dickinson, Oxford, UK). The binding of an isotypic control antibody was taken as non-specific binding and subtracted from the observed geometric mean fluorescence.

Calibration beads, consisting of a mixture of four different populations of 2 μm diameter latex beads, each with a different defined amount of murine antibody per bead, were used to estimate the number of GPIIb/IIIa, GPIb and GPIa/IIa receptors per platelet. The singlet bead populations were gated according to their forward and side scatter. Histograms of the geometric mean fluorescence intensity of 10,000 events were recorded and used to plot a log-log graph of the mean fluorescence intensity versus the number of antibodies attached to each bead. The number of platelet-bound antibodies was estimated from this graph on the basis of the geometric mean fluorescence intensity of the sample. After subtraction of nonspecific binding and assuming mono-valent binding, the number of specifically bound antibody molecules was taken as the number of the relevant glycoprotein receptors per platelet.

Urinary 11-dehydro TXB_2 and $iPF_{2\alpha}$-III

Urine was collected and stored at -20°C. The principle urinary metabolite of TXA_2 (11-dehydro-TXB_2) and the isoprostane $iPF_{2\alpha}$-III were measured by liquid chromatography coupled to a triple quadripole mass spectrometer (LC-MSMS) (Sciex 3000. Perkin Elmer, CA, USA). Briefly, urine (2 ml) was spiked with authentic deuterated internal standards for both metabolites. Following solid phase extraction, the samples were dried down and reconstituted in mobile phase. Quantitation was performed using MRM (Multiple Reaction Monitoring) operated in negative ion electrospray mode. The MRM transitions were 367 > 305 and 353 > 193 for endogenous thromboxane and isoprostane, respectively. Similarly MRM transitions for the tetradeuterated internal standards were 371 > 309 and 357 > 197. Results were corrected for creatinine excretion and reported as pg/mg creatinine.

Genotyping

Following DNA extraction [209] genotyping for PI^{A2} was performed by restriction digest. A 266 base pair (bp) segment of DNA containing the PI^{A} polymorphism site was initially amplified by the Polymerase Chain Reaction (PCR) using the following primers: 5' TTC TGA TTG CTG GAC TTC TCT T 3' and 5' TCT CTC CCC ATG GCA AAG AGT 3'. The PCR protocol consisted of an initial denaturation step of 94°C for 5 minutes, followed by the amplification cycle (94°C for 60 seconds, 57°C

for 90 seconds and 72°C for 90 seconds). The amplification cycle was repeated a total of 35 times before a final extension step of 72°C for 10 minutes.

The 266 bp fragment was then digested by the Msp1 restriction enzyme when incubated together at 37°C for 4 hours. PI^{A2} introduces a second Msp1 restriction digest site, such that in patients who have the $PI^{A1/A1}$ genotype 221 and 45 bp fragments are generated versus 177 and 46 and 45 bp fragments in patients with the $PI^{A2/A2}$ genotype and 221, 177, 46 and 45 bp fragments with the $PI^{A2/A1}$ genotype. These fragments were detected after electrophoresis of digestion products in a 3% agarose gel stained with 1 mg/ml ethidium bromide (Figure 2.3).

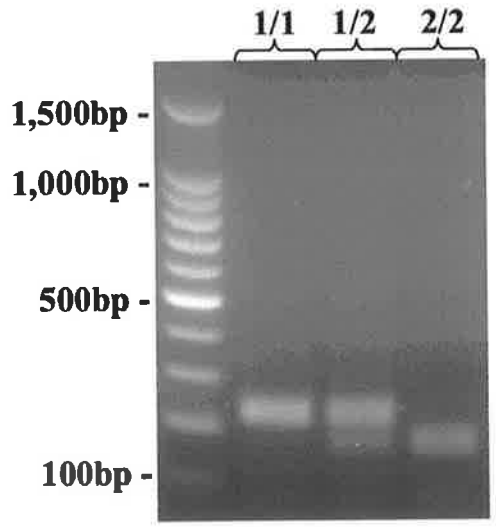

Figure 2.3. Electrophoresis gel showing patient samples with the $PI^{A1/A1}$ (1/1), $PI^{A1/A2}$ (1/2), and $PI^{A2/A2}$ (2/2) genotypes.

Statistical Analysis

Data are presented as counts and proportions or medians and interquartile ranges (IQR). Clinical characteristics were compared using a two-sided chi-square test for categorical variables. For continuous variables we used the unpaired t test. A value of $p < 0.05$ in the two-tailed test was considered statistically significant. Correlation between PFA-100 measurements and serum TXB_2 was performed using Spearman's rank correlation. Platelet function data was compared using the nonparametric Mann-Whitney test. A value of $p < 0.05$ was again considered statistically significant. Correction for multiple comparisons between aspirin responders and non-responders was peformed using the false discovery rate procedure [210].

Results

Phase I

172 (86%) of the 199 patients analysed were treated with aspirin 75 mg once daily and the remainder with doses of 100 to 300 mg daily. We used serum levels of TXB_2 as a surrogate for aspirin compliance. In 24 non-aspirinated healthy volunteers (mean age 26 ± 6 yrs, 25% male) median serum TXB_2 was 294 ng/ml (IQR 215 - 390 ng/ml). The lowest serum TXB_2 measured 67 ng/ml. We therefore defined a serum TXB_2 value ≥ 50 ng/ml as likely to indicate aspirin non-compliance. The normal closure time on the PFA-100 of 95 to 193 seconds was confirmed in the same group of untreated healthy volunteers. Aspirin non-responsiveness with

the PFA-100 was therefore defined as a closure time ≤ 193 seconds despite a serum TXB_2 level < 50 ng/ml. Using this definition 8 (4%) patients were non-compliant, 161 (81%) were responsive to aspirin, and 30 (15%) patients were non-responsive (Figure 2.1). There were no significant clinical predictors of aspirin non-responsiveness as measured by the PFA-100 on univariate analysis (Table 2.1).

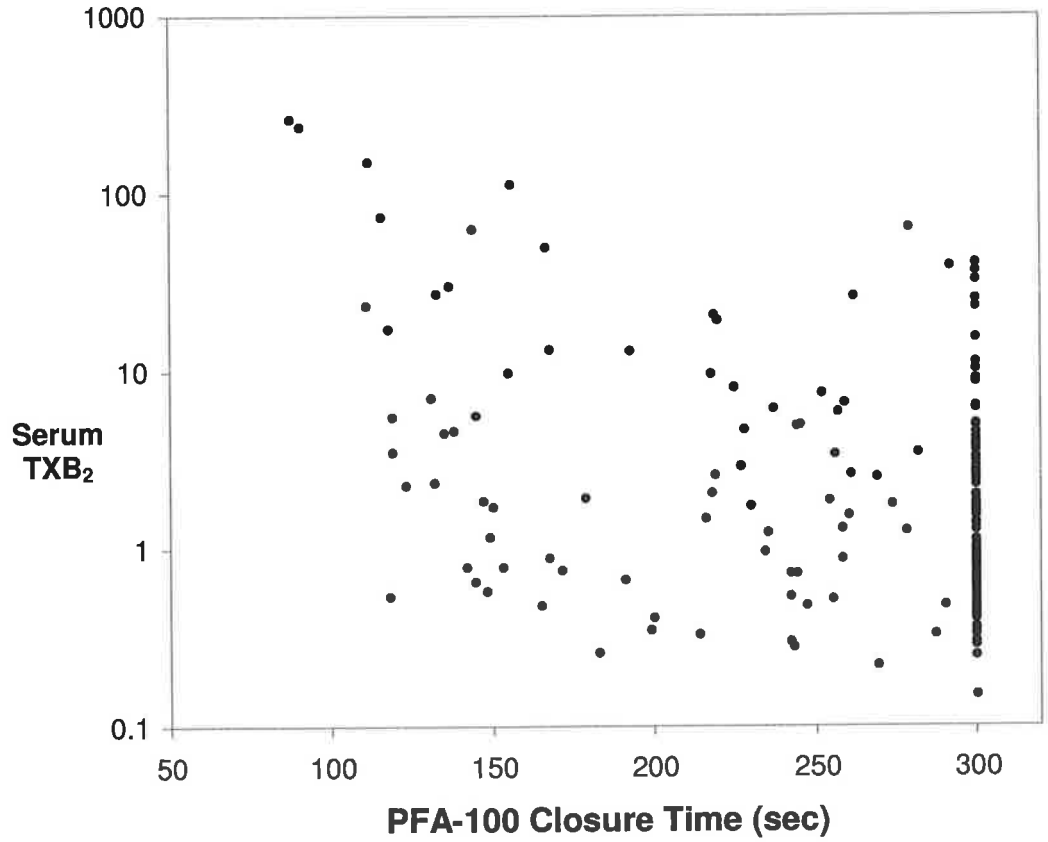

Figure 2.4. Graph of PFA-100 closure time versus serum TXB_2. There is weak correlation between PFA-100 measurements and serum TXB_2, r = -0.2836, 95% CI - 0.4104 to - 0.1460, p < 0.0001.

Table 2.1. Clinical characteristics in PFA-100 defined aspirin responders and non-responders. EC = enteric coated aspirin.

Characteristics	Aspirin Responder (n = 161)	Aspirin Non- Responder (n = 30)	Significance
Mean age (years)	62.4	63.5	p = 0.57
Gender (male)	108 (67%)	21 (70%)	p = 0.83
EC Aspirin 75 mg	134 (83%)	26 (86%)	p = 0.79
Smoking	28 (17%)	8 (26%)	p = 0.31
Diabetes Mellitus	8 (5%)	3 (10%)	p = 0.38
Hypercholesterolaemia	122 (76%)	24 (80%)	p = 0.82
Hypertension	87 (54%)	14 (47%)	p = 0.55
MI	63 (39%)	15 (50%)	p = 0.31
PCI	42 (26%)	7 (23%)	p = 0.82
CABG	46 (29%)	6 (20%)	p = 0.38
β Blockers	88 (55%)	19 (63%)	p = 0.55
ACE inhibitors/ARBS	60 (37%)	16 (53%)	p = 0.11
Calcium Blockers	47 (30%)	8 (27%)	p = 0.83
Nitrates	38 (24%)	6 (20%)	p = 0.81
Statins	109 (69%)	20 (67%)	p = 0.83

There was a weak correlation between PFA-100 closure times and serum TXB_2 levels in all patients, r = - 0.2836, 95% confidence interval (CI) - 0.4104 to - 0.1460,

$p < 0.0001$ (Figure 2.4). Although substantially inhibited in both groups, median levels of serum TXB_2 were higher in aspirin non-responders compared to aspirin responders (2.32 versus 1.24 ng/ml, $p = 0.02$). We have previously shown that serum TXB_2 levels of > 2.2 ng/ml are associated with suboptimal inhibition of platelet COX-1 by aspirin [40]. 16 (53%) of the 30 aspirin non-responders had TxB_2 levels > 2.2 ng/ml consistent with suboptimal suppression of COX-1 compared to 54 (27%) of the 161 aspirin responders ($p = 0.06$).

Phase II

25 aspirin non-responders and 25 age and sex matched aspirin-responsive controls from the original cohort returned 3 to 13 months later for more detailed platelet function analysis. The results are summarised in Table 2.2. We repeated the PFA-100 closure time and serum TXB_2 levels in all 50 patients. A short closure time was reproduced in 40% of the original aspirin non-responder cases versus 4% of controls ($p < 0.05$). There was no difference between repeat serum TXB_2 levels in aspirin non-responders versus responders (median 1.03 vs 1.11 ng/ml, p = NS). Although repeat serum TXB_2 levels were lower on repeat testing compared to initial values in aspirin non-responders, this difference was also not statistically significant (1.03 vs 2.27 ng/ml, p = NS). Arachidonic acid aggregation (> 20%) did not occur more frequently in aspirin non-responders than responders (8 vs 4, p = NS). Similarly, there was no difference in aggregation to collagen in cases versus

Table 2.2. Platelet function data in matched cases (PFA-100 aspirin non-responders) and controls in phase II. Data expressed as median (25th, 75th centile). Data available for * 22 cases and 20 controls, ‡ 24 cases and 22 controls.

	Cases (n = 25)	Controls (n = 25)	P value
Initial PFA-100 (sec)	147 (123,167.5)	300 (300, 300)	
Repeat PFA-100 (sec)	300 (176, 300)	300 (300, 300)	$p < 0.05$
Initial Serum TxB_2 (ng/ml)	2.27 (0.89, 5.63)	1.29 (0.67, 2.76)	NS
Repeat Serum TxB_2 (ng/ml)	1.03 (0.63, 2.85)	1.11 (0.66,2.33)	NS
Arachidonic Acid aggregation (%)	6 (4, 64)	6 (4, 9)	NS
Epinephrine aggregation (%)	38 (24, 69)	23 (15, 31)	$p < 0.05$
Collagen aggregation (%)	8 (6, 12)	6.5 (5.5, 9.3)	NS
P-selectin (% platelets positive)	17 (6, 27)	10 (5, 16)	NS
P-selectin TRAP (% platelets positive)	66 (61, 72)	56 (50, 65)	$p < 0.05$
GPIIb/IIIa receptors (number)	55020 (50704, 60585)	49403 (43666, 55158)	NS
GPIb receptors (number)	39780 (37788, 44087)	36858 (34829, 40139)	NS
GPIa/IIa receptors (number)	4058 (3683, 5194)	4234 (3091, 6435)	NS
urinary 11-dehydro-TxB_2* (pg/mg creatinine)	141 (99, 236)	117 (79, 227)	NS
urinary $iPF_{2\alpha}$-III ‡ (pg/mg creatinine)	475 (296, 646)	521 (304, 848)	NS

PI^{A2} controls. However, aggregation to epinephrine was significantly higher in cases (median level of aggregation 38% vs 23%, $p < 0.05$).

The percentage of stimulated platelets expressing the activation marker and adhesive ligand, p-selectin was higher in aspirin non-responders than controls (median 66% vs 56%, $p < 0.05$) (Table 2.2). Although there was a trend towards higher median levels of GPIIb/IIIa (55020 vs 49403) and GPIb (39780 vs 36858) receptors expressed per platelet, these differences were not statistically significant after correction for multiple comparisons. There was no difference in the levels of the collagen receptor GPIa/IIa between groups (median 4058 vs 4234, p = NS). Activation of the TP receptor by non-platelet derived thromboxane or isoprostanes may result in platelet activation despite inhibition of COX-1. However there were no differences in urinary levels of 11-dehydro-TXB_2 and $iPF_{2\alpha}$-III between cases and controls (Table 2.2).

Genotyping for PI^{A2} was successful in 194 of 199 patients. In the remaining 5 patients the quality of DNA was inadequate for PCR amplification and restriction digest analysis. The wildtype $PI^{A1/A1}$ genotype was present in 149 (77%) patients. 43 (22%) were heterozygous for PI^{A2}, and 2 (1%) were homozygous for PI^{A2}. 5 (17%) of 30 non-responders were PI^{A2} carriers versus 42 (26%) of 161 of aspirin responders (p = 0.36). There was no difference in PFA-100 closure times in PI^{A2} heterozygotes (median 300, IQR 237 - 300 secs) or homozygotes (median 300, IQR 300-300 secs) compared to wildtype controls (median 300, IQR 231 - 300 secs). Similarly there was no difference in TXB_2 generation in PI^{A2} heterozygotes

(median 1.27, IQR 0.48 - 5.0 ng/ml) or homozygotes (median 7.22, IQR 4.2 - 10.24 ng/ml) compared to wildtype controls (median 1.36, IQR 0.66 - 3.79 ng/ml).

Discussion

In this study 15% of patients with stable cardiovascular disease had continuing platelet reactivity as measured by the PFA-100, despite treatment with aspirin. Incomplete suppression of COX-1 and TXB_2 generation appears to contribute to some, but not all of the PFA-100 measured aspirin non-responsive cases. Almost half (47%) of the original 30 patients with short PFA-100 closure times demonstrated serum TXB_2 levels $\leq$ 2.2 ng/dl, a threshold which may indicate optimal inhibition of platelet cyclooxygenase-1 by aspirin [40]. The presence of enhanced platelet reactivity despite adequate inhibition of cyclooxygenase-1 in these patients suggests that COX independent as well as COX-dependent mechanisms are responsible for the observed variability in the antiplatelet response to aspirin. Although we did not test whether increasing the aspirin dose would convert some aspirin non-responders to responders, this has been observed in other studies [46,48,49]. In fact improved aspirin compliance may have contributed to the high number of patients (60%) who converted from aspirin non-responder to responder on repeat PFA-100 testing in the second phase of the study. Consistent with this are the lower levels, albeit not statistically significant, of serum TXB_2 seen in aspirin non-responders on repeat testing.

Comprehensive repeat testing of platelet function was performed in aspirin non-responders and matched responder controls to determine the precise mechanisms and relative importance of COX dependent and independent factors associated with PFA-100 measured aspirin resistance. On repeat testing there was no significant difference in serum TXB_2 levels or platelet aggregation to arachidonic acid between aspirin non-responders and controls suggesting equivalent inhibition of cyclooxygenase by aspirin in both groups. Moreover, the urinary excretion of the predominant thromboxane metabolite, 11-dehydro-TxB_2 was similar irrespective of the PFA-100 closure time, suggesting an equivalent *in vivo* effect of aspirin. However, we found evidence for increased activity of COX independent pathways in aspirin non-responder patients detected by the PFA-100. Firstly, there was enhanced sensitivity to epinephrine in aspirin non-responders. This is not that surprising as there is a coating of epinephrine on the collagen membrane in the PFA-100 cartridge. Nevertheless epinephrine sensitivity may be an important predictor of the observed variability in the response to aspirin therapy. There is evidence that epinephrine and thromboxane work in synergy to induce aggregation in aspirin treated platelets [211]. A recent study showed that detection of increased sensitivity to low dose epinephrine is reproducible (ICC = 0.81) and present in 14% of healthy volunteers [50]. In addition, increased sensitivity to epinephrine has been detected in patients presenting with acute coronary syndromes [212]. Platelet hypersensitivity to low-dose epinephrine may be heritable and associated with a common G-protein spliced variant [213].

Secondly, there was increased activation of platelets in aspirin non-responder patients as evidenced by the higher levels of platelet surface p-selectin expression after stimulation with TRAP. P-selectin is expressed on the platelet surface following platelet activation. It plays a critical role in haemostasis, binding polymorphonuclear leukocytes and attracting procoagulant microparticles to developing thrombus [214]. TRAP is a peptide fragment of the protease-activated receptor 1 (PAR-1) for thrombin, and is used experimentally to mimic the effects of thrombin on platelets without causing fibrin clot formation. In addition to directly causing platelet activation and aggregation, thrombin and TRAP induce platelet production of arachidonic acid [215]. Increased platelet activation has previously been observed in aspirin treated patients despite significant cyclooxygenase inhibition and was attenuated in those treated with clopidogrel, suggesting involvement of an ADP-dependent pathway [31]. Although this study does not identify involvement of a specific platelet activation pathway, it is consistent with up-regulation of a COX-independent pathway in certain patients.

Activation of the platelet surface receptors is the final step in platelet adhesion and aggregation. The expression of the platelet collagen receptor GPIa/IIa varies up to ten-fold in normal individuals [216]. The presence of a collagen coated membrane in the PFA-100 had led us to speculate initially that this marked variation in the expression of the platelet GPIa/IIa would affect the closure time and explain many cases of enhanced platelet reactivity with this device. However there was no difference between expression of the collagen GPIa/IIa receptor or collagen

aggregometry in cases versus controls in this study. We measured the levels of two other platelet receptors, GPIIb/IIIa and GPIb in the study population, which vary up to two-fold in the normal population [217]. GPIIb/IIIa is the platelet fibrinogen receptor and mediates platelet aggregation and thrombus formation [218]. The GPIb receptor is responsible for platelet adhesion to the vessel wall in high shear conditions. It binds collagen indirectly through von Willebrand factor (vWF) [219]. There was a non-significant increase in the platelet expression of GPIIb/IIIa and GPIb receptors in aspirin non-responders.

We had also speculated that activation of the thromboxane (TP) receptor by non-platelet derived thromboxane or isoprostanes might explain some cases of non-responsiveness to aspirin. But levels of their urinary metabolites were similar in aspirin responsive and non-responsive individuals. In addition, we did not observe an association between the PI^{A2} polymorphism and aspirin resistance.

Seven of the aspirin non-responder group and seven of the control group had TXB_2 levels > 2.2 ng/ml on repeat testing in phase II. If these patients had been excluded from the analysis, epinephrine induced platelet aggregation (median 29 vs 23, p = 0.048) and activated p-selectin expression (median 67.5 vs 55.5, p = 0.002) would have remained significantly higher in cases versus controls and there would have been a non-significant trend towards increased GPIIb/IIIa (median 57641 versus 48726, p=0.08), and GPIb (39771 vs 36727, p=0.15) receptor expression. This is consistent with the data from phase I of the study suggesting that there is up-

regulation of COX independent pathways in a proportion of patients with PFA-100 defined aspirin non-responsiveness resulting in increased platelet activation.

Of concern is the large intra individual variation between initial and repeat PFA-100 closure times. 15 (60%) of the 25 patients who were initially aspirin non-responders became responders in phase II, and 1 (4%) of the 25 patients who was initially a responder became a non-responder. Therefore 32% of patients changed status on repeat testing. An intraindividual variation of 9% has been reported for the PFA-100 [220]. Although one study reported a lower cross-over rate from aspirin non-responder to responder (10% over several months), another study reported a high cross-over from aspirin non-responder to responder (74% at 1 year) [221, Poulsen, 2007 #295]. As already discussed, it is possible that improved compliance in Phase II of our study explains some of this difference: bloods were drawn at the time of recruitment in the initial screening phase, whereas patients were alerted several days prior to their second visit and reminded to take their aspirin. Consistent with this are the lower measurements, albeit not statistically significant, of serum TXB_2 seen in aspirin non-responders on repeat testing. However, it is also likely that aspirin non-responsiveness is a dynamic phenomenon and the factors influencing it may be similar to those for platelet aggregation, including diet, time of day, exercise, and intercurrent infection [222-224]. In addition, significant variability in PFA-100 closure times have been observed related to disposable cartridge batch, time of blood draw, and vWF levels [225].

There are a number of limitations to this study. Firstly we used the PFA-100 for assessment of aspirin resistance. Although there is no agreed definition of aspirin resistance, there is mounting consensus that it is best characterized by demonstration of incomplete inhibition of COX by aspirin. The PFA-100 is a COX-independent assay and therefore does not specifically measure the antiplatelet effects of aspirin, as demonstrated by the poor correlation between PFA-100 and serum TXB2 in this and other studies [47 226]. In addition, as we did not measure PFA-100 closure times off aspirin therapy in this study, it is possible that the aspirin non-responders identified also had increased platelet reactivity off therapy that was not entirely reversible with aspirin. Indeed this may underlie the observed weak correlation between serum TXB_2 levels and PFA-100 readings (Figure 2.3). Another possible criticism of this study is the somewhat arbitrary definition of aspirin non-compliance in this study. Our cut-off for serum $TXB_2 \geq 50$ ng/ml may have resulted in an underestimation of the number of non-compliant patients. Data from healthy volunteers suggests that a level of approximately 50 ng/ml discriminates well between aspirin use and non-use [36,227]. Measurement of salicylate levels are a more established measure of aspirin compliance, although this is also problematic because of overlap between salicylate levels in aspirin treated and non-aspirin treated individuals [228]. Finally, there was inconsistency in the preparation and doses of aspirin taken by participants in the study. Nevertheless, the majority (84%) of all patients were treated with enteric-coated aspirin 75 mg daily, with similar proportions in the aspirin responder and non-

responder groups. Although the high proportion of complete suppression of TXB_2 in the aspirin non-responder group suggests that higher doses of aspirin would be unlikely to prolong PFA-100 closure times in all of these patients, further testing of PFA-100 closure times and platelet activation in aspirin non-responders on higher doses of aspirin would be required to determine this with absolute certainty.

Conclusion

Aspirin non-responsiveness as defined by the PFA-100 assay was observed in 15% of stable cardiovascular disease patients. Although approximately half of non-responders appeared to have suboptimal inhibition of platelet COX-1 by aspirin, the other half demonstrated complete suppression, suggesting that both inadequate aspirin dosing and COX independent pathways of platelet activation are responsible for aspirin non-responsiveness detected by the PFA-100. Neither endogenous ligands of the TP receptor or fibrillar collagen mediated platelet activation were associated with aspirin non-responsiveness. However, aspirin non-responsiveness measured with the PFA-100 was associated with increased platelet sensitivity to epinephrine and increased platelet activation.

~ Chapter 3 ~

Platelet Inhibition During Tirofiban Treatment in Patients with Acute Coronary Syndromes

Introduction

GPIIb/IIIa antagonists decrease cardiovascular events and mortality in patients with acute coronary syndromes (ACS) undergoing percutaneous intervention (PCI) [77,78]. However their efficacy in patients with ACS who do not undergo PCI is uncertain despite 6 large scale randomized trials [6]. Whereas treatment with eptifibatide and tirofiban has been associated with modest reductions in recurrent cardiac events in this population, abciximab showed no benefit versus placebo in the GUSTO IV-ACS trial [92,96,97]. As with any drug, proper dosing is critical to the efficacy of the GPIIb/IIIa anatagonists and it is possible that differences in the levels of platelet inhibition achieved by different dosing regimens is responsible, at least in part, for the differences observed between the agents and in different settings. Early studies of GPIIb/IIIa antagonists suggested that greater than 80% receptor occupancy, with nearly complete inhibition of platelet aggregation is necessary to prevent platelet-induced coronary thrombosis [103,104]. The importance of higher levels of platelet inhibition with GPIIb/IIIa antagonists during PCI was confirmed in the GOLD trial [112]. This study measured the levels of platelet inhibition in patients treated with abciximab during PCI and confirmed that higher levels of platelet inhibition were

associated with lower levels of adverse cardiac outcomes. Similarly the 3 to 4 times higher dose of eptifibatide used in the ESPRIT study was likely responsible for the observed improvement in outcome compared to the earlier IMPACT-II trial (significant 35% risk reduction vs non-significant 19% risk reduction) [72,74].

In PRISM-PLUS, treatment with tirofiban in ACS patients who did not routinely undergo PCI significantly reduced the risk of death, MI or refractory ischaemia at 30 days and 6 months by 17% and 14% respectively [96]. The same dosing schedule in patients undergoing PCI is associated with a modest and statistically non-significant 16% reduction in ischaemic endpoints at 30 days [73]. Recent pharmacodynamic studies of tirofiban have suggested that platelet inhibition in the setting of PCI is suboptimal, particularly in the first 6 hours of treatment [106-109].

This study was designed to address the platelet inhibitory effects of a prolonged infusion of tirofiban in an ACS population undergoing initial conservative management. In addition to determining whether there was adequate inhibition of platelet aggregation we also assessed for the effect of tirofiban on platelet activation. In order to determine the differential effects of varying durations of tirofiban treatment on platelet function, patients were randomised to 24, 48, 72 and 96 hour infusions.

Methods

Study Population

Between February 2001 and November 2002, 21 patients were enrolled in this single centre study at St. James's Hospital, Dublin. The protocol was reviewed and approved by the Irish Medicines Board and the institutional Ethics Committee. All of the subjects gave written informed consent. Patients were suitable for enrolment if they were aged between 21 and 80 years and had a clinical diagnosis of unstable angina or non-ST-segment-elevation MI based on chest pain of at least 10 minutes at rest in the previous 12 hours, plus one of the following: ST depression > 1mm in at least 2 ECG leads, elevated troponin T (> 0.1 ng/ml), or confirmed diagnosis of coronary artery disease on previous coronary angiography.

Exclusion criteria for participation in the study included planned percutaneous coronary intervention within 5 days of enrollment; administration of clopidogrel or ticlopidine; administration of a GPIIb/IIIa antagonist in the previous 35 days; active internal bleeding or history of hemorrhagic diathesis; major surgery or serious trauma within the previous 6 weeks; gastrointestinal or genitourinary bleeding of clinical significance within the previous 6 weeks; history of cerebrovascular accident within the previous 2 years or any cerebrovascular accident with a residual neurological deficit; administration of oral anticoagulants within the previous 7 days unless prothrombin time ≤ 1.2 times control (or international normalized ratio (INR) ≤ 1.4), or ongoing treatment with oral anticoagulants; platelet count < 100,000 cells/μl; serum creatinine value greater than 220 μmol/l;

intracranial neoplasm, arteriovenous malformation, or aneurysm; confirmed hypertension with systolic blood pressure > 180 mmHg or diastolic blood pressure > 100 mmHg; participation in another clinical research study involving the evaluation of another investigational drug or device within 16 weeks prior to enrollment.

Study Design

Patients were pre-randomised to 4 different durations of treatment with tirofiban (24, 48, 72 and 96 hours) using the randomisation function in excel. Tirofiban was administered as a bolus dose of 10 µg/kg over 3 minutes followed by a maintenance infusion of 0.1 µg/kg/min. All patients received aspirin 150 to 300 mg daily. Heparin was co-administered to achieve a target APPTr of 2.0. All other treatment was at the discretion of the attending physician.

Blood samples were taken at baseline and 6, 24, 48, 72 and 96 hours following infusion commencement. 9 parts blood was collected into 1 part sodium citrate 3.2%. Later in the study when we learnt of the potential for calcium chelation with citrate anticoagulant to cause overestimation of the inhibitory effect of tirofiban we acquired duplicate blood samples in PPACK anticoagulant, a direct thrombin inhibitor, in 3 patients. Platelet function was measured at baseline, 6, 24, 48, 72 and 96 hours.

Receptor Occupancy

GPIIb/IIIa receptor number and occupancy were quantified by flow cytometry using the GPIIb/IIIa receptor occupancy kit (Biocytex, Marseille, France), which contains the anti-GPIIIa monoclonal antibodies mAb1 (clone LYP18), mAb2 (clone 4F8), isotypic control antibody, and calibration beads [229]. As previously described, mAb1 and mAb2 bind to GPIIb/IIIa and are differentially displaced by abciximab and small molecular weight antagonists [230]. These antibodies may therefore be used to monitor receptor number and occupancy during administration of a GPIIb/IIIa antagonist. Samples were immuno-stained and fixed in 1% formaldehyde within 2 hours of blood collection and stored at 4°C. Antibody binding was quantified by flow cytometry (FACScalibur; Becton Dickinson, Oxford, UK) within 24 hours of staining at 488 nm excitation [230]. Platelet and calibration bead populations were gated according to their forward and side light scatter. Histograms were generated using 10,000 counts and geometric mean fluorescence was calculated using the CELLQUEST software of the FACScan system (Becton Dickinson). The binding of an isotypic control antibody was taken as non-specific binding and was subtracted from the observed geometric mean fluorescence. Calibration beads, consisting of a mixture of 4 different populations of 2 μm diameter latex beads, each with a different defined amount of murine antibody per bead, were used to estimate the number of antibodies bound per platelet. Histograms of the geometric mean fluorescence intensity of 10,000 events were recorded and used to plot a log-log graph of the mean fluorescence intensity versus the number of antibodies attached

to each bead. The number of platelet-bound mAb1 and mAb2 molecules was estimated from this graph on the basis of the geometric mean fluorescence intensity of the sample. After subtraction of non-specific binding and assuming monovalent binding, the number of specifically bound antibody molecules was taken as the number of bound sites for either mAb1 or mAb2. Receptor occupancy was calculated using the equation: (baseline mAb2 sites - time point mAb2 sites)/(baseline mAb2 sites) x 100.

Platelet Light Transmission Aggregometry

Platelet aggregation studies were performed within 2 hours of blood sampling as previously described in the methods section of Chapter 2. Briefly, platelet aggregation was determined following the addition of ADP 20 μM to PRP at 37°C by light transmission (Biodata PAP-4, Biodata Corporation). Aggregation was not adjusted for the platelet count of each sample; however, the aggregation at the different time points on drug treatment was expressed as a percentage of baseline aggregation, before the administration of the study drug.

Platelet Function Analyser -100

PFA-100 analysis was performed as previously described in the methods section of Chapter 2. Samples were analysed within 4 hours of blood collection. Testing was performed with the collagen-ADP cartridge (normal range 71-118 seconds). Results were expressed as percentage prolongation of the baseline closure time

with a maximal closure time of ≥ 300 seconds taken as 100% prolongation (prolongation (%) = [(closure time-baseline closure time) / (300-baseline closure time)] x 100).

Platelet Activation Markers

Platelet activation was quantified by measuring the expression of platelet surface activation markers CD62p (p-selectin) and CD63 by flow cytometry as previously described in the methods section of Chapter 2. Briefly, 20 µl monoclonal antibodies to CD62, CD63, or isotype (Becton Dickinson) were incubated with. 5 µl whole blood for 20 minutes at room temperature. A similar tube was also prepared except for the addition of TRAP (5 µM) to whole blood 10 minutes prior to the addition of CD62p or CD63 or isotype. Immunostained samples were fixed in 1% formaldehyde within 2 hours of blood collection, and stored at 4°C. Flow cytometric analysis of the fixed sample was then performed within 24 hours at 488 nm excitation.

Genotyping

Following DNA extraction [209] genotyping for PI^{A2} was performed by restriction digest as previously described in the methods section of Chapter 2. Briefly, a 266 bp segment of DNA containing the PI^{A} polymorphism site was initially amplified by PCR. The 266 bp fragment was then digested by the Msp1 restriction enzyme. PI^{A2}

introduces a second Msp1 restriction digest site that permits discrimination of PI^{A2} carriers versus wildtype controls.

Statistical Analysis

Data are presented as medians and interquartile ranges (IQR). Data was compared using the non-parametric Wilcoxon rank sum test for unmatched pairs and Wilcoxon matched-pairs signed-ranks test for matched pairs. Where more than 2 values were compared the Kruskal-Wallis nonparametric one-way analysis of variance was performed. A two sided p value of < 0.05 was considered significant. Correlation was calculated using Spearman's non-parametric rank correlation coefficient. Statistical analysis and generation of box plots was performed using JMP Statistical Discovery Software from SAS.

Results

Of the 21 patients who took part in the study, 6 were randomised to the tirofiban 24-hour infusion arm and 5 each to the 48, 72 and 96-hour treatment groups. The patients' background characteristics are presented in Table 3.1. There were no major bleeding events. 2 patients were lost to follow up at 30 days. The 30 day event rate for the other 19 patients was 42% and consisted exclusively of percutaneous coronary intervention procedures that were performed during the index hospitalisation.

Table 3.1. Baseline characteristics of study patients.

	Number
Male Gender	16 (76%)
Mean Age (years)	57.9 ± 11.0
Mean Weight (kg)	84.0 ± 14.1
Hypertension	8 (38%)
Hyperlipidaemia	12 (57%)
Current Smoker	6 (29%)
Diabetes	4 (19%)
Troponin T +ve	8 (38%)
ECG changes (ST shift only)	8 (38%)

Receptor Occupancy

Receptor occupancy levels were complete for 64 of 72 (89%) sampling times during tirofiban infusion. Median receptor occupancy on treatment was 80% (IQR 73 - 85.75%). Receptor occupancy levels of 80% or greater were recorded at 34 of 64 (53%) sampling times during treatment. Two thirds of patients (14/21) had receptor occupancy levels less than 80% at some time during their tirofiban infusion. There was no difference between the level of receptor occupancy at 6, 24, 48, 72 and 96 hrs, $p = 0.58$ (Figure 3.1). The number and proportion of patients with $\geq 50\%$, $\geq 80\%$ and $\geq 90\%$ receptor occupancy at the different timepoints are presented in Table 3.2.

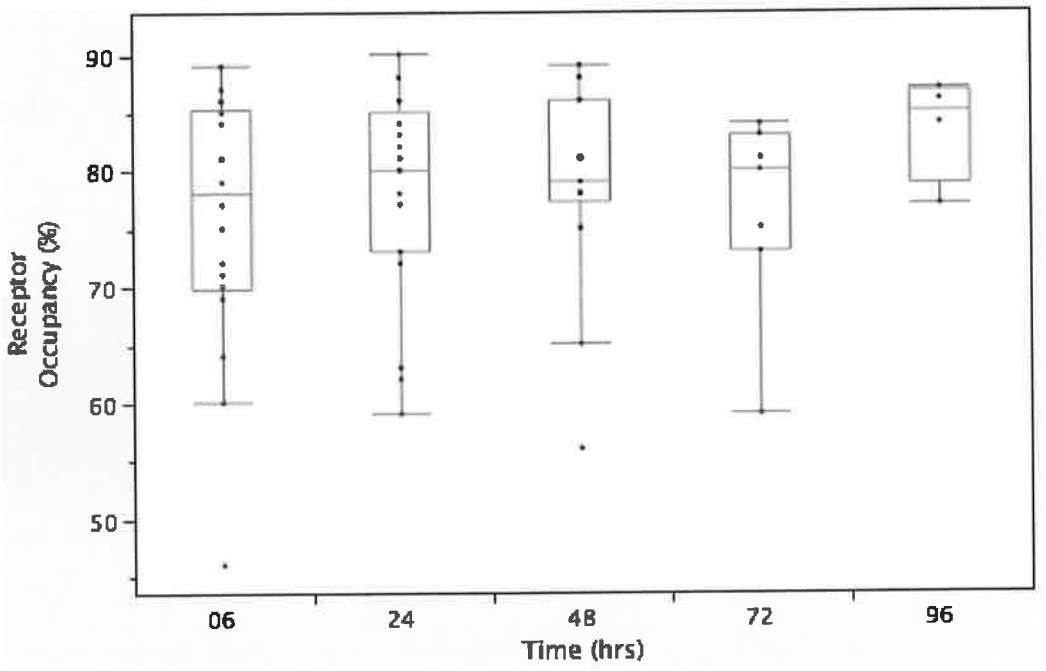

Figure 3.1. Box plots illustrate receptor occupancy at 6, 24, 48, 72 and 96 hrs. The boxes define the interquartile range (IQR) with the median indicated by the crossbars. The whiskers extend from the ends of the box to the outermost data point that falls within the distances computed: upper quartile + 1.5*(IQR), lower quartile - 1.5*(IQR).

Table 3.2. Level of inhibition measured by receptor occupancy.

	Level of receptor occupancy		
Timepoint	≥ 50%	≥ 80%	≥ 90%
6 hrs (n = 18)	17 (94%)	8 (44%)	0 (0%)
24 hrs (n = 21)	21 (100%)	13 (62%)	1 (5%)
48 hrs (n = 14)	14 (100%)	6 (43%)	0 (0%)
72 hrs (n = 7)	7 (100%)	4 (50%)	0 (0%)
96 hrs (n = 4)	4 (100%)	3 (75%)	0 (0%)
Total (n = 64)	63 (98%)	34 (53%)	1 (2%)

Platelet Aggregometry

Platelet aggregation data was complete for 67 of 72 sampling times during tirofiban infusion. Median inhibition of ADP-induced platelet aggregation was 81% (IQR 74.75 - 90%). Inhibition of aggregation levels of 80% or greater were recorded at 44 of 66 (67%) sampling times during treatment. Two thirds of patients (14/21) had levels less than 80% at some time during the tirofiban infusion. Median inhibition of platelet aggregation was greater at later time points during the infusion, p = 0.04 (Figure 3.2), and measured 81% (IQR 76 -90%) at 6 hrs, 80% (IQR 67 -83.5%) at 24 hrs, 82.5% (IQR 75.25 - 87.5) at 48 hrs, 87.5% (IQR 80 - 94%) at 72 hrs, and 90% (IQR 82.25 - 94.75%) at 96 hrs. In pairwise comparisons of the different time points, inhibition of platelet aggregation was higher at 72 and 96 hours than at 24 hours (p = 0.02 for both). Although there was a trend towards a decrease in

inhibition of platelet aggregation from 6 to 24 hours, this difference was not statistically significant (p = 0.14). The number and proportion of patients with ≥ 50%, ≥ 80% and ≥ 90% inhibition of platelet aggregation at the different timepoints are presented in Table 3.3.

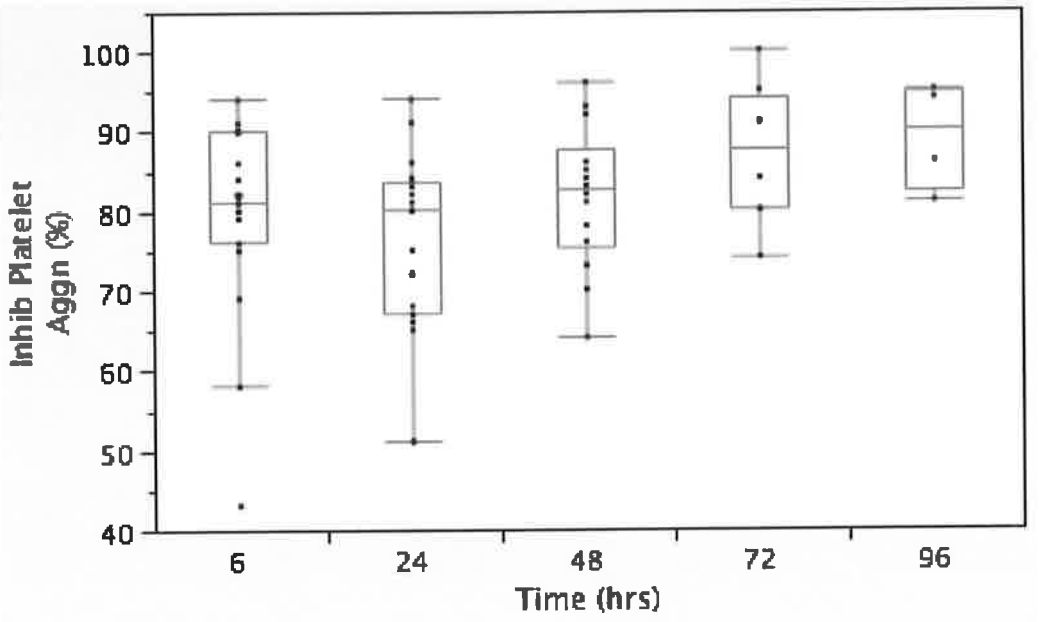

Figure 3.2. Box plots illustrate inhibition of platelet aggregation to ADP at 6, 24, 48, 72 and 96 hrs. Inhibition of platelet aggregation was higher at 72 and 96 hours than at 24 hours (p = 0.02 for both).

Correlation Between Receptor Occupancy and Aggregometry

Although both receptor occupancy and platelet aggregometry both detected lower levels of platelet inhibition at many timepoints, these assays showed poor agreement for identification of suboptimal platelet inhibition with tirofiban (Figure 3.3). There was a weak and non-significant correlation between receptor occupancy and inhibition of aggregation to ADP ($r = -0.17$, $p = 0.1835$).

Table 3.3. Level of inhibition measured by platelet aggregometry.

	Level of inhibition of platelet aggregation to ADP 20μM		
Timepoint	≥ 50%	≥ 80%	≥ 90%
6 hrs (n = 19)	18 (95%)	13 (68%)	6 (32%)
24 hrs (n = 21)	21 (100%)	11 (52%)	2 (10%)
48 hrs (n = 14)	14 (100%)	9 (64%)	3 (21%)
72 hrs (n = 8)	8 (100%)	7 (86%)	4 (43%)
96 hrs (n = 4)	4 (100%)	4 (100%)	2 (33%)
Total (n = 66)	65 (98%)	44 (67%)	17 (26%)

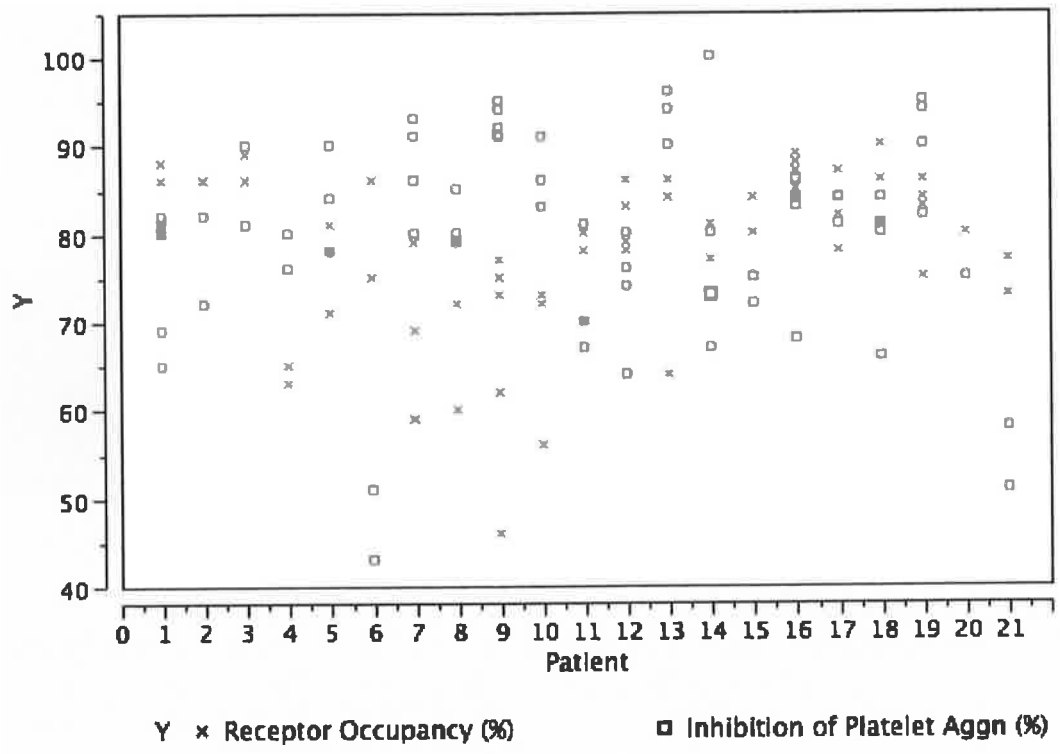

Figure 3.3. Receptor occupancy and inhibition of platelet aggregation plotted by patient.

Interindividual Variation in Receptor Occupancy and Aggregometry

There was significant interindividual variation in the level of platelet inhibition achieved as measured by receptor occupancy ($p = 0.0017$) and inhibition of platelet aggregation ($p = 0.0014$) (Figure 3.3). However there was discrepancy between the two assays in predicting which patients had a poor response to treatment, as predicted by the absence of correlation between them as described above. Some patients (e.g. patients 4, 7, 8, 9, 10) with lower levels of receptor occupancy had

higher levels of inhibition to ADP-induced platelet aggregation, whereas others (e.g. patients 1, 2, 6, 12, 15, and 21) with lower levels of inhibition of platelet aggregation had high levels of receptor occupancy. Both interindividual variation detected by receptor occupancy and inhibition of platelet aggregation were not related to patients baseline clinical characteristics or PI^{A2} genotype (18 of 21 typed, 5 PI^{A2} carriers). However, the study was insufficiently powered for this analysis.

PFA-100

PFA-100 data was complete for 59 of 72 sampling times during tirofiban infusion. Median inhibition measured on the PFA-100 was 100% (IQR 95 - 100%). 53 of 59 sampling times on treatment showed 100% inhibition of the closure time. Inhibition of closure times on the PFA-100 correlated with inhibition of ADP-induced platelet aggregation during tirofiban treatment ($r = 0.46$, $p = 0.0002$), reaching maximum values between 60 to 70% inhibition of ADP-induced platelet aggregation (Fig. 3.4).

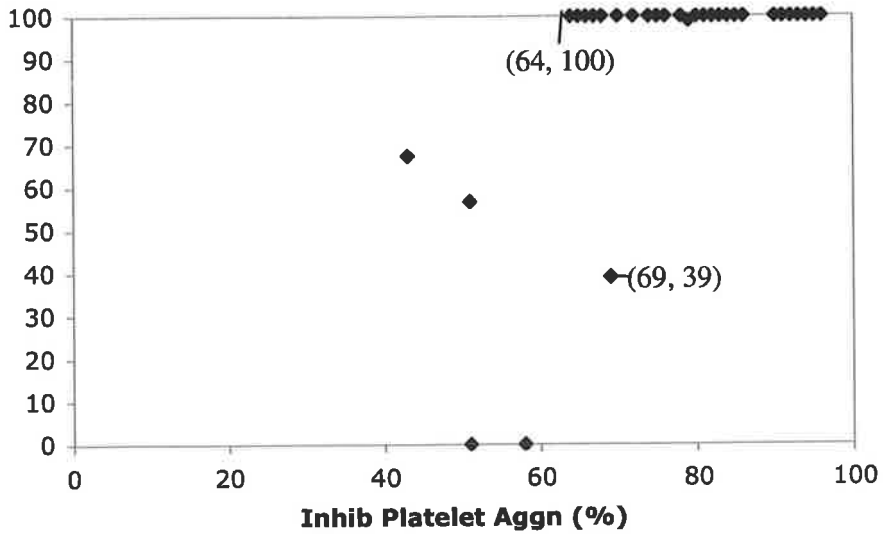

Figure 3.4. Correlation of inhibition of the PFA-100 closure time versus inhibition of platelet aggregation in patients during tirofiban infusion.

Overestimation of Platelet Inhibition by Citrate

Chelation of calcium by sodium citrate anticoagulant may lead to overestimation of the platelet inhibitory effects of GPIIb/IIIa antagonists. In the final 3 patients (7 measurements) we compared the platelet inhibition observed when blood was collected in sodium citrate 3.2% versus PPACK, a specific thrombin inhibitor. Median (range) receptor occupancy was 79% (73 - 84%) versus 65% (40 - 82%), p = 0.02, for citrate and PPACK anticoagulated blood respectively. Similarly, median (range) inhibition of platelet aggregation was higher in citrate anticoagulated samples: 82% (51 - 95%) versus 60% (29 - 79%), p = 0.03 (Figure 3.5).

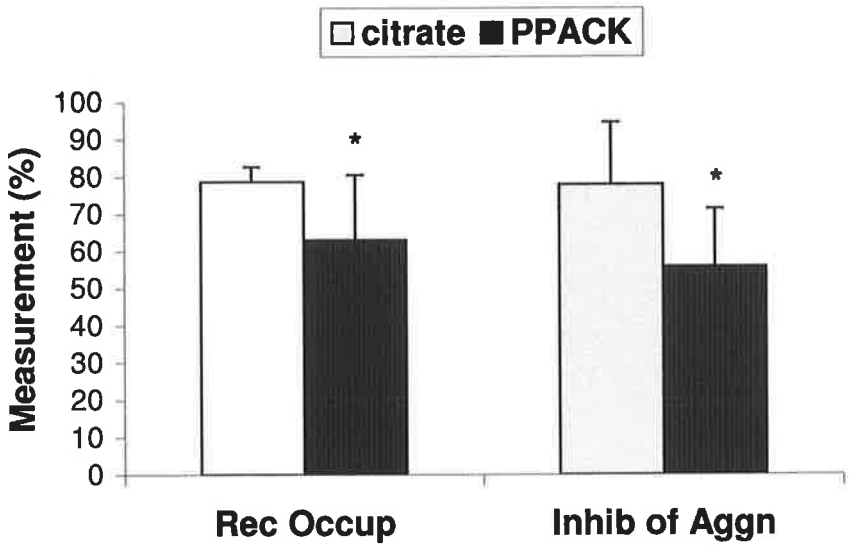

Figure 3.5. Collection of blood samples in PPACK versus sodium citrate anticoagulant was associated with a significant decrease in the measured receptor occupancy and level of inhibition of platelet aggregation to ADP during treatment with tirofiban. * $P < 0.05$.

Platelet Activation Markers

Unstimulated CD63 and CD63 expression was generally low (median = 0 for both) and not affected by drug therapy. However, non-parametric one-way analysis of variance (ANOVA) showed significant variation in the percentage of platelets expressing CD62 after stimulation with TRAP across all timepoints from baseline to 96 hrs ($p = 0.01$) (Figure 3.6). Pairwise analysis showed a significant increase in

TRAP stimulated CD62 expression from baseline to 24 hours (median 37% vs 67%, p = 0.0008) and from 6 to 24 hours (median 47.5% vs 67%, p = 0.02).

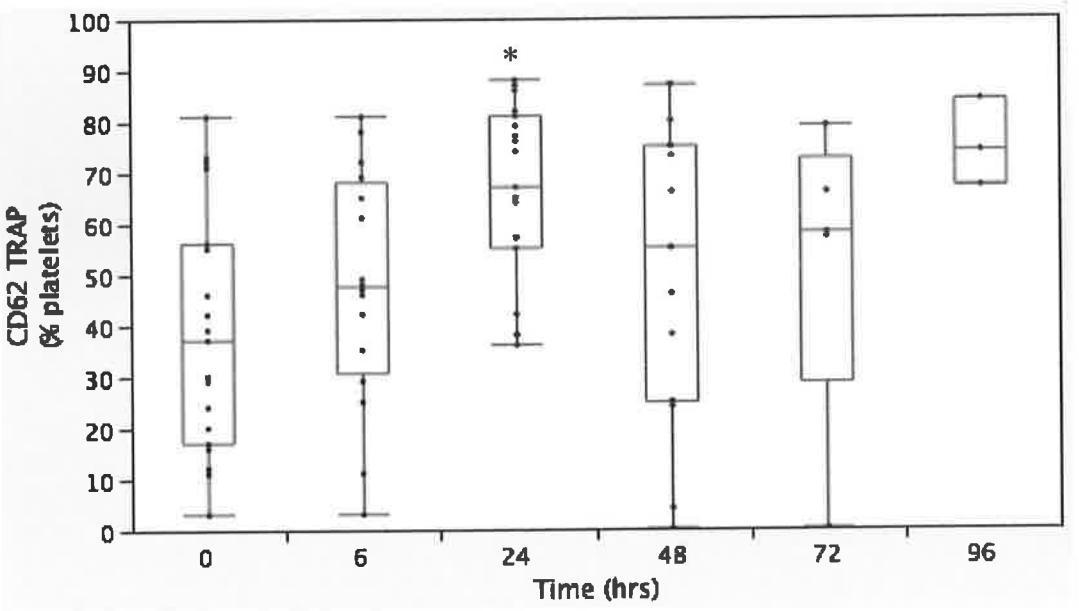

Figure 3.6. Box plot of TRAP induced CD62 (p-selection) expression pre tirofiban therapy at baseline (0 hrs) and during tirofiban therapy at 6, 24, 48, 72, and 96 hrs. *P < 0.001 vs 0 hrs, p < 0.05 vs 6 hrs.

There was no significant variation in the percentage of platelets expressing CD63 after stimulation with TRAP across all timepoints from baseline to 96 hrs (p = 0.11). Pairwise analysis showed no significant increase in TRAP stimulated CD63 expression from 0 to 24 hours (median 26% vs 43%, p = 0.12). However there was a significant increase in TRAP stimulated CD63 expression from 6 to 24 hours (25.5% vs 43%, p = 0.012). We also compared TRAP stimulated platelet activation

markers in patients pre, during, and post tirofiban therapy (Figure 3.7). The median percentage of stimulated platelets expressing CD62 during therapy was 61% (IQR 38 -76%) compared to 37% (IQR 17 - 56%) pre and 38% (IQR 27 - 62.5%) post (p = 0.004). Pairwise comparisons showed that the number of stimulated platelets expressing CD62 during therapy was significantly higher than pre and post therapy, p = 0.009 and p = 0.006 respectively. The median percentage of platelets expressing CD63 during therapy was 39% (IQR 24 - 61%) compared to 26% (13 - 58%) pre and 31% (6.5 - 42%) post (p = 0.04). Pairwise comparisons showed that the number of stimulated platelets expressing CD63 during therapy was significantly higher than post therapy (p = 0.01), but not when compared to pre therapy (p = 0.28).

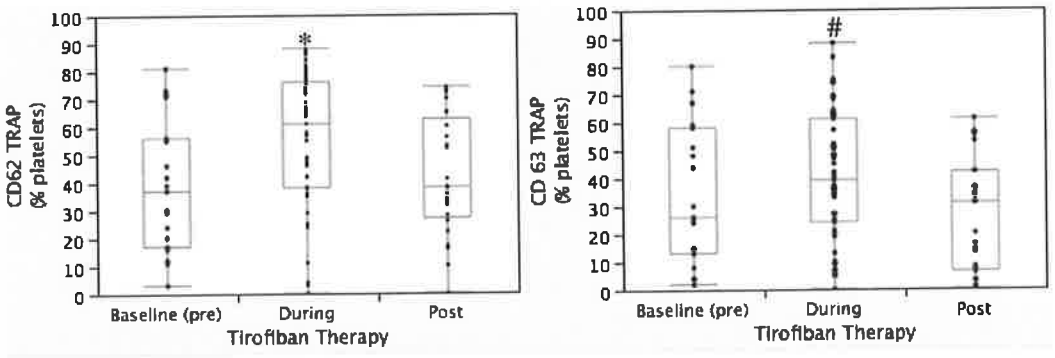

Figure 3.7. Box plots of TRAP induced CD62 and CD63 expression before, during and after tirofiban therapy. * P < 0.01 vs pre and post therapy. # P = 0.01 vs post therapy. No difference vs pre therapy.

Discussion

The majority of patients (two thirds) failed to achieve high levels of platelet inhibition during tirofiban infusion when measured both by receptor occupancy levels and inhibition of platelet aggregation, despite overestimation of the drug effect due to collection of blood samples in sodium citrate. Although both inhibition of platelet aggregation and receptor occupancy predicted similar rates of suboptimal platelet inhibition during tirofiban infusion, there was no evidence of significant correlation between the assays. This means that receptor occupancy and platelet aggregation to ADP predicted insufficient drug response in different patients. Only one study patient (24 hour group) had 80% or greater inhibition of both platelet aggregation and receptor occupancy throughout infusion of the study drug. Although good correlation has previously been described ($r = 0.8$) between receptor occupancy of small molecule GPIIb/IIIa antagonists detected by mAb2 and inhibition of ADP induced aggregation over a wide range of GPIIb/IIIa antagonist concentrations [230], it would appear that the agreement is poor over a narrower therapeutic range of the drug as observed in this analysis. In contrast the PFA-100, which correlated significantly with inhibition of platelet aggregation to ADP during tirofiban infusion, suggested high levels of platelet inhibition during drug therapy at virtually all time points. Therefore this point-of-care assay appears to be overly sensitive to the platelet inhibitory effects of tirofiban and useful only in detecting very low levels of platelet inhibition by GPIIb/IIIa antagonists in general.

It is of particular interest that expression of platelet activation markers CD62 and CD63 were increased during treatment with tirofiban. Binding of the GPIIb/IIIa receptor to its natural ligands, fibrinogen and vWF, results in outside-in signalling and enhanced platelet activation [116]. It would appear that engagement of the GPIIb/IIIa receptor with its antagonists also results in outside-in signalling and GPIIb/IIIa antagonist-induced platelet activation [117]. Enhanced expression of the platelet activation marker CD63 was also observed during treatment with orbofiban in the OPUS-TIMI 16 trial [118]. This would appear to be particularly important at lower doses of GPIIb/IIIa antagonists, where platelet activation occurs without significant inhibition of platelet aggregation, thereby generating a partial agonism effect. For example doses of abciximab, eptifibatide and tirofiban that incompletely inhibit platelet aggregation *in vitro* enhance sCD40L generation, although higher doses reduce its levels [119].

Finally, we noted significant interindividual variation in the antiplatelet response of tirofiban. Whether this could be overcome by increasing the drug dose in certain patients has not been tested, but warrants further study. Although our sample size was small we did not find any clinical predictors, including age and weight, for decreased platelet inhibition with tirofiban. In another study, only the presence of thrombus on coronary angiography was predictive of decreased response to GPIIb/IIIa antagonists in certain individuals [12]. In addition, we did not find the PI^{A2} polymorphism in GPIIIa to be a predictor of response to tirofiban.

A major limitation of this study was the use of sodium citrate for collection of blood samples. Although there was evidence prior to initiation of this study that the inhibitory effect of another small molecule GPIIb/IIIa antagonist, eptifibatide, was overestimated due to calcium chelation by the anticoagulant sodium citrate[105], similar findings for tirofiban were not published until the later stages of this trial [106]. In the case of eptifibatide, this finding led to an additional randomized clinical trial using a higher drug dose with an associated improvement in clinical outcome [72,74]. Although we were only able to compare sodium citrate and PPACK anticoagulant in 3 patients, our findings were consistent with the previously published data for overestimation of platelet inhibition by tirofiban with sodium citrate anticoagulant [106]. Even though suboptimal inhibition with tirofiban was common in our analysis, it is highly likely that the problem is even more prevalent when overestimation of the platelet inhibitory effect of the drug by sodium citrate is taken into account. The current approved dose of tirofiban patients with unstable angina or non-ST-elevation MI is a bolus infusion of 0.4 mcg/kg/min for 30 minutes followed by maintenance infusion of 0.1 μg/kg/min continued until 12-24 hours after percutaneous coronary intervention and is based on the findings from the PRISM-PLUS study, which showed a statistically significant decrease in the composite primary endpoint of death, MI, or refractory ischemia at 7 days in patients treated with heparin and tirofiban versus heparin alone [96]. Although one very small study (n=25) showed improved platelet inhibition with higher bolus and maintenance infusion doses of tirofiban (15 μg/kg over 3 minutes followed by maintenance

infusion of 0.2 μg/kg/minute), higher doses have not been evaluated in a clinical trial for safety or efficacy [109]. In view of this, the current approved dosing of Tirofiban is recommended.

Conclusion

It appears that many ACS patients fail to achieve adequate levels of platelet inhibition while undergoing treatment with a standard regimen of the GPIIb/IIIa antagonist tirofiban. This is despite the use of sodium citrate anticoagulant, which overestimates the platelet inhibitory effects of this drug. Higher or tailored doses of tirofiban may be necessary to achieve levels of platelet inhibition, which have been previously shown to optimally inhibit coronary thrombosis. Monitoring and dose titration of GPIIb/IIIa antagonism in PCI and ACS patients with a point-of-care device may also be beneficial. However, both higher and/or tailored dosing of tirofiban require further evaluation in large clinical trials before any changes to current practice can be recommended. One such point-of-care device, the PFA-100, is overly sensitive to platelet inhibition by tirofiban and therefore does not appear to be a suitable assay for monitoring GPIIb/IIIa antagonist treatment.

~ Chapter 4 ~

Functional Analysis Of The Linkage Between The PI^{A2} and Promoter Variants Of Integrin Subunit β_3 In Cardiovascular Disease

Introduction

The Platelet Antigen (PI^{A}) single nucleotide polymorphism results in a leucine (PI^{A1}) to proline (PI^{A2}) substitution in GPIIIa and has a carrier frequency of around 20% in Caucasians [135,136]. It is discussed in detail in Chapter 1. A number of small case control studies have demonstrated that the PI^{A2} polymorphism increases the risk of arterial thrombosis and acute myocardial infraction, however a recent meta-analysis suggests that this association is weak (RR 1.10, 95%CI 1.03-1.18) [139]. There is also evidence for an interaction between antiplatelet treatment and the PI^{A2} genotype. A genetic sub-study of the OPUS-TIMI-16 trial demonstrated an interaction between the PI^{A2} genotype and treatment with an oral GPIIb/IIIa antagonist, orbofiban [165]. PI^{A2} carriers treated with orbofiban had less bleeding and an increased risk of MI, suggesting that the variant attenuated the response to the GPIIb/IIIa antagonist.

Attempts to define the effect of the PI^{A2} polymorphism on platelet function have yielded conflicting results. The largest of these, a cohort of 1,422 participants in the Framingham Offspring study, concluded that PI^{A2} was associated with increased platelet reactivity as evidenced by enhanced sensitivity to epinephrine and ADP [148].

While one smaller study reported similar findings [146], other studies have failed to show an effect on platelet function [144,149,150]. To overcome the problem of heterogeneity in human platelets, two groups have examined the function of the PI^{A2} isoform of GPIIb/IIIa expressed in cell lines. Whereas one showed increased adhesion, cell spreading and signalling in cells expressing the PI^{A2} variant [143], a second study failed to reproduce the finding in a different cell line [144].

Linkage disequilibrium is one possible explanation for inconsistent results in case-control and functional studies of gene variants, if different patterns are present in different populations. Three polymorphisms have recently been identified in the promoter region of the IIIa gene at positions -468 (G/A), -425 (A/C), and -400 (C/A)[231]. In a preliminary report, these promoter polymorphisms were found to be associated with the differential expression of the GPIIb/IIIa receptor on the platelet surface [208]. There is substantial variation in the number of GPIIb/IIIa receptors expressed, with between 30,000 and 100,000 copies per platelet [217]. Although this degree of variation is not known to result in a functional effect, major quantitative or qualitative deficiencies in GPIIb/IIIa as seen in patients with Glanzmann's thrombasthenia lead to impaired platelet aggregation and increased bleeding [232].

In this study we sought to determine the effect of the PI^{A2} and promoter polymorphisms of GPIIIa on platelet expression of GPIIb/IIIa. In addition, we examined the influence of the GPIIIa promoter variants on clinical outcome in patients enrolled in the OPUS-TIMI-16 trial.

Methods

Study Population

Blood was drawn from a total of 207 subjects (130 with a history of coronary artery disease, 77 patients with a history of hypertension) for platelet receptor density analysis and genotyping of GPIIIa variants. The protocol was approved by the institutional ethics committees and all of the subjects gave written informed consent.

In addition, we examined the influence of the promoter variants on clinical outcome in patients who had previously donated a genetic sample during their participation in the OPUS-TIMI-16 trial. The design of OPUS-TIMI-16 and its genetic substudy (n = 1,014) have previously been described [165]. Briefly, this was a phase III multi-centre randomized controlled trial of the oral GPIIb/IIIa anagonist, orbofiban, in patients presenting with acute coronary syndromes. Consent was obtained after randomisation for analysis of genetic variants in relation to cardiovascular disease and therapy. Patient data is maintained in a database free of patient identifiers to protect anonymity. We restricted the analysis of the effect of the promoter variants in this population to Caucasian patients on whom there was complete genotypic data (n = 887).

Genotyping

Following DNA extraction [209] genotyping for PI^{A2} was performed by heteroduplex analysis as previously described [233]. This rapid and automated technique involves

initial amplification of the gene sequence containing the mutation site by a single PCR reaction that is then followed by its hybridisation with a synthesised DNA oligomer possessing a specified deletion in the vicinity of the mutation. The resulting heteroduplexes show different migration patterns on capillary electrophoresis depending on the genotype (ABI Prism 310 Genetic Analyser). The promoter polymorphisms were identified by direct sequencing in the population in whom receptor density was quantified. A 284 bp segment of the 5'UTR incorporating positions -468, -425 and -400 (Figure 4.1) was amplified from genomic DNA using the forward primer: 5'-AAGCTTGGGATGTGGTCTTG-3', and reverse primer: 5'-TGGGCACTTGGTGTGCTTA-3'. The resultant PCR product was purified using the QIAquick PCR purification kit (Qiagen) prior to cycle sequencing using BigDye Terminator chemistry. Capillary electrophoresis of forward and reverse primer generated sequence was performed on the ABI Prism 310 genetic analyser (Applied Biosystems). Promoter polymorphisms in the OPUS study were genotyped using the Amplifluor™ assay (Kbiosciences, Basildon, Essex, UK).

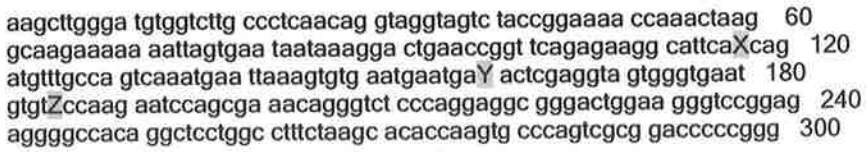

Figure 4.1. GPIIIa promoter polymorphisms at positions - 400 (X, c→a), - 425 (Y, a→c), and - 468 (Z, g→a) from the ATG site.

Sequence Homology

Analysis of sequence homology of human and mouse GPIIIa promoter sequence was performed following alignment of the respective ATG sites using NCLUSTAL and Graph Align software (http://srs.ebi.ac.uk and http://www.swbic.org) with a window size of 21.

Quantitative Receptor Expression

GPIIb/IIIa and GPIb receptor numbers were analysed by flow cytometry (Platelet GPIIb/IIIa-GPIb kit, Biocytex, Marseille, France) as previously described in the methods section of Chapter 2. Briefly. 20 µl whole blood, after 1 in 4 dilution in Biocytex buffer, was incubated with 20 µl anti-GPIIIa (p18) or anti-GPIb (SZ2) antibody (10 µg/ml) at room temperature for 20 minutes. Antibody binding was determined using fluorescein isothyocyanate (FITC) labelled $F(ab)_2$ fragments of human Ig absorbed, sheep anti-mouse IgG (H+L) antibodies. The samples were diluted with 2 ml of Biocytex buffer after 10 minutes incubation and then analysed by flow cytometry (FACScalibur, Becton Dickinson, Oxford, UK) at 488 nm excitation.

Statistical Analysis

Medians were compared using a Kruskal-Wallis rank sum test for absolute GPIIb/IIIa receptor numbers and for adjusted GPIIb/IIIa levels. The 95% confidence intervals for the medians were calculated using a binomial method that makes no

assumptions as to the underlying distribution of the variable. In the absence of genotypes from isolated chromosomes one relies on algorithms to infer haplotype frequencies. We imputed haplotype frequencies using a maximum likelihood method that embedded a log-linear model in the expectation algorithm, based on iterative proportional fitting. The EM algorithm handles the phase uncertainty and the log-linear modelling allows testing for linkage disequilibrium. We tested for Hardy Weinberg Equilibrium (HWE) using a goodness of fit χ^2 statistic. The linkage disequilibrium coefficient D measures deviation from random association between alleles at different loci. We calculated the standardized pairwise linkage disequilibrium statistic D' where the value of D is standardized by its maximal value, giving it a range from -1 to +1. Pearson's correlation statistic was used to measure the linear association between GPIIb/IIIa and GPIb. The associations between genotypic variants in GPIIIa and clinical outcome in the OPUS study were calculated using logistical regression. Statistical analysis used Stata Release 8.0 (Stata Corporation).

Results

SNP and Haplotype Analysis

The frequencies of the 4 polymorphisms in our study group are presented in Table 4.1. All the polymorphisms satisfied HWE. The predicted haplotype frequencies are presented in Table 4.2. Because of the strong association between genotypes, the 3 most common haplotypes accounted for almost 95% of the total number of

haplotypes; haplotype 1 (5'-GACT-3') 74%; haplotype 2 (5'-AAAT-3') 11% and haplotype 3 (5'-GCCC-3') 9%. All 4 polymorphic variants were in linkage disequilibrium (Table 4.3). PI^{A2} (T→C) was associated with the less frequent allele at position - 425 (C) and the more frequent alleles at positions - 400 (G) and - 468 (C).

Table 4.1. Polymorphisms in GPIIIa (n = 414 chromosomes) which observe Hardy-Weinberg equilibrium.

SNP	Allele 1	Allele 2	heterozygosity
G - 468 A	361 (87%)	53 (13%)	0.246
A - 425 C	370 (89%)	44 (11%)	0.193
C - 400 A	368 (89%)	46 (11%)	0.213
T1565C ($PI^{A1/2}$)	367 (89%)	47 (11%)	0.208

Table 4.2. Estimated frequencies of the 6 common haplotypes, n = 414. *GACT indicates the haplotype -468G, -425A, -400C, 1565T.

Hapl 1*	Hapl 2	Hapl 3	Hapl 4	Hapl 5	Hapl 6
5'-GACT-3'	5'-AAAT-3'	5'-GCCC-3'	5'-GACC-3'	5'-AACT-3'	5'-GCCT-3'
308 (74%)	46 (11%)	38 (9%)	9 (2%)	7 (2%)	6 (1%)

Table 4.3. Linkage disequilibrium between the 4 polymorphisms expressed as standardized pairwise linkage disequilibria statistic D'. [a] p = 0.02, [b] p < 0.00001, [c] p = 0.04, [d] p = 0.06

Allele	- 425C	- 400A	1565C
- 468A	-0.56[a]	0.45[b]	-0.50[a]
- 425C		-0.49[c]	0.41[b]
- 400A			-0.43[d]

Platelet Receptor Expression Analysis

The median receptor density was 56,418 (range 28,089 to 109,669). As the - 425 variant was strongly associated with PI^{A2} (in only 3% of the 6 haplotypes shown in Table 4.3 is the rare allele at each of these positions not in association), it is not surprising that receptor density was similar with both genotypes. The other two promoter variants, -468A and - 400A, were very strongly associated with each other, and their rare alleles were mainly found on a PI^{A1} background. Again however, neither group of variants showed any clear association with receptor density (Table 4.4). The number of GPIIb/IIIa receptors per platelet depends on platelet volume. Therefore, we used GPIb to correct for confounding effects of platelet volume. GPIb receptor density was strongly correlated with that of GPIIb/IIIa ($r^2 = 0.5$, $p < 0.0001$) (Figure 4.2).

Table 4.4. GPIIb/IIIa receptor expression among carriers and non-carriers of specified GPIIIa alleles. Median (95% confidence interval).

Allele	Carriers	Non-carriers	p-value
- 468A	56874 (54151, 59182)	56368 (55133, 57569)	0.52
- 425C	55896 (51539, 58606)	56810 (55464, 57712)	0.59
- 400A	56940 (53646, 59331)	56367 (55357, 57558)	0.52
1565C (PI^{A2})	56368 (51936, 58693)	56613 (55362, 57654)	0.93

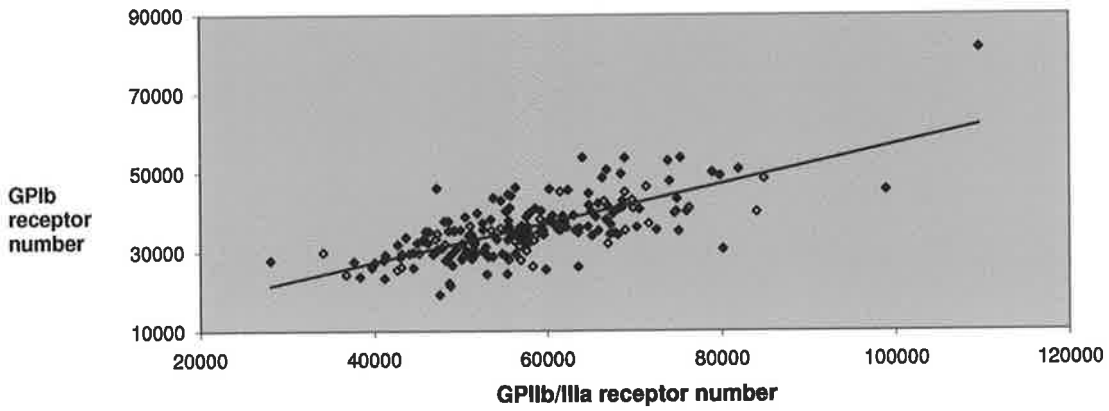

Figure 4.2. Scatter diagram showing the correlation between GPIIb/IIIa and GPIb. Carriers of the - 468A polymorphism (◊) are distinguished from non-carriers (♦).

Adjusted GPIIb/IIIa levels were slightly higher in carriers of the - 468A polymorphism, but this increase of approximately 4% in receptor densities did not

reach statistical significance (59200 versus 57000, p = 0.07) (Table 4.5). As might be expected, the strongly associated - 400 variant showed a similar trend, although again this was not statistically significant (p = 0.15). Even correcting for the density of GPIb receptors, there was no differences in the platelet expression of GPIIb/IIIa between Pl^{A2} carriers and non-carriers (p = 0.47).

Table 4.5. Adjusted GPIIb/IIIa receptor expression [GPIIb/IIIa - (GPIb - average(GPIb)] among carriers and non-carriers of specified GPIIIa alleles. Data expressed as median (95% confidence interval).

Allele	Carriers	Non-carriers	p-value
- 468A	59200 (56200, 61000)	57000 (55100, 58200)	0.07
- 425C	56700 (53400, 59200)	57500 (56400, 59100)	0.31
- 400A	58700 (55600, 61100)	57200 (55500, 58500)	0.15
1565C (Pl^{A2})	56800 (53200, 59800)	57500 (56300, 59000)	0.47

Human-Mouse GPIIIa Promoter Sequence Homology

Analysis of sequence homology for the first 540 base pairs (from the ATG start site) of the human and mouse GPIIIa promoter (Figure 4.3) showed that the region encompassing the three promoter polymorphisms was not highly conserved. The area of the - 468 polymorphism was the most conserved and showed 65% identity between human and mouse DNA.

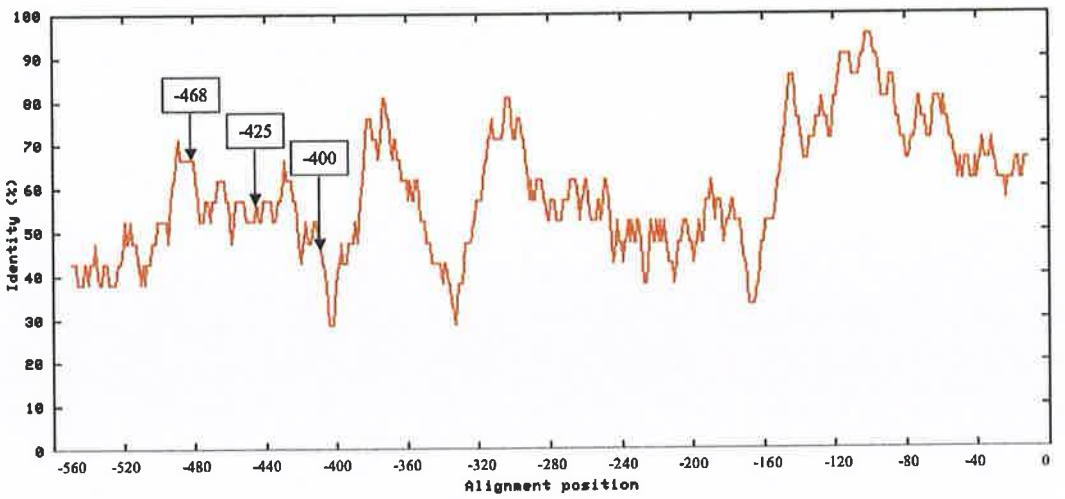

Figure 4.3. Human GPIIIa sequence homology with mouse gene.

OPUS Analysis

In a substudy of OPUS, PI^{A2} carriers had a significant increase in MI during follow up with a relative risk of 2.71 (95%CI, 1.37 to 5.38, p = 0.004)[165]. We examined whether there were genotypic effects of the IIIa locus additional to PI^{A2} that impacted on outcomes in the OPUS population. Neither of the two variants (- 400A, -468A) that are largely independent of PI^{A2} was associated with MI (p = 0.175 and 0.368 respectively). Moreover, the variant (- 425C) that is in linkage disequilibrium with PI^{A2} was not associated with MI (relative risk [RR] 0.90, 95% confidence interval [CI] 0.36 to 2.26, p = 0.8234). The strongest effect observed was for the - 425 variant, which protected against bleeding in patients receiving orbofiban (RR

0.63, 95%CI 0.38 to 1.05, p = 0.0656) (Table 4.6), an effect that has already been demonstrated more persuasively for the PI^{A2} genotype [165].

Table 4.6. Interaction between promoter genotype and therapy on bleeding events in OPUS.

Variant	Therapy	OR bleeding (95%CI)	P value
- 400A	Placebo	0.65 (0.30-1.41)	0.2587
	Orbofiban	1.15 (0.75-1.75)	0.5293
- 425C	Placebo	1.42 (0.70-2.89)	0.3425
	Orbofiban	0.63 (0.38-1.05)	0.0656
- 468A	Placebo	0.53 (0.24-1.16)	0.0970
	Orbofiban	1.17 (0.78-1.76)	0.4561

Discussion

As variants in the promoter region of genes may influence gene expression, we investigated whether the 3 polymorphisms of the GPIIIa promoter influenced receptor density in patients with cardiovascular disease. These variants are not in highly conserved regions, although the - 468 variant is within a short motif that shows approximately 65% identity with an aligned region of the mouse GPIIIa promoter. We have shown that PI^{A2} is strongly associated with 1 variant in the promoter region of GPIIIa, while two other variants (- 468 and - 400) in the promoter

are in tight disequilibrium. However, neither PI^{A2} nor the - 468 variant strongly influence platelet GPIIb/IIIa expression. Using GPIb receptor numbers to correct for non-genetic variation in GPIIb/IIIa expression is crude, but does suggest that the - 468 polymorphism may have at most a minor effect.

The results are consistent with studies on the region encompassing position -1159 to +1 of GPIIIa [234,235]. Jin et al. showed that the promoter sequence contained both positive and negative regulatory elements by examining a series of deletion constructs in transient transfection assays for reporter gene activity. Regions of the promoter including nucleotides - 468, - 425 and - 400 did not appear to influence promoter activity in megakaryocyte lines expressing GPIIIa. We are not aware of any other common genetic variants in the promoter region of GPIIIa. If there are genetic modifiers of the IIb/IIIa receptor density in Caucasians, they are likely to lie outside the immediate promoter and protein coding regions of the GPIIIa gene.

In the OPUS trial, the PI^{A2} polymorphism was associated with an increased risk of MI, particularly in those treated with orbofiban [165]. In addition there was a reduced risk of bleeding in PI^{A2} carriers in the treatment arm. Analysis of the promoter variants in this population did not demonstrate any additional effects beyond those seen with PI^{A2} in terms of either risk of MI or bleeding events. The association between - 425C variant and lower bleeding events in patients receiving orbofiban was weaker than that previously noted with PI^{A2} and is in all likelihood explained by the linkage disequilibrium between - 425C and PI^{A2}. It is therefore concluded that

the promoter variants do not confer any significant additional modification of bleeding or MI risk on patients, to that conferred by PI^{A2}.

Conclusion

The promoter of the GPIIIa gene contains several polymorphisms. One of these, the - 425C variant, is in strong linkage disequilibrium with the PI^{A2} variant, which in turn has been associated with acute myocardial infarction. However, none of the promoter variants were found to markedly influence platelet expression of GPIIb/IIIa or add to the predictive value of PI^{A2} on clinical outcome in the OPUS-TIMI-16 study.

~ Chapter 5 ~

Functional Genetics of PI^{A2}; Effects on Receptor Function and the Response to GPIIb/IIIa Antagonists

Introduction

As previously discussed in Chapters 1 and 4, the PI^{A2} polymorphism of integrin β_3 (GPIIIa) has been extensively studied, although many questions remain about its effects, both on coronary artery disease risk and on platelet function. In the OPUS study, a clinical trial of an oral GPIIb/IIIa antagonist orbofiban, PI^{A2} carriers randomized to treatment with orbofiban had a more than fourfold increased risk of MI versus non-carriers (RR 2.46 vs. 0.59, p = 0.08) [165]. One explanation for this finding is that GPIIb/IIIa antagonists act as partial agonists [118], particularly in carriers of PI^{A2}.

The purpose of this study was to determine the effect of the GPIIIa PI^{A2} polymorphism on GPIIb/IIIa receptor function and signalling. A key hypothesis was that the PI^{A2} polymorphism modifies the interaction of GPIIb/IIIa antagonists with the receptor, by influencing the propensity for these compounds to act as partial agonists.

Methods

Study Population

This study was approved by the local ethics committees (six hospital ethics committees and the RCSI ethics committee for the healthy volunteer subgroup) and all individuals consented to participate. Using an anonymised genetic database operated by Surgen Ltd. in the Royal College of Surgeons in Ireland, 1109 patients with a history of premature cardiovascular disease from 14 centres in Ireland were genotyped for the PI^A variant of GPIIIa. Inclusion criteria for this genetic database included a history of an acute coronary syndrome occurring in males 55 years and females 60 years or younger. An older age of onset (male under 60, female under 65) was permitted for patients who had a sibling with early onset ACS. We wrote to a total of 654 patients who were recruited from Beaumont Hospital (n = 234), Tallaght Hospital (n = 64), and St James's Hospital (n = 90), Dublin; the Royal Victoria Hospital (n = 111), Belfast; Cork University Hospital (n = 122) and Mallow General Hospital (n = 33), Cork to see if they would be willing to provide another blood sample for detailed platelet function analysis. 400 (61%) patients responded and indicated that they would be willing to participate in the research study. From this patient sample, a third party in Surgen not directly involved in the study generated a list of PI^{A2} carriers and controls for further study. Patients and investigators remained blinded to each participant's genotype. Genetic and phenotypic data were linked at the conclusion of the study by Surgen, whilst simultaneously all patient identifiers were removed. We also genotyped 43 healthy volunteers (postgraduate students and departmental staff) from the Royal College of Surgeons in Ireland, who had no history of cardiovascular disease. In the case of

healthy volunteers both investigators and participating individuals had access to PI^{A2} genotype information.

Genotyping

Following DNA extraction [209] genotyping for PI^{A2} was performed by restriction digest as previously described in the methods section of Chapter 2. Briefly, a 266 bp segment of DNA containing the PI^{A} polymorphism site was initially amplified by PCR. The 266 bp fragment was then digested by the Msp1 restriction enzyme. PI^{A2} introduces a second Msp1 restriction digest site that permits discrimination of PI^{A2} carriers versus wild type controls.

GPIIb/IIIa Receptor Affinity for Fibrinogen

GPIIb/IIIa receptor affinity for fibrinogen was compared in the different groups by measuring binding of a fluorescently labelled antibody, which distinguishes bound fibrinogen using a receptor induced binding site (RIBS), by flow cytometry (Biocytex fibrinogen kit, Marseille, France) [236]. Initially a 1 in 4 dilution of 20 μl whole blood in Biocytex buffer was performed. 3 similar tubes were prepared except for an additional 1 in 4 dilution with inhibitory reagent or ADP 2 μM or TRAP μM. The total volume of all 4 tubes was 80 μl. 20 μl of each tube was then individually incubated with 20 μl of fluorescently labelled mouse monoclonal anti-human bound fibrinogen antibody (9F9) at room temperature for 10 minutes, after which samples were

diluted with 2 ml of Biocytex buffer and then analysed by flow cytometry at 488 nm excitation.

GPIIb/IIIa Antagonist-Induced Platelet Activation

Platelet p-selectin (CD62) expression was measured by flow cytometry after incubation of whole blood with saturating concentrations of the GPIIb/IIIa antagonists orbofiban (10 μM), tirofiban (0.1 μM) and abciximab (2.5 μg/ml) for 30 minutes. Signalling through integrins is accommodated by prior clustering of the receptor [237]. We also performed the above analyses in the after were clustering of platelet GPIIb/IIIa receptors with the antibody 4F8 (Biocytex, Marseille, France). 4F8 (Mab2) binds to an epitope on GPIIb/IIIa which behaves as a ligand-attenuated binding site (LABS) and is displaced by small molecule GPIIb/IIIa antagonists such as orbofiban [238]. Although binding of 4F8 to GPIIb/IIIa does not prevent fibrinogen binding, it prevents platelet aggregation suggesting disappearance of the 4F8 epitope is required for full platelet activation. Monoclonal antibody to CD62 was purchased from Becton Dickinson. 5 μl whole blood was incubated with 20 μl CD62p or isotype for 20 minutes at room temperature. Similar tubes were also prepared except for the addition to whole blood of saturating concentrations of orbofiban ± 4F8, tirofiban ± 4F8, abciximab ± 4F8, or 4F8 alone for 30 minutes prior to the addition of CD62p or isotype. Incubation of an additional blood sample with TRAP (5 μM) for 10 minutes served as a positive control. Samples were

immunostained within 2 hours of blood collection and then fixed in 1% formaldehyde and stored at 4°C.

Flow Cytometry

Flow cytometric analysis of the fixed samples was performed within 2 hours. Platelet populations were gated according to their forward and side light scatter. Histograms were generated using 10,000 counts and geometric mean fluorescence and percentage of platelets positive for CD62p was calculated using the CELLQUEST software of the FACScalibur system (Becton Dickinson, Oxford, UK

Platelet Thromboxane Generation

Thromboxane B_2 was measured in non-aspirinated individuals ($PI^{A2/A2}$ n = 5, $PI^{A1/A1}$ n = 5, $PI^{A1/A2}$ n = 4). Washed platelets were re-suspended in the physiological buffer JNL at a concentration of $1x10^5$ platelets/μl and then incubated with 4F8 and saturating concentrations of orbofiban (10 μM), tirofiban (0.1 μM) and abciximab (2.5 μg/ml) for 30 minutes. Samples were then snap frozen and platelet thromboxane generation was measured by ELISA (Assay Designs, Inc., Michigan, USA).

sCD40L

Soluble plasma CD40L generation was measured in 3 PI^{A2} homozygotes and 3 $PI^{A1/A1}$ wild type controls after aggregation with TRAP 5 μM under stirred conditions

for 30 minutes. A similar analysis was performed after pre-incubation of PRP for 5 minutes with scaled doses of the GPIIb/IIIa anatagonists, abciximab (final concentrations 2.5, 3.2, 5, and 10 μg/ml) and orbofiban (final concentrations 0.1, 0.32, 1, and 10 μM). The supernatant from the aggregation samples were stored at - 80°C and levels of sCD40L were later measured by ELISA (Alexis Corporation).

Statistical Analysis

Data is expressed as mean ± standard deviation (SD). Comparisons of measurements within individuals were made using Student's paired t-test. Comparisons of measurements between individuals were made using Student's t-test for normally distributed data and the Wilcoxon rank sums test for non-parametric data. Data was analysed using the statistics software program JMP by Stata. A p value of < 0.05 in the two sided test was considered statistically significant.

Results

1094 of 1109 patients from the ACS database were successfully genotyped. There were 17 PI^{A2} homozygotes (1.5%), 259 PI^{A2} heterozygotes (23.5%) and 828 (75%) wild type ($PI^{A1/A1}$) patients. All 43 healthy volunteers were successfully genotyped with identification of 3 PI^{A2} homozygotes (7%), 9 PI^{A2} heterozygotes (21%), and 31 wild types (72%). We were provided with an enriched list of CAD patients by SURGEN that included 4 PI^{A2} homozygotes, 5 wild type controls, and 1 PI^{A2}

Table 5.1. Clinical characteristics of study participants. * 22 = PI^{A2} homozygote, 21 = PI^{A2} heterozygote, 11 = wild type control ($PI^{A1/A1}$).

No.	PI^{A*}	ASA	Age (yrs)	Gender	CVD	Cigarette	D.M.
1	22	0	70	M	+	Ex	0
2	22	0	47	M	0	Ex	0
3	22	0	22	F	0	0	0
4	22	0	25	F	0	0	0
5	22	0	24	F	0	0	0
6	22	+	56	F	+	Ex	0
7	22	+	65	M	+	Ex	0
8	22	+	71	M	+	Ex	0
All	PIA^{22}	3 (37.5%)	47.5	50% Male	4 (50%)	50% ex smokers	0 (0%)
9	11	0	54	M	+	Ex	0
10	11	0	51	M	+	Ex	+
11	11	0	21	F	0	0	0
12	11	0	25	F	0	0	0
13	11	0	22	F	0	0	0
14	11	+	55	F	+	Ex	0
15	11	+	65	M	+	0	0
16	11	+	71	M	+	Ex	0
All	PIA^{11}	3 (37.5%)	45	50% Male	5 (62.5%)	50% ex smokers	1 (12.5%)
17	21	0	70	M	+	Ex	0
18	21	0	22	F	0	0	0
19	21	0	24	F	0	0	0
20	21	0	24	F	0	0	0
All	PIA^{22}	0 (0%)	35	25% Male	1 (25%)	25% ex smokers	0 (0%)

heterozygote. In addition we performed platelet function analyses on 4 PI^{A2} homozygotes, 3 wild type controls, and 3 PI^{A2} heterozygotes from the healthy

volunteer group. This resulted in a total population of 8 PI^{A2} homozygotes, 8 wild type controls and 4 PI^{A2} heterozygotes. The study participants were successfully matched for aspirin usage and gender. The majority of patients were matched for age and presence of cardiovascular disease (CVD). However, two PI^{A2} homozygotes failed to match with wild type controls for age and CVD respectively. Clinical characteristics of the patients are presented above (Table 5.1).

PI^{A2} and GPIIIa Receptor Number and Fibrinogen Binding

There was no difference between GPIIb/IIIa receptor numbers in PI^{A2} homozygotes (42,036 ± 3,467/platelet) versus PI^{A2} heterozygotes (36,892 ± 2,019) and wild type controls (41,097 ± 2,171). Fibrinogen binding to GPIIb/IIIa was determined by detection of a receptor-induced conformational change in bound fibrinogen with a fluorescently labelled monoclonal antibody. 44.1 ± 8.3% and 46.8 ± 9.9% of platelets showed evidence of fibrinogen binding in the inhibited and resting states respectively. This increased to 70.5 ± 18.6% and 82.1 ± 11.8% after stimulation with ADP (2 μM) and TRAP (20 μM) respectively. There was no difference between PI^{A2} carriers and non-carriers.

PI^{A2} and Platelet Activation

The mean percentage of resting platelets expressing p-selectin was 12.1 ± 9.6% and was no different between PI^{A2} homozygotes (10.8 ± 5.8%), heterozygotes (15.5 ± 11.7%) and wild type controls (11.0 ± 12.1%). Similarly there was no difference

by genotype in platelet activation measured by p-selectin expression after stimulation with TRAP. Mean p-selectin expression was 52.9 ± 13.3% in PI^{A2} homozygotes versus 50.3 ± 8.7% in heterozygotes and 50.4 ± 21.8% in wild type controls.

PI^{A2} and Outside-In Signalling with the GPIIb/IIIa Antagonists

P-selectin expression

There was a significant increase from baseline in the percentage of platelets expressing p-selectin after incubation of whole blood with the GPIIb/IIIa antagonists orbofiban (28.2 ± 13.8%, $p < 0.0001$), tirofiban (29.9 ± 13.4%, $p = 0.0004$) and abciximab (19.0 ± 9.3%, $p = 0.01$) (Figure 5.1).

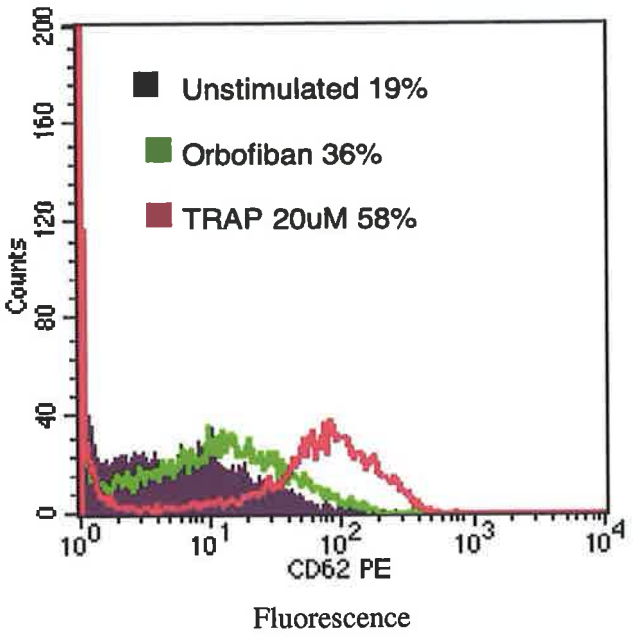

Figure 5.1. Flow cytometry shows increased fluorescent intensity compared to baseline after pre-incubation of whole blood with orbofiban, but less than seen after TRAP stimulation.

The small molecule GPIIb/IIIa antagonists orbofiban and tirofiban were associated with greater expression of p-selectin than abciximab ($p = 0.02$ for both). There was also a larger increase in platelet activation in individuals homozygous for the PI^{A2} polymorphism in GPIIIa (Figure 5.2).

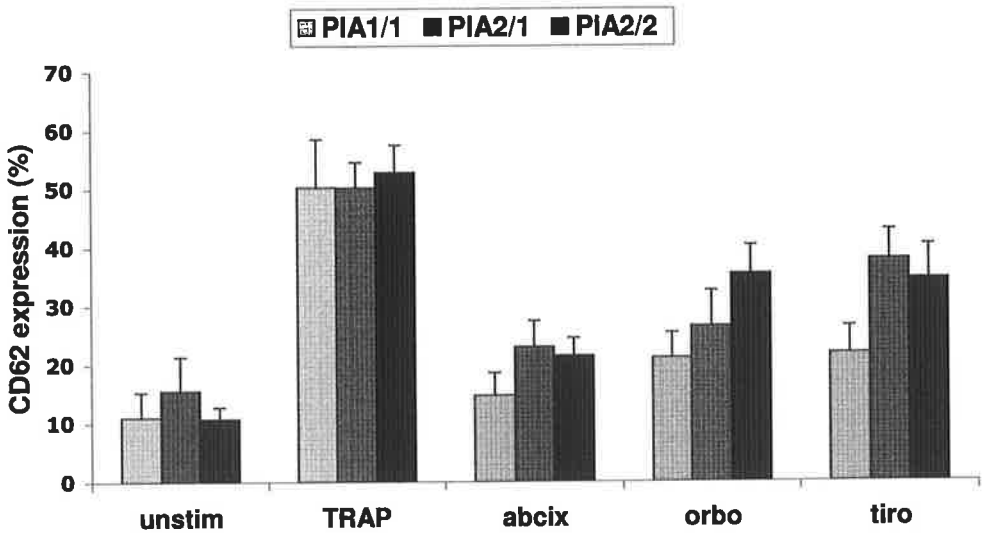

Figure 5.2. Bar graph showing platelet p-selectin expression at baseline and after stimulation with TRAP or GPIIb/IIIa anatagonists according to PI^{A2} genotype.

The platelets of PI^{A2} homozygotes incubated with orbofiban expressed more p-selectin compared to those of wild type controls after correction for baseline platelet activation by subtraction of unstimulated p-selectin measurements (24.8% vs. 10.1%, p = 0.02) (Figure 5.3). Similar, but non-significant trends were seen after incubation of PI^{A2} homozygote platelets with tirofiban (22.8% vs. 14.3%, p = 0.26) and abciximab (10.2% vs. 6.7%, p = 0.34).

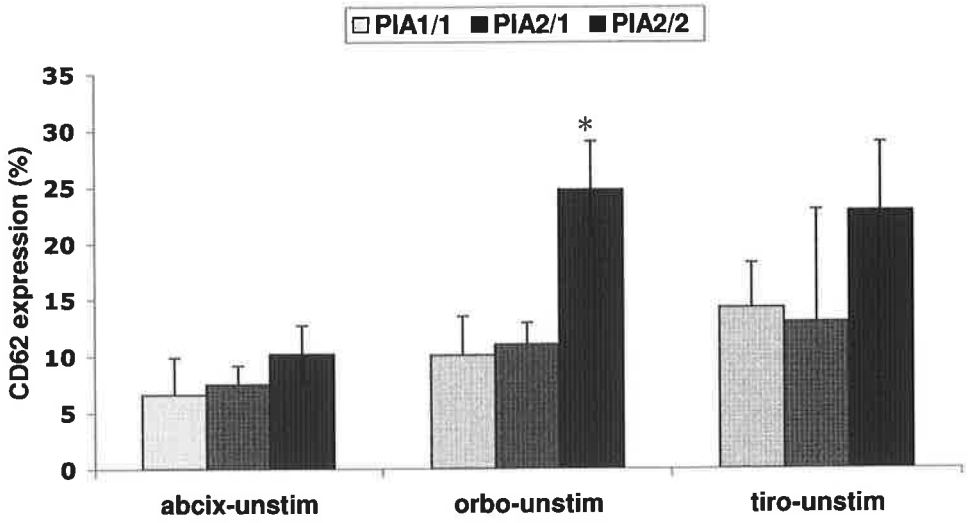

Figure 5.3. Platelet p-selectin expression (corrected for baseline expression) after stimulation with GPIIb/IIIa antagonists. * P = 0.02 versus $PI^{A1/A1}$ matched controls.

Analysis of the PI^{A2} heterozygote genotype was limited because of the small number of patients studied (n = 4). However, when PI^{A2} heterozygotes were compared only with matched PI^{A2} homozygotes and wild type controls, the absolute increase in p-selectin expression after incubation with orbofiban was significantly different in heterozygotes compared to both homozygotes and wild type controls (11.0% vs. 26.3%, p = 0.02 and 11.0% vs. 6.0%, p = 0.03 respectively) (Figure 5.4).

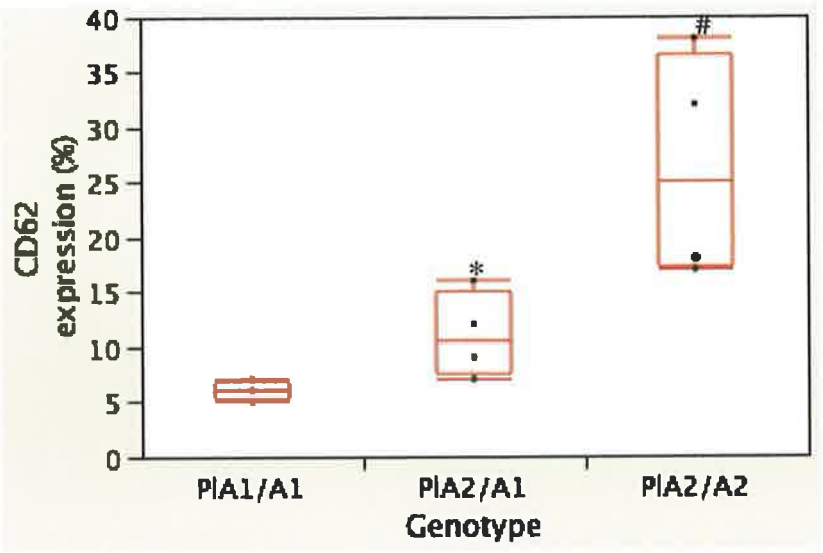

Figure 5.4. Box plot showing the percentage of platelets expressing p-selection by PI^{A2} genotype after incubation with orbofiban. Only matched patients included in the analysis. P-selection expression is corrected for baseline (unstimulated) values. * p = 0.02 vs. $PI^{A1/A1}$. # p = 0.03 vs. $PI^{A2/A1}$ and p = 0.02 vs. $PI^{A1/A1}$.

We also looked at platelet activation in the presence of the GPIIb/IIIa activating compound 4F8 in the presence and absence of GPIIb/IIIa antagonists. There was a small but significant increase in platelet p-selectin expression from baseline after incubation of samples with 4F8 (19.2 ± 10.5% vs. 12.1 ± 9.6%, p = 0.02). Co-incubation of whole blood with 4F8 and the GPIIb/IIIa antagonists resulted in even greater expression of p-selectin, which was again even higher for the small molecule antagonists orbofiban and tirofiban (Figure 5.5). There was a trend toward greater p-selectin expression in PI^{A2} homozygotes versus wild type controls after incubation with 4F8 alone, but this difference was not statistically significant

(23.2% vs. 14.7%, p = 0.25). There was no difference between p-selectin expression in PI^{A2} homozygotes versus wild type controls after incubation with 4F8 and abciximab (30.9% vs. 25.9%), 4F8 and orbofiban (60.8% vs. 58.4%) and 4F8 and tirofiban (57.3% vs. 49.3%).

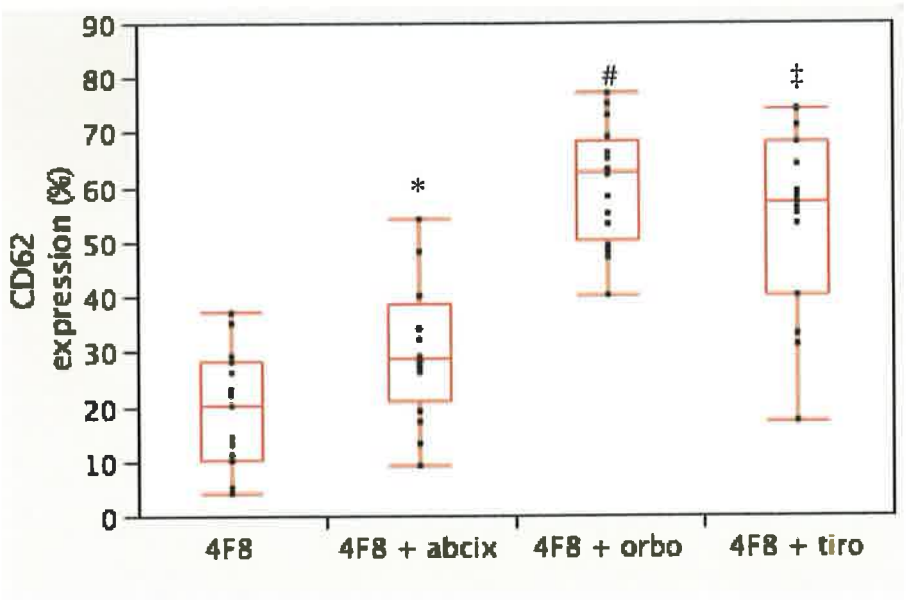

Figure 5.5. Box plot showing the percentage of platelets expressing p-selection by PI^{A2} genotype after incubation with 4F8 alone, 4F8 and abciximab, 4F8 and orbofiban, and 4F8 and tirofiban. P < 0.0001 by non-parametric analysis of variance for all measurements. * P = 0.005 vs. 4F8 alone. # P < 0.0001 vs. 4F8 alone and vs. 4F8 + abciximab; p = 0.01 vs. 4F8 and tirofiban. ‡ P < 0.0001 vs. 4F8 alone, p = 0.0004 vs. 4F8 + abciximab.

Platelet Thromboxane Generation

Platelet TXB_2 generation was measured in PI^{A2} homozygotes (n = 5), PI^{A2} heterozygotes (n = 4), and wild type controls (n = 5) who were not on aspirin therapy. There was minimal TXB_2 generation by platelets alone (3.4 ± 2.1 ng/ml) and in platelets incubated with saturating concentrations of abciximab (3.3 ± 2.4 ng/ml), orbofiban (3.9 ± 3.5 ng/ml) and tirofiban (3.6 ± 2.3 ng/ml). Similarly there was minimal TXB_2 generated when platelets were incubated with the GPIIb/IIIa activating compound 4F8 (3.0 ± 2.4 ng/ml). There was a significant increase in TXB_2 generation after co-incubation of platelets with 4F8 and the small GPIIb/IIIa antagonists orbofiban (89.4 ± 49.8 ng/ml, p = 0.0004) and tirofiban (90.0 ± 48.3 ng/ml, p = 0.0003), but not abciximab (2.8 ± 2.2 ng/ml). TXB_2 generation was not affected by PI^{A2} status (Figure 5.6). Although there was a trend towards lower levels of TXB_2 generation in PI^{A2} homozygotes versus wild type controls after incubation with orbofiban and 4F8 (64.6 ng/ml vs. 104.6 ng/ml, p = 0.46) and tirofiban and 4F8 (65.6 ng/ml vs. 117.9 ng/ml, p = 0.14), these differences were not statistically significant.

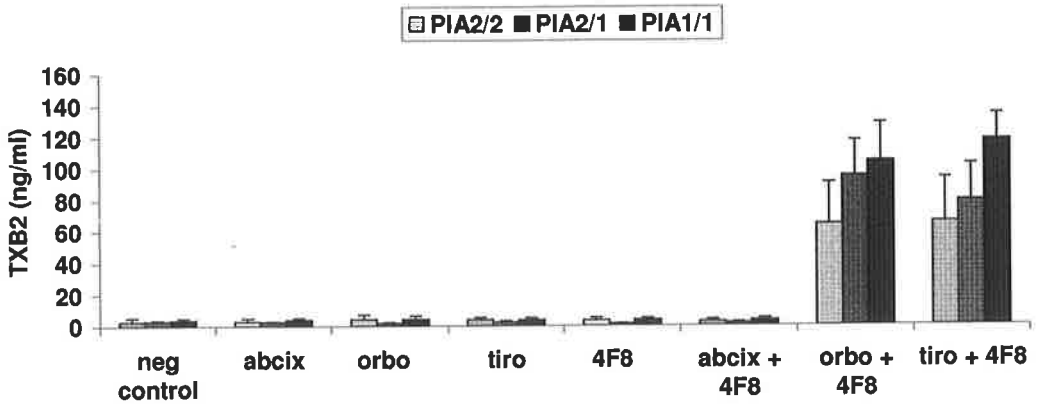

Figure 5.6. Platelet TXB_2 generation by genotype after incubation with saturating concentrations of abciximab, orbofiban, and tirofiban and/or the GPIIb/IIIa activating compound 4F8.

<u>sCD40L</u>

Soluble CD40L generation was measured in 3 PI^{A2} homozygotes and 3 $PI^{A1/A1}$ wild type controls. Mean ± SD plasma sCD40L after TRAP induced platelet aggregation measured 3971 ± 1768 pg/ml. There was a non-significant increase in sCD40L concentration after pre-incubation with low dose abciximab (2.5 μg/ml) to 4733 ± 2618 pg/ml ($p = 0.3$). Soluble CD40L decreased with higher concentrations of abciximab (2548 ± 2618 pg/ml, 2440 ± 1770 pg/ml, and 1364 ± 1611 pg/ml for 3.2, 5, and 10 μg/ml of abciximab respectively, $p < 0.05$ versus no pre-incubation with abciximab for each). Although there was no difference in the absolute levels of sCD40L generation between PI^{A2} carriers and controls, there was a trend to higher generation of sCD40L in PI^{A2} carriers after correction for baseline values in each

case (Figure 5.7). However the numbers of comparisons are too small to be statistically significant.

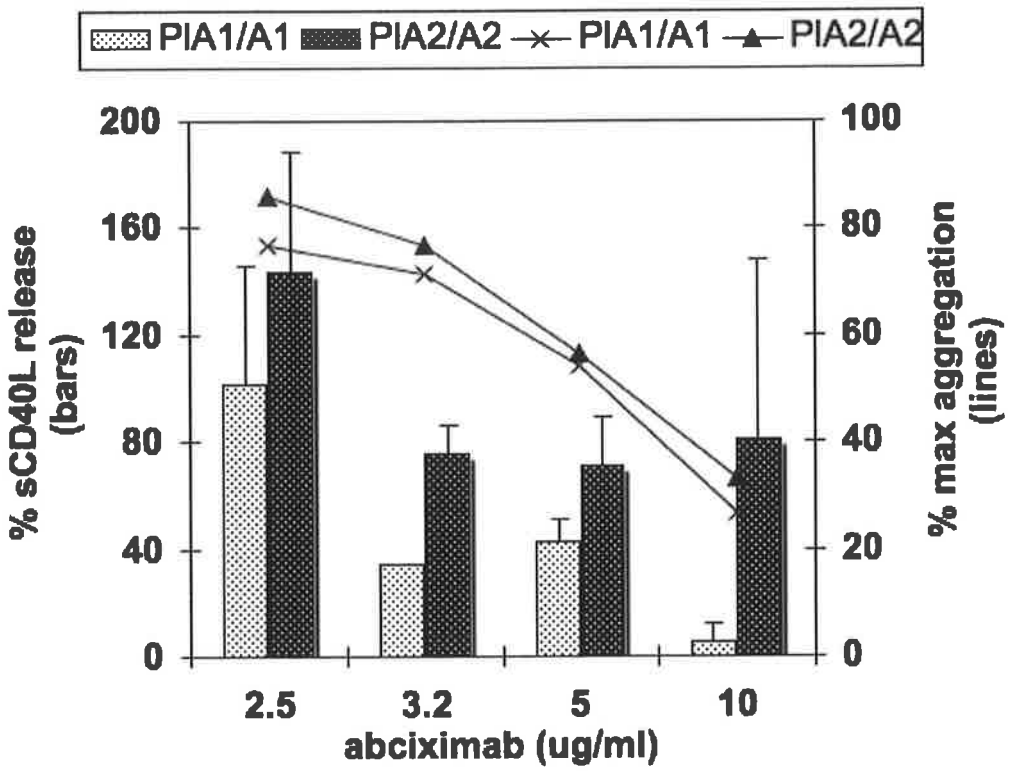

Figure 5.7. Soluble CD40L expression in TRAP aggregated platelets after incubation with varying doses of abciximab. There is comparable inhibition of platelet aggregation in PI^{A2} homozygotes and controls. However there is a trend to increased production of sCD40L in PI^{A2} homozygotes after correction for baseline values.

In contrast to abciximab, sCD40L generation was reduced at all doses of orbofiban tested (3406 ± 1780 pg/ml, 814 pg/ml (n =1), 1983 ± 1366 pg/ml and 345 ± 478 pg/ml for 0.1, 0.2, 0.32 and 1 μM of orbofiban respectively, $p = 0.06$ for 0.1 and 0.32 and $p < 0.007$ for 1 μM orbofiban versus no pre-incubation). There was no difference between PI^{A2} homozygotes compared to PI^{A1} controls at all concentrations of orbofiban, even after correction for baseline values.

Discussion

Previous studies of the effect of the PI^{A2} polymorphism on the platelet phenotype have largely compared PI^{A2} heterozygotes to wild type controls. Our aim was to generate a large sample size of PI^{A2} homozygotes to better determine the effect of PI^{A2} on platelet function and interaction with GPIIb/IIIa antagonists. Despite genotyping a large database of patients with coronary artery disease, our final analysis included only a modest number of patients homozygous for the PI^{A2} polymorphism (n = 8), limiting the statistical power of this study. The relatively low number of PI^{A2} homozygotes with CAD recruited (n = 4) is explained by the fact that many patients did not consent to partake in the study and that ultimately we could only recruit patients who lived locally or were willing to travel to us because of the time constraints involved in doing the platelet function analyses. Indeed we were fortunate to be able to supplement the study population with 4 PI^{A2} homozygote healthy volunteers.

We did not demonstrate any differences in GPIIb/IIIa receptor number, fibrinogen binding, or resting or induced platelet activation in PI^{A2} homozygotes versus wild type controls. Cox et al. have previously demonstrated evidence of partial agonism *in vivo* during treatment with the GPIIb/IIIa antagonist orbofiban [118]. Partial agonism likely occurs due to outside-in signalling of the GPIIb/IIIa receptor after ligand binding [239,240]. Outside-in signalling is dependent on clustering of the receptor [241] (feasible only with ligands with multiple binding sites such as fibrinogen) and linkage of the cytoplasmic tail to intracellular proteins [116]. They detected an increase in platelet activation measured by CD63 expression. In addition, they demonstrated increased platelet thromboxane generation *in vitro* after incubation of samples with an antibody that causes clustering of the GPIIb/IIIa receptors (4F8) and saturating concentrations of orbofiban. We similarly found an increase in the expression of another platelet surface activation marker, CD62 (p-selectin), after incubation of samples with saturating concentrations of orbofiban and another small molecule GPIIb/IIIa antagonist, tirofiban. There was a significant but smaller increase in p-selectin expression after incubation of samples with abciximab, suggesting that partial agonism is a property of all GPIIb/IIIa antagonists, but may be more pronounced with the small molecule agents. Indeed the small molecule antagonists induce a different conformational change in the GPIIb/IIIa receptor compared to the monoclonal antibody, abciximab [230]. Consistent with this differential effect was the finding of increased platelet thromboxane generation with saturating concentrations of orbofiban and tirofiban, but not abciximab after

GPIIb/IIIa receptor clustering with the monoclonal antibody 4F8. Additionally, in a small number of subjects we observed a non-significant increase in platelet sCD40L release with lower concentrations of abciximab, but not orbofiban. Although we did not find an effect of PI^{A2} on platelet activation in the resting state or after stimulation with a strong agonist (TRAP 5 μM), there was evidence of enhanced GPIIb/IIIa antagonist induced platelet activation in PI^{A2} homozygotes. Platelet p-selectin expression was significantly higher in PI^{A2} homozygotes versus wild type controls after incubation with orbofiban and a similar trend was observed for tirofiban and abciximab. However, we did not observe a similar effect of PI^{A2} genotype on thromboxane generation after incubation with 4F8 and either orbofiban or tirofiban. In fact there was a tendency for thromboxane generation to be lower in PI^{A2} homozygotes. But there was a trend towards higher sCD40L generation in PI^{A2} homozygotes after incubation with abciximab. However the small number of samples tested for sCD40L limits the power of this analysis.

This data confirms the platelet activating properties of GPIIb/IIIa antagonists observed in other studies [117-119,242-244]. Although partial agonism with these drugs is unlikely to be of importance when administered at the therapeutic doses that are associated with high levels of inhibition of platelet aggregation, it is a concern at lower drug levels when there is weak or no inhibition of platelet aggregation. In fact this may explain the failure of abciximab to reduce major cardiac events in the GUSTO-IV ACS study [92] and the higher rates of myocardial infarction and mortality observed in the trials of the oral GPIIb/IIIa antagonists [101]. A substudy of OPUS, a

clinical trial of an oral GPIIb/IIIa antagonist orbofiban, suggested that PI^{A2} carriers had an even higher risk of coronary thrombosis than non-carriers [165].

Conclusion

The results from this study provide a mechanism whereby PI^{A2} may contribute to an increase in adverse events during particular treatment scenarios with GPIIb/IIIa antagonists. Specifically the greater activation observed in PI^{A2} homozygote platelets compared to wild type controls after incubation with three different GPIIb/IIIa antagonists may indicate a greater propensity for outside-in signalling in patients with this variant, that puts them at significant increased risk of platelet mediated thrombosis when the doses of GPIIb/IIIa antagonist are inadequate to cause suppression of platelet aggregation. Genotyping of the PI^{A2} polymorphism should be considered in future prospective studies of the GPIIb/IIIa antagonists to confirm its potential adverse effect on outcome in patients treated with these agents.

~ Chapter 6 ~

Conclusion and Future Directions

This body of research showed that variability in the antithrombotic response to aspirin is likely related to COX-dependentand COX-independent mechanisms. We also demonstrated that the GPIIb/IIIa antagonist, tirofiban, appears to cause inadequate inhibition of platelet aggregation at it's currently approved intravenous dose. Finally we confirmed partial agonism with a number of GPIIb/IIIa antagonists and demonstrated *in vitro* for the first time that this effect is more pronounced in patients with the PI^{A2} polymorphism of GPIIIa. However, we did not find evidence for an association between the PI^{A2} polymorphism and aspirin resistance or GPIIb/IIIa expression.

There is no accepted definition for aspirin resistance, however there appears to be increasing acceptance that it is probably best characterized by demonstration of incomplete inhibition of cyclooxygenase, the primary target of aspirin (M Cattaneo. European Heart Journal. 2007: 28:1673-1675. Maree et al. Circulation 2007;115;2196-2207. Tantry et al. Progress in Cardiovascular Diseases. 2009; 52:141-152.). Aspirin resistance has been observed in a large number of studies using a variety of platelet function assays [25,28]. The PFA-100, a point of care device, examines platelet function at high shear in whole blood thereby simulating physiological conditions [114]. We confirmed the presence of aspirin resistance using

this device in a population of patients with coronary artery disease. Although we did not determine the clinical consequence of aspirin resistance in this population, there is some evidence from a number of small studies that aspirin resistance measured with the PFA-100 is associated with a higher risk of subsequent cardiovascular events [57-59]. Our data suggests that the causes of aspirin resistance are multifactorial. After excluding non-compliant patients, we found that approximately half of aspirin resistant patients had evidence of incomplete suppression of platelet thromboxane generation, most likely representing under-dosing with aspirin. In another study involving this population of patients we showed that increasing patient weight, younger age, and a previous history of MI were associated with lower levels of suppression of thromboxane generation by aspirin [37]. We have also shown that genetic variants in COX-1 determine the suppression of thromboxane generation by aspirin [40]. However, although some patients have evidence of a decrease in the antithrombotic response to aspirin because of COX-1 dependent mechanisms, we also found evidence for COX-1 independent mechanisms of aspirin resistance. Specifically we found that aspirin resistance measured with the PFA-100 was associated with increased platelet sensitivity to epinephrine and increased platelet activation. There is evidence for a subpopulation of patients with increased sensitivity to epinephrine activation [50]. Although there is evidence for a genetic basis to this enhanced sensitivity, it may also be affected by environmental or clinical variables [213]. There are still many questions to be answered regarding the existence, prevalence, predictors, and

clinical importance of aspirin resistance. Aspirin resistance appears to be multifactorial. Large scale clinical trials, using an approach similar to ours using a combination of COX-dependentand COX-independent assays of aspirin are likely to yield the best results in answering these questions. It is likely that evidence of both incomplete suppression of thromboxane and up regulation of cylooxygenase-independent pathways of platelet activation are independently predictive of adverse clinical events. It then will remain to be seen whether increasing the dose of aspirin or adding additional antiplatelet drugs, such as clopidogrel can improve the prognosis of patients with aspirin resistance.

Tirofiban is licensed for use in patients with acute coronary syndromes, including those undergoing PCI. In a small clinical study we demonstrate that tirofiban appears to provide suboptimal inhibition of platelet aggregation at its current approved dosage, a finding that has been observed in several other small studies that were published after approval of tirofiban by the regulatory authorities [106-109]. This finding is consistent with the apparent superiority of another GPIIb/IIIa antagonist, abciximab in indirect and direct comparisons of these two drugs [75]. Higher dosing schedules with tirofiban have been suggested. Although it is unlikely to be undertaken, a new randomized clinical trial of tirofiban at a higher and more effective dose should be performed. The modest clinical benefits with tirofiban therapy in the PRISM-PLUS study were observed prior to the results from the CURE study showed that addition of clopidogrel to aspirin was of benefit in ACS patients [96,99]. So in addition to the testing of a higher dose of tirofiban, there is an

argument for newer clinical trials of GPIIb/IIIa antagonist therapy compared to dual antiplatelet therapy with aspirin and clopidogrel, or even newer and more potent oral antiplatelet drugs such as prasugrel and ticagrelor. In addition we found evidence for enhanced platelet activation *in vivo* during tirofiban infusion by measurement of platelet expression of CD62 and CD63. Enhanced expression of CD63 was also observed in the OPUS trial, during oral administration of the GPIIb/IIIa antagonist orbofiban [118]. Unfortunately the partial agonist effects of GPIIb/IIIa antagonists were only detected after the negative results of the clinical trials of the oral agents. But this data, plus the results from our *in vitro* analysis suggests that partial agonism is a property of all GPIIb/IIIa antagonists, and not just the small molecule agents. Our finding that PI^{A2} appears to potentiate the partial agonist effects of GPIIb/IIIa antagonists supports the observation from the OPUS study of an increased incidence of myocardial infarction in PI^{A2} patients on orbofiban therapy [165]. In addition it suggests that PI^{A2} may have a more widespread effect on platelet function, by modifying outside-in signalling of the GPIIb/IIIa receptor in response to ligand binding. Unfortunately the published studies attempting to determine the effect of PI^{A2} on the platelet phenotype, including ours, have been underpowered to detect anything less than large differences in gene effect. In addition, confounding due to genetic heterogeneity and environmental factors makes it difficult to generate reproducible results in studies of single nucleotide polymorphisms such as PI^{A2}. Although at this time, based on the accumulated data, it appears that PI^{A2} probably does not play a significant role in

platelet aggregation or clinical thrombotic events, newer approaches including genotype-wide SNP arrays and haplotype analysis will allow clearer distinction of the roles of specific genetic variants in the future [245,246].

Publications Arising From Thesis

Full Scientific Journal Publications

O'Halloran AM, Patterson CC, Horan P, Maree A, Curtin R, Stanton A, McKeown PP, Shields DC. Genetic polymorphisms in platelet-related proteins and coronary artery disease: investigation of candidate genes, including N-acetylgalactosaminyltransferase 4 (GALNT4) and sulphotransferase 1A1/2 (SULT1A1/2). J Thromb Thrombolysis. 2009; 27: 175-84.

O'Halloran AM, Curtin R, O'Connor F, Dooley M, Fitzgerald A, O'Brien JK, Fitzgerald DJ, Shields DC. The impact of genetic variation in the region of the GPIIIa gene on PlA2 expression bias and GPIIb/IIIa receptor density in platelets. Br J Haematol. 2006; 132: 494-502. (O'Halloran and Curtin joint first authors).

Maree AO, Curtin RJ, Dooley M, Conroy RM, Crean P, Cox D, Fitzgerald DJ. "Platelet response to low-dose enteric-coated aspirin in patients with stable cardiovascular disease." J Am Coll Cardiol. 2005; 46: 1258-1263. (Maree, Curtin, Cox and Fitzgerald joint first authors).

Maree AO, Curtin RJ, Chubb A, Dolan C, Cox D, O'Brien J, Crean P, Shields DC, Fitzgerald DJ. "Cyclooxygenase-1 haplotype modulates platelet response to aspirin." J Thromb Haemost. 2005; 3: 2340-2345. (Maree and Curtin joint first authors).

Scientific Abstracts

Curtin R, Maree A, Dooley M, Crean P, Shields DC, Cox D, Fitzgerald DJ. "Low dose enteric coated aspirin fails to inhibit platelet cyclooxygenase in heavier cardiovascular patients." Ir J Med Science 2004; 173(Suppl. 1): 27. (Irish Cardiac Society, YIA Session).

Maree AO, Curtin R, Dooley M, O'Brien J, Crean P, Cox D, Fitzgerald DJ. "Genetic determinants of epinephrine induced platelet aggregation and aspirin resistance." Eur Heart J. 2004; 25: (Abstract Suppl.): 238.

Maree A, Curtin R, Dooley M, Crean P, Shields DC, Cox D, Fitzgerald DJ. "Aspirin resistance in cardiovascular disease; underdosing of overweight patients." J Am Coll Cardiol 2004; 43(Suppl. 2): 532A. (ACC, Young Investigator Award Session).

Curtin R, Manganello J, Phillips D, Fitzgerald DJ. "GPIIb/IIIa antagonist-induced activation is enhanced by the PIA2 polymorphism." Ir J Med Science 2003; 172(Suppl. 1): 27. (Irish Cardiac Society, Moderated Poster Award).

Curtin R, Maree A, Dooley M, Crean P, Fitzgerald DJ. "Using the PFA-100 and serum thromboxane B2 to measure aspirin resistance in patients with cardiovascular disease." Eur Heart J. 2003; 24 (Abstr. Suppl.): 119. (ESC Young Investigator Award).

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Abbreviations

AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
ACS	Acute Coronary Syndrome
ADP	Adenosine Di-phosphate
ANOVA	Analysis Of Variance
APTT	Activated Partial Thromboplastin Time
CD	Cluster of Determinants, eg CD62
COX	Cyclooxygenase
CRP	C-Reactive Protein
CY	Cytochrome
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Emergency Room
FDA	Food and Drug Administration
FITC	Fluoroscein Isothyocyanate
GP	Glycoprotein, eg GPIIb/IIIa
GUSTO	Global Utilisation of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries (Study Group)
HR	Hazard Ratio
HWE	Hardy Weinberg Equilibrium

IC	Inhibitory Concentration
ICC	Intraclass Correlation Coefficient
IL	Interleukin
IP	Isoprostane
IQR	Interquartile Range
LD	Linkage Disequilibrium
LIBS	Ligand Induced Binding Site
MAb	Monoclonal Antibody, eg MAb1
MACE	Major Adverse Cardiovascular Events
MI	Myocardial Infarction
NSAID	Non-Steroidal Anti-Inflammatory Drug
NSTEMI	Non-ST elevation MI
PAI	Plasminogen Activator Inhibitor
PCI	Percutaneous Coronary Intervention
PDGF	Platelet Derived Growth Factor
PFA	Platelet Function Analyser
PG	Prostaglandin
PI^A	Platelet Antigen
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethylketone
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
RIBS	Receptor Induced Binding Site

RPFA	Rapid Platelet Function Analyser
RR	Relative Risk
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
TIMI	Thrombolysis in Myocardial Infarction (Study Group)
TRAP	Thrombin Receptor Agonist Peptide
TX	Thromboxane
UA	Unstable Angina

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