Post-$P2Y_{12}$-receptor signalling mechanisms and platelet responses to clopidogrel

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Abstract

Anti-aggregatory agents such as clopidogrel limit platelet responses to adenosine diphosphate (ADP) by blocking the $P2Y_{12}$ receptor and have pivotal roles in the prevention of coronary artery stent thrombosis. However anti-aggregatory responses to such agents vary between individuals and this may predispose to stent thrombosis: the bases for this variablility are incompletely understood. Loss of function CYP2C19 genotypes, impeding clopidogrel bioactivation, have received considerable attention in the literature. However, this alone does not account for the increased rates of lack of response to clopidogrel commonly seen in diseases such as diabetes, obesity and acute coronary syndromes. Furthermore, assessment of aggregation on treatment, while clinically useful, does not precisely measure response to anti-aggregatory therapy, and standardisation of drug dosing may lead to relative underdosing in obese individuals.

$P2Y_{12}$ receptor activation by ADP is linked via Giα protein, to inhibition of adenylate cyclase and thus suppression of cyclic AMP formation. This results in nett pro-aggregatory effects. Hence clopidogrel acts in part by reversing this cascade.

A number of prostanoids including prostacyclin and prostaglandin E_1 function as physiological activators of adenylate cyclase and have been shown to potentiate the effects of $P2Y_{12}$ inhibitors. Furthermore the integrity of the prostanoid/adenylate cyclase signalling pathway is impaired in both cardiovascular disease states and diseases predisposing to cardiovascular disease such as obesity and diabetes. Cyclic AMP formation contributes, together with

that of cyclic GMP, to phosphorylation of vasomotor stimulated phosphoprotein (VASP) an effector pathway for ADP effects and their inhibition.

The principal hypothesis tested in this thesis was that adenylate cyclase signalling integrity predicted 7 day response to clopidogrel.

This was tested by measuring response to clopidogrel (both regarding changes in aggregation and in VASP phosphorylation) in both normal subjects and patients with known ischaemic heart disease. Integrity of prostanoid/adenylate cyclase signalling was measured via pre-clopidogrel platelet response to PGE_1. Putative determinants of clopidogrel response, including genotype and cGMP formation, were evaluated via univariate followed by multivariate analyses, and it was found that PGE_1 response (but not genotype) strongly predicted clopidogrel response.

In related analyses, it was also shown that

(i) weight adjusted clopidogrel dosing could be utilised to show that variable BMI does not markedly influence response to clopidogrel and that

(ii) presence of symptomatic cardiac disease was not a significant cause of variability in response.

Finally, exploratory analyses were undertaken to evaluate:-

(i) the role of insulin resistance

(ii) the putative involvement of the phosphoinositide 3-kinase (PI3-kinase) pathway and

(iii) the matricellular protein thrombospondin-1 (TSP-1) as further modulators of clopidogrel effect.

Overall the data demonstrate marked inter-individual heterogeneity of responsiveness to clopidogrel, which is engendered at least in part, by variable post-receptor signal transduction.

Declaration

I, Nicola Leigh Hurst, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Nicola Hurst

September 2014

Scholarships and Awards related to this thesis

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Vivek Nooney, Nicola Hurst, John Horowitz, Jeffrey Isenberg, Yuliy Chirkov, Thrombospondin-1 exerts bidirectional effects on prostacyclin siganlling in human platelets: implications regarding clopidogrel resistance, World Congress of Cardiology Meeting, Melbourne Australia, 2014

List of abbreviations

ABCB1	ATP Binding Casette B1
ACS	Acute coronary syndrome
ADP	Adenosine Diphosphate
ADMA	Asymmetric dimethylargine
Akt	Previously Protein kinase B (PKB)
ANCOVA	Analysis of covariance
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BSA	Body surface area
cAMP	Cyclic adenosine monophosphate
CAD	Coronary Artery Disease
CBE	Complete Blood Examination
CCB	Calcium channel blocker
CCF	Congestive cardiac failure
cGMP	Cyclic guanosine monophosphate
CHD	Coronary Heart Disease
CV	Coefficient of Variance
CYPs	Cytochrome P450s
DAG	Diacylglycerol
DAPT	Dual anti-platelet therapy
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribosenucleic acid
ENT 1	Equilibrative nucleoside transporter 1
E-NTPDases	Ectonucleoside triphosphate dephosphorylases
GP	Glycoprotein
GPCR	G protein coupled receptor
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HTPR	High on treatment platelet reactivity
IHD	Ischaemic heart disease
IMS	Invaginated membrane system
IP	Prostacyclin receptor
IP_3	Inositol (1,4,5) trisphosphate
IR	Insulin Resistance
MACE	Major Adverse Coronary Events
MFI	Median Fluorescence Intensity
MI	Myocardial Infarction
MLCk	Myosin light chain kinase
NO	Nitric Oxide
P-AM	Prasugrel active metabolite
PARs	Protease-activated Receptors.
PCI	Percutaneous coronary investigation
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase chain reaction
PDK-1	Phosphadylinositol-dependent kinase 1

PGE_1	Prostaglandin E_1
PGI_2	Prostaglandin I_2 or prostacyclin
PI3-kinase	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein Kinase G
PLC	Phospholipase C
Plk	Polo like kinase
PPIs	Proton pump inhibitors
PRI	Platelet reactivity index
PRP	Platelet Rich Plasma
PVD	Peripheral vascular disease
ROC	Receiver Operating Curve
SAP	Stable Angina Pectoris
SD	Standard Deviation
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SQ22536	9-(Tetrahydro-2-furanyl)9*H*-purin-6-amine
SPA	Spontaneous Platelet Aggregation
STEMI	ST elevation myocardial infarction
TF	Tissue Factor
T2DM	Type 2 diabetes mellitus

TSP-1	Thrombospondin-1
VASP	Vasomotor-stimulated phosphoprotein
vWF	Von Willebrand Factor
WBA	Whole Blood Aggregometry

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CHAPTER 1: INTRODUCTION

1.1 Role of platelets in cardiovascular homeostasis

Platelets play a crucial role in atherothrombotic cardiac disease. They have been described as proatherogenic, creating a bridge between leucocytes and endothelium in the early stages of atherosclerosis[1] and have been shown to have a key role in the ongoing inflammatory response[2,3]. Activated platelets promote coagulation and smooth muscle cell migration[4] and proliferation[5].

Platelets are pivotal to the process of arterial thrombus formation. In acute coronary syndromes (ACS), the resultant clot formed when the atheromatous plaque ruptures is platelet rich, formed as a result of exposure to collagen and von Willebrand Factor (vWF) that initiates platelet adhesion and activation. When human atheromatous plaque material was exposed to blood, thrombosis occurred in two discrete steps. Firstly, collagen induced Glycoprotein VI (GPVI) mediated platelet adhesion and aggregation occurred, followed by a delay where thrombin and fibrin was formed which was driven by the presence of plaque tissue factor[6]. Given this crucial role of the platelet in the pathogenesis of atherothrombotic ischaemic heart disease, considerable attention has been given to role of antiplatelet agents in the prevention of thrombotic cardiac events.

Platelets from patients with ischaemic heart disease[7] and diseases with a greater risk of developing ischaemic heart disease including diabetes[8] and polycystic

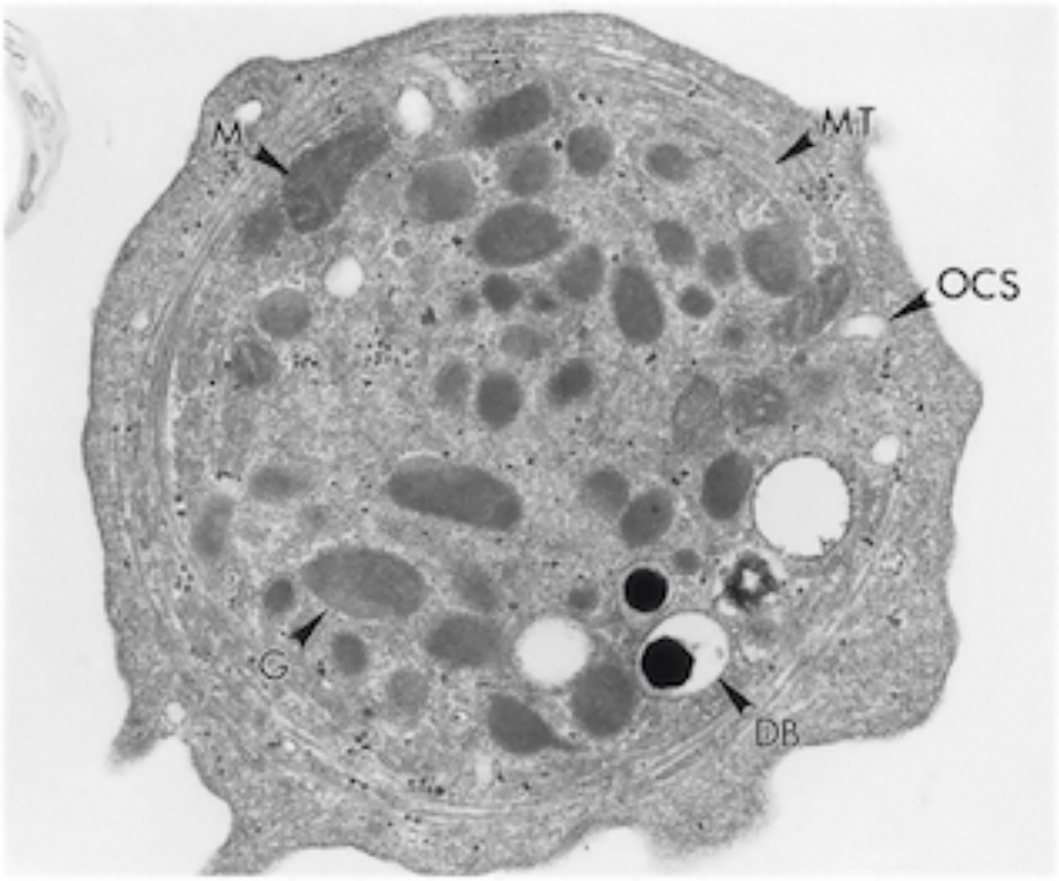
ovarian syndrome[9,10] (PCOS) have been demonstrated to be hyperaggregable when compared with healthy subjects[11]. Furthermore, platelets in patients with ischaemic heart disease have been demonstrated to be less sensitive to anti-aggregatory agonists such as prostacyclin (PGI_2) and nitric oxide (NO) [12-15] and to have associated blunted nucleotide responses (cAMP and cGMP respectively) in response to these agonists[12,14].

1.2 Platelet structure and function

Platelets are anucleate, discoid cells with a circulating life span of 7 to 10 days. They are formed by fragmentation of polyploid megakaryocyte cytoplasm which occurs largely in the bone marrow[16] with some megakaryocytes also found in the lungs. The megakaryocyte begins a maturation stage in which the cytoplasm fills with platelet specific proteins, organelles and membrane systems. During this stage of maturation, distinct ultrastructural features appear including the assembly of an invaginated membrane system (IMS), dense tubular system and intraplatelet (alpha (α) and delta (δ)) granules[17]. The relationship between the ultrastructure of platelets and their various physiological roles has been investigated extensively. Specifically the IMS is thought to develop into the surface connected open canalicular system (OCS) which serves as the pathway for the transport of substances into platelets and as conduits for the discharge of alpha granules during platelet activation[18]. The dense tubular system is an internal smooth endoplasmic reticulum membrane system involved in platelet prostanoid synthesis and calcium storage and release[19]. The α granules contain

predominantly platelet factor-4, β thromboglobulin, platelet-derived growth factor, fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor-1, P-selectin and von Willebrand factor (vWF). The δ granules are rich in calcium, serotonin and adenosine diphosphate (ADP). (Figure 1.1)

Figure 1.1: Electron Microscopy cross-section of a resting human platelet. Disk is supported by coil of microtubules (MT), lying just under the cell membrane. Organelles including alpha granules (G) and dense granules (DB) are randomly dispersed in the cytoplasm. OCS: open canalicular system, M: mitochondria. Magnified x36 000 (Reproduced from[20]).



Megakaryopoiesis and thrombopoiesis are regulated at multiple levels by different cytokines, however the full development of megakaryocytes requires IL-3 and thrombopoietin (TPO) with TPO the principle regulator[21]. Platelets are then released into the circulating blood in response to regulatory mediators such as interleukins (IL-3, IL-6), thrombopoietin and nitric oxide. Their non-activated discoid shape is due to an actin filament system, which also has a major contribution to the platelets ability to change and become activated.

Platelet aggregation: first described

Platelet aggregation was first recognised in the late 1800s, in a number of pioneering studies including by Osler. However it was Bizzozero, using intravital imaging, who clearly established that small elements of the blood, clumped together at sites of vessel damage[22]. He was the first to demonstrate their role in promoting thrombosis and coagulation. He described the changes platelets underwent in contact with foreign surfaces including the formation of aggregates and "white thrombi" and clearly described the plugging of small vascular punctures by thrombi which was recognised as a major discovery at the time. He further described the platelet adhesion and aggregation sequence followed by fibrin deposition "numerous blood platelets, plus a few red and white corpuscles, stick to the thread, covering it with thick layers. Thereafter, fibrin is deposited on top of the platelet layer which forms flowing, owing to the continuing flow, in long fibrillar bundles". This description of platelet plug formation has been crucial to our understanding of the importance of platelets in arterial thrombosis.

1.3 Physiological modulators of platelet function

Platelets are required to respond appropriately to environments that may be anti- or pro- aggregatory. Their behaviour is influenced by the physiological (and pharmacological) platelet modulators available to them in the blood vessel and their ability to respond to those modulators.

1.3.1 Anti-aggregatory modulators

While anti-aggregatory autocoids may originate within formed elements of blood (including platelets and erythrocytes) and from the adjacent vascular endothelium, healthy endothelium notably produces the anti-aggregatory autocoids prostacyclin[23] and nitric oxide[24]. There is substantial evidence for release of endothelium derived NO[25,26] and PGI_2[26] into the vascular lumen and for their potential interaction with platelet reactivity.

1.3.2 Endothelium

The vascular endothelium is a regulatory organ composed of a monolayer of endothelial cells, which line the luminal surface of every blood vessel. These endothelial cells cover a large surface area which has been estimated as up to 7 metres squared[27]. This creates a dynamic interface between the blood and the muscle wall of the blood vessel that is present in the vessels of every organ system. However, the endothelium of these diverse tissues is heterogeneous with respect to surface phenotype and protein expression[28]. A major role of the endothelium is to produce vasoactive molecules that relax or constrict vessels and

controls vascular tone and diameter. The vasoactive molecules nitric oxide[29] (NO discussed Chapter 1.3.4) and prostacyclin[30] (discussed Chapter 1.3.3) are produced particularly by the endothelium in response to shear stress, the frictional force produced at the endothelial surface by flowing blood (reviewed[31]). In healthy conduit arteries, this increase in arterial flow stimulates "flow mediated dilatation" an increase in the luminal diameter which acts to limit the effect of shear stress. Flow mediated dilatation has emerged as an important indicator of endothelial function in cardiovascular disease (reviewed[32]). Another important endothelial function is to create pro- and anti- aggregatory environments through variable cellular adhesion. Under normal circumstances, quiescent endothelium presents a highly thromboresistant surface to flowing blood.

1.3.2.1 Endothelial regulation of platelet activity.

The endothelium contributes to the regulation of platelet activity by the release of biologically diverse molecules from endothelial cells. Under normal physiological conditions, circulating platelets do not interact with non-activated endothelium. The anti adhesive phenotype of endothelial cells is controlled by three intrinsic pathways (1) the nitric oxide pathway (Chapter 1.3.4), (2) the eicosanoid, arachidonic acid prostacyclin pathway (Chapter 1.3.3) and (3) the ecto-ADPase/CD39/NTPDase pathway (Chapter 1.3.5). During cellular activation nitric oxide is released continuously[33] whereas prostacyclin is released transiently[34]. In healthy vessels the glycocalyx also fulfils a vasculoprotective role. The glycocalyx is formed by the endothelium and is a complex gel located

between the blood plasma and the endothelium that functions as a barrier[35]. Its composition and dimensions fluctuate as it continuously replaces material sheared by flowing plasma[36]. Glycoproteins and proteoglycans create a luminal mesh providing the bulk of the glycocalyx[37]. It provides endothelial cells with a framework to bind plasma proteins and soluble glycoaminoglycans, creating a physiologically active endothelial layer. It has a nett negative charge and repels negatively charged molecules as well as white and red blood cells and platelets[35]. Formation is influenced by shear stress with increased thickness in areas of high shear and thickness increasing in arterial vessels of large diameter[38] compared with those with smaller diameters[39]. There is evidence that damage to the glycocalyx has a role in both acute and chronic vascular pathologies. Acutely glycocalyx "shedding" has been stimulated by release of B-type natriuretic peptide[40] and documented in acute myocardial infarction[41] as well as during coronary revascularisation[42]. It is also increasingly likely that propensity towards atherothrombosis, and especially abnormal platelet-endothelial interactions, may be modulated by impaired homeostasis at the level of the glycocalyx, which lines the luminal surface of the endothelium. For example, in patients with Type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD), there is a significant decrease in arterial endothelial surface charge density compared with controls, findings which are compatible with reduced glycocalyx volume[43]. A mechanism likely to contribute to decreased endothelial surface charge is the shedding of sialic acid from the glycocalyx.

Upon activation endothelium secretes Weibel-Palade bodies, which are storage granules[44] containing P-selectin and von Willebrand factor (vWF), with the latter known to be an important ligand of platelet glycoproteins Ibα and GP IIb/IIIa involved in platelet adhesion and aggregation during vascular injury. This endothelial release of vWF dramatically increases the localisation of platelets to the vessel wall through an interaction with GPIbα[45].

Endothelial Dysfunction

Endothelial dysfunction is a term that encompasses a generalised defect in the homeostatic mechanisms provided by a healthy endothelium. When this balance is disrupted, the vasculature is predisposed to vasoconstriction, platelet activation and thrombosis, the development of atherosclerosis is promoted and increases the likelihood of plaque instability and rupture. Although initial experiments suggested that endothelial dysfunction might be engendered primarily by impaired nitric oxide signalling[46], it is now clear that variable endothelial reactivity also reflects impact of prostanoids, endothelin-1, and possibly other autocoids including endothelium-derived hyperpolarising factor (reviewed[28]). It has been implicated in the pathogenesis and clinical course of all known cardiovascular diseases and is associated with further adverse cardiovascular events[47]. Examples of various causes of endothelial dysfunction are provided in Table 1.1.

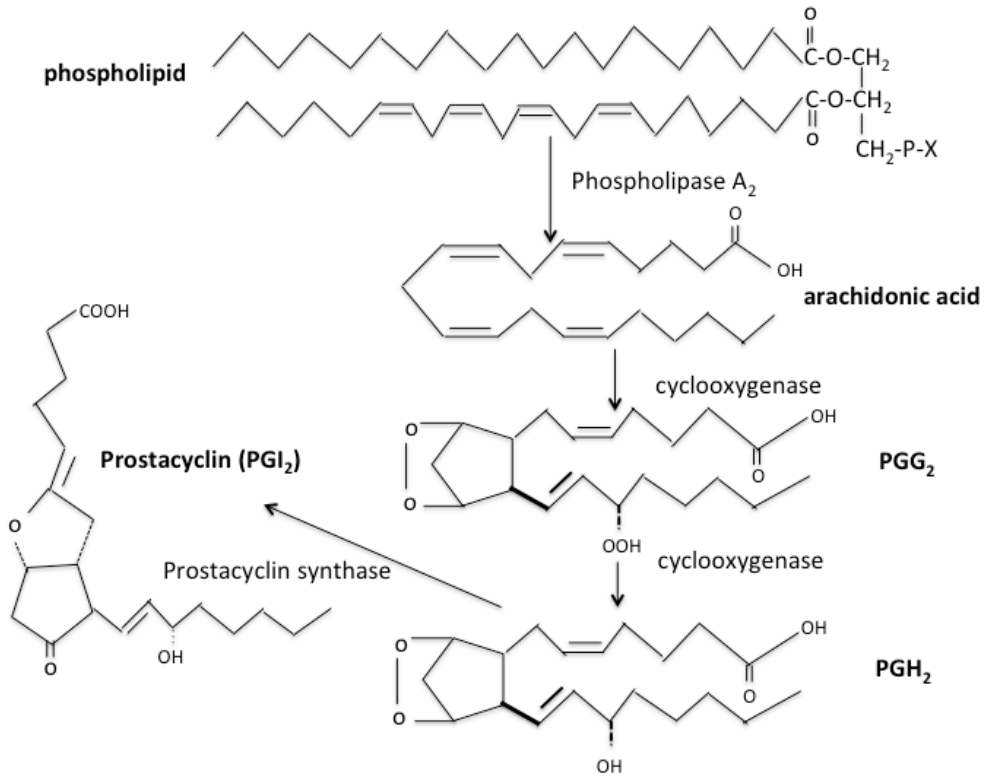
Table 1.1 Mechanisms of endothelial dysfunction.

Mechanism	Examples
A. Decreased formation of NO	• Increased concentrations of asymmetric dimethyl arginine (ADMA) (CAD, CCF, PCOS, aortic stenosis). • Activators of arginases (CAD, T2DM).
B. Increased NO clearance	• O2- release (hyperglycaemia).
C. Decreased response of soluble guanylate cyclase	• Pulmonary hypertension.

1.3.3 Prostacyclin

Prostaglandin synthesis, including prostacyclin (PGI_2) begins with the release of arachidonate acid from membrane phospholipids by phospholipase A_2[48]. Arachidonic acid is then oxidised to hydroxyperoxy endoperoxide (PGG_2) by cyclooxygenase, followed by reduction of PGG_2 to hydroxyendoperoxide (PGH_2) again by cyclooxygenase[23]. The final step is the enzyme prostacyclin synthase converts PGH_2 to PGI_2[48]. As with other prostaglandins and eicosanoids, PGI_2 is a 20-carbon unsaturated carboxylic acid with a cyclopentane ring. (Figure 1.2).

Figure 1.2: Metabolism of prostacyclin from membrane phospholipids.



Following its synthesis and release PGI_2 has effects only locally and is rapidly converted by non-enzymatic processes to an inactive metabolite. PGI_2-producing endothelial cells are positioned between vascular smooth muscle on the basal side and platelets on the luminal side[49]. Its release by endothelial cells affects its local environment, on the basal side of the vessel it causes relaxation and on the luminal side preventing platelet adherence to the vessel wall. Mechanical or chemical perturbations of cell membranes leads to the formation and release of prostaglandins[50], including prostacyclin[51], which are not stored by cells. In the endothelial cell, prostacyclin is generated by pulsatile pressure, and endogenous mediators. Endogenous mediators include plasma-derived substances such as thrombin and bradykinin and chemical stimulants derived from stimulated platelets such as serotonin, platelet derived growth factor (PDGF), interleukin-1 (IL-1) and adenine nucleotides[52].

Prostacyclin binds to the platelet prostacyclin (IP) receptor which recognises both the cyclopentane ring and the side chains of prostaglandins. The PGI_2 binding pocket in the IP is composed of the first to second (ring recognition) transmembrane domains[53] and the seventh (side chain recognition) transmembrane domain[54]. The IP receptor is coupled to a stimulatory G protein (Gs_α) which, via a conformational change, activates adenylate cyclase to produce cyclic adenosine $3',5'$-monophosphate (cAMP). This further activates protein kinase A (PKA). This intraplatelet rise in cAMP[55] has a significant anti-aggregatory effect via its downstream effectors.

1.3.4 Nitric Oxide

Nitric Oxide (NO) is primarily biosynthesised from L-arginine by endothelial nitric oxide synthases (eNOS) in endothelium and platelets, and also transported in/unloaded from erythrocytes[56]. Furthermore, it is now recognised that, especially under hypoxic conditions, plasma nitrate may be reduced by a range of nitrate reductases to form NO[57 58]. NO diffuses across the platelet membrane and acts primarily by binding to the haeme moiety of soluble guanylate cyclase (sGC). Haeme binding activates guanylate cyclase increasing cyclic guanosine 3′,5′ monophosphate (cGMP) which in turn activates protein kinase G (PKG). Recent studies performed in sGC-deficient mice have demonstrated that most of the effects of NO are indeed mediated by sGC and cGMP[59] despite the existence of "sGC-independent" effects the latter mediated by protein nitrosylation[60].

1.3.5 Adenosine

Adenosine is generated following inflammation[61] hypoxia[62] or ischaemia[63], or after injury to cell membranes which leads to marked elevations in extracellular ATP[64] (Figure 1.3). It is a purine nucleoside, generated from the breakdown of ATP released after cellular injury with ectonucleoside triphosphate dephosphorylases (E-NTPDases) such as CD39 serving to dephosphorylate ATP to adenosine 5′ -diphosphate (ADP) to adenosine 5′-monophosphate (AMP)[65] and ectopyrophosphatases/phosphodiesterases (E-NPP) hydrolyse ATP directly to AMP[66]. Adenosine may also be generated via ATP and ADP released from erythrocytes[67,68], platelet dense granules[69] and vascular endothelium[61,70].

Adenosine signals via four G-protein coupled receptors which in turn inhibit or stimulate adenylate cyclase (reviewed[71]). The A_1 and A_3 receptors are coupled to the inhibitory Go and Gi proteins with activation leading to decreased cAMP or increased calcium in the case of A_1 (reviewed[72]). Both A_2 receptors A_{2A} and A_{2B} are coupled to Gs leading to stimulation of adenylate cyclase and a subsequent rise in cAMP. Only A_{2A} and A_{2B} receptors have been found on platelets. Bidirectional adenosine transporters, such as the concentrative transporters (CNTs) and the equilibrative nucleoside transporters (ENTs) facilitate the transport of intracellular adenosine out of cells[72]. They further contribute to its short half-life by rapidly mediating the cellular uptake of adenosine. Adenosine is taken up via erythrocyte ENTs where it is degraded to inosine and hypoxanthine[67] or rephosphorylated to AMP and ADP to ATP by adenosine kinase, ecto-adenosine kinase and nucleoside disphosphate (NDP) kinase respectively (reviewed[73]). Blockade of the ENT-1 receptor has been demonstrated to increase adenosine levels and contribute to a greater antiplatelet effect[74].

Figure 1.3: Adenosine formation after ATP release and uptake and breakdown by erythrocytes.

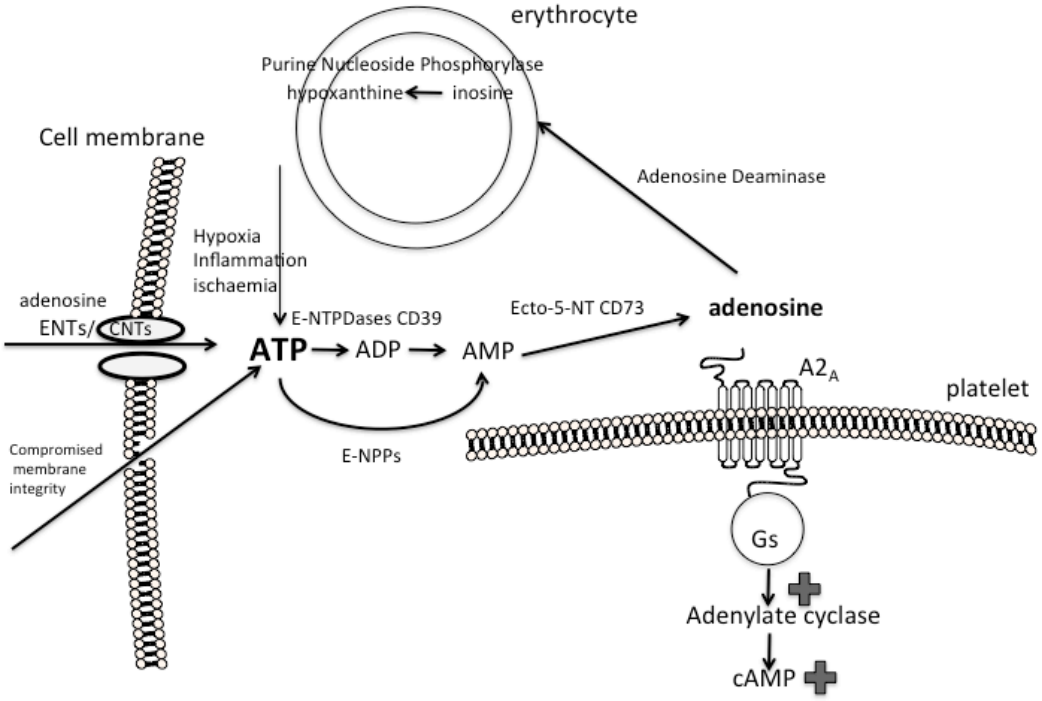


Figure 1.3: Signalling through adenosine receptors $A2_A$ and $A2_B$ leads to increased cAMP. Modified from Johnston-Cox[73] and Heptinstall[67]. Note the current schematic does not include rephosphorylation of adenosine to AMP, ADP and ATP by adenosine kinase, ecto-adenosine kinase and nucleoside diphosphate (NDP) kinase respectively.

A number of other endothelial-derived autocoids have been shown to exert anti-aggregatory effects, although the physiological significance of these is ill defined at present. These include hydrogen sulphide[75] and carbon monoxide[76].

1.4 Pro-aggregatory modulators

Under normal physiological conditions, platelets do not interact with the endothelium because of endothelial anti-aggregatory modulators[23,77]. However once the vessel wall is breached, platelets are required to form a haemostatic plug to prevent excess blood loss. Subendothelial matrix proteins become exposed to the bloodstream, especially collagen and von Willebrand Factor (vWF) and activate specific platelet receptors, GP VI[78] and GP1bα respectively[79]. This tethers the platelets to the breached surface where further binding via collagen and the subendothelial matrix proteins laminin and fibronectin[80] occurs. Once adherent, GP IIb/IIIa is upregulated and binds multiple ligands including fibrinogen[81], fibrin, fibronectin[82] and vWF[83].

Platelets also provide a surface for thrombin generation, which is dependent on the presence of tissue factor (TF)[84]. Thrombin is a potent stimulator of platelet aggregation through its protease activated receptors (PARs) and also generates fibrin, which aids in clot stabilisation. Collagen exposure via its receptor GPVI induces the release of the soluble agonists thromboxane and ADP which act to amplify platelet aggregation including thrombin production. ADP induces a phosphoinositide 3-kinase pathway dependent thrombin generation. Generated

thrombin activates protease-activated receptor 1 (PAR-1) and mediates dense granule secretion and the second wave of platelet aggregation[85]

When an atheromatous plaque ruptures, collagen and TF become exposed to the blood stream and platelet aggregation is initiated[6], leading to thrombosis within the coronary vessel. Attempts to limit this process include the use of dual anti-platelet therapy (DAPT) with aspirin and $P2Y_{12}$ receptor antagonists when combined with coronary artery stenting.

1.4.1 Physical basis for platelet aggregation – focus on $P2Y_1$ and $P2Y_{12}$ receptor based, ADP mediated aggregation.

Platelet aggregation leading to clot formation is a multistep process, which has not yet been entirely elucidated. It is known that ADP plays an important role, and once secreted from the platelet-dense granules, amplifies the responses induced by other platelet agonists and assists in stabilising platelet aggregates. Adenine nucleotides within the platelet dense granules are present in very high concentrations and are released when platelets are exposed to thrombin, collagen or thromboxane A2[86].

ADP based platelet aggregation - importance of $P2Y_1$ and $P2Y_{12}$ receptors.

Platelets possess two P2Y receptors for ADP and co-activation of the $P2Y_1$ and $P2Y_{12}$ receptors is required by ADP for platelet aggregation to occur. The $P2Y_1$ gene is located on chromosome 3q25.2 near the genes coding for $P2Y_{12}$ (3q25.1) $P2Y_{13}$ (3q24) and $P2Y_{14}$ (3q21-25)[87,88]. It has a widespread tissue distribution [89] and is a G coupled protein receptor which stimulates the Gq receptor[90] in response to ADP.

The $P2Y_{12}$ receptor was first cloned by Hollopeter [91] with Northern blotting detecting abundant expression in human platelets and distributed within the amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra and thalamus of the brain[91]. Further studies revealed its expression in vascular smooth muscle cells [92], pancreatic islet cells[93] and other haematopoietic cells such as lymphocytes, monocytes and stem cells[94] and macrophages[95].

The $P2Y_{12}$ receptor is a G protein coupled receptor (GPCR) composed of 342 amino acids. It contains four extracellular cysteines at positions 17, 97, 175 and 270[88, 96]. $P2Y_{12}$ receptors exist as homo oligomers situated in lipid rafts and on treatment with the active metabolite of clopidogrel these are disrupted into non-functional dimers and monomers sequestered outside the lipid raft[97]. The $P2Y_{12}$ receptor has 5 common polymorphisms, 4 in complete linkage disequilibrium and these 4 polymorphisms determine two haplotypes[98] H1 and H2.

Figure 1.4: ADP stimulated platelet aggregation

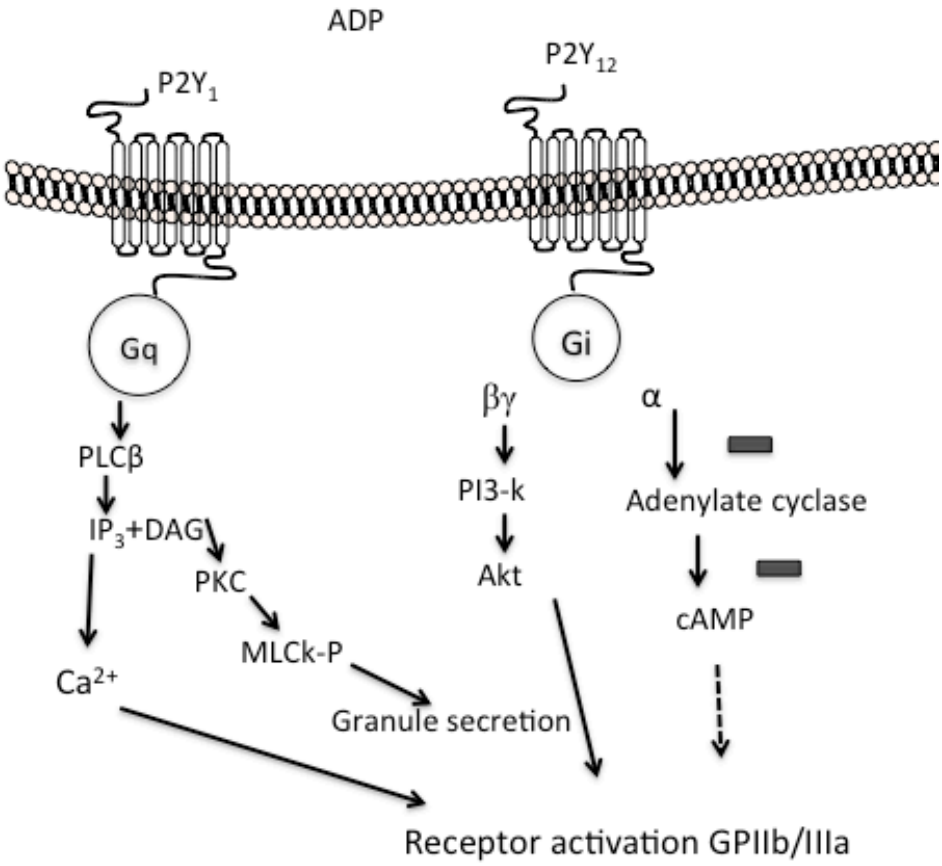


Figure 1.4: Schematic outlining ADP co-stimulated $P2Y_1$ and $P2Y_{12}$ platelet aggregation. Stimulation of the $P2Y_1$ receptor by ADP results in platelet shape change and calcium release and stimulation of the $P2Y_{12}$ receptor results in Giα mediated inhibition of adenylate cyclase and Gβγ activation of PI3-kinases leading to receptor activation of GPIIb/IIIa.This is fully described below in 1.4.1 Role of ADP in platelet aggregation..

1.4.1.1 Role of ADP in platelet aggregation.

ADP is secreted from platelet dense granules after adherence to exposed sub-endothelial collagen[99] and is released from erythrocytes[100]. As outlined in Figure 1.4, the platelet aggregating effects of ADP are mediated via the ADP receptors $P2Y_1$ and $P2Y_{12}$ which are intracellularly coupled to activation of phospholipase Cβ (PLC) and inhibition of adenylate cyclase activity via Gq and Gi respectively[101]. The stimulation of $P2Y_1$/Gq by ADP results in the shape change seen in platelets as they become activated. Co-stimulation of the $P2Y_1$ and $P2Y_{12}$ receptors by ADP is required for sustained platelet aggregation. Both ADP receptors appear to contribute to the activation of the intravascular tissue factor system[102]. Although ADP alone cannot cause the release of platelet dense granules, it greatly amplifies platelet secretion induced by thromboxane[103] and thrombin activating peptide[104].

Activation of the $P2Y_1$ receptor causes a transient increase in the concentration of intracellular ionised calcium, partly by influx of calcium from the medium, but mainly by release of intracellular stores[105]. Through its coupling to Gq[90] and activation of phospholipase Cβ (PLC)[106] this pathway is responsible for the formation of inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG), an activator of protein kinase C (PKC).

The $P2Y_{12}$ receptor is negatively coupled to adenylate cyclase activity through activation of Giα protein[107]. Platelet adenylate cyclase is regulated via G proteins. Agents that increase platelet adenylate cyclase activity and cAMP, such as

prostacyclin and PGE_1 do so through the Gsα subunit while platelet agonists such as ADP and thrombin, which inhibit adenylate cyclase, do so via Giα or in the case of adrenaline Gzα.

Phosphoinositide (PI) 3-kinase (PI3-kinase) is also "downstream" of the $P2Y_{12}$ receptor and its continuous activation through ADP stimulated $P2Y_{12}$ signalling coupled to Giβγ is required for maintaining GP IIb/IIIa receptors in their high affinity state for sustained platelet aggregation[108]. PI3-kinase is also involved in clot stability and is activated by other platelet agonists including thrombin[108]. All Class 1 PI3-kinase enzymes are expressed in platelets[109] and form the lipid second messenger phosphatidylinositol 3,4,5 triphosphate ($PtdIns(3,4,5)P_3$) and phosphatidyl inositol 3,4-bisphosphate ($PtdIns(3,4)P_2$) at the plasma membrane[110]. These PI3-kinase products $PtdIns(3,4)P_2$ and PtdIns 3,4,5 trigger the simultaneous phosphorylation of Akt by phosphadylinositol-dependent kinase 1 (PDK 1)[111]. However, the physiological role of Akt in platelet aggregation is still poorly understood: it is thought to contribute to platelet granule secretion and hypothesised as a regulator of GP IIb/IIIa[112].

Partial blockade of the $P2Y_{12}$ receptor allows ongoing function of these distal signalling pathways. However the relative contributions of each component distal pathway to overall platelet aggregation remains uncertain: indeed it is even less certain to what extent inter-individual variability in distal signalling may occur, and the importance of this putative variability.

1.5 Vasomotor Stimulated Phosphoprotein (VASP)

VASP was first isolated from human platelets[113] but is also highly expressed in variety of other cells and tissues, including vascular endothelial cells[114], vascular smooth muscle cells[114] and fibroblasts[113]. It belongs to a highly conserved family of proline-rich proteins that include the Drosophila protein Enabled (Ena), its mammalian otholog Mena and the Ena-Vasp like protein Evl[115]. These proteins are composed of a central proline-rich domain, and N- and C-terminal domains which are highly homologous and named Ena-VASP homology (EVH) domains 1 and 2[115]. Purified human platelet VASP was discovered to bind to the actin binding proteins zyxin[116] and profilin[117]. Further experiments determined that the EVH1 domain mediates binding of Ena/VASP proteins to proline rich ligands such as vinculin and zyxin[118], whilst the proline rich domain binds to profilin[119].

The platelet cytoskeleton and VASP

The platelet cytoskeleton is composed of three major structures, an actin filament system with a spectrin-based membrane skeleton, a cytoplasmic "core" cytoskeleton and a microtubule-formed ring structure around the platelet plasma membrane edge[17]. The platelet actin cytoskeleton contributes to the disc like shape of the resting platelet, the regulation of shape change, formation of F-actin-based filopodia and lamellopodia and movement of subcellular compartments during platelet adhesion and activation.

Platelet shape change is essential for platelets to become spherical and adherent rather than remaining discoid and non-adherent, and requires remodelling of the resting cytoskeleton and the assembly of new cytoplasmic actin filaments[120]. Actin polymerises to form filaments, and is found in platelets as the globular (G-actin) or filamentous (F-actin) forms[121]. During platelet activation, the proportion of F-actin greatly increases. F-actin has also been shown to regulate dense granule secretion[122].

VASP is found at the leading edge of lamellopodia during the later stage of platelet spreading and is found at the tips of filopodia. VASP promotes F-actin stress fibre assembly and elongation by antagonising capping proteins[123], reduces actin branching activities[124], promoting the transfer of G-actin bound to profilin at the barbed end of growing filaments[123] and increasing the bundling of filaments[125] .

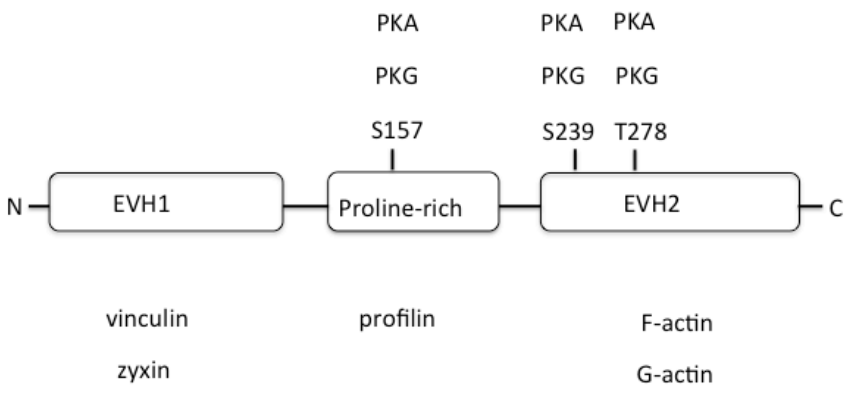
1.5.1 VASP phosphorylation

The phosphorylation of VASP has been demonstrated to abolish its anti-capping activity[123] reduce its association with actin and have negative effects on actin polymeration[126]. Lamellipodial structures retract upon VASP phosphorylation[127] Furthermore, phosphorylation of VASP in human platelets correlates with GP IIb/IIIa receptor inhibition[128].

In response to the cyclic nucleotide-regulating platelet inhibitors NO and prostacyclin, VASP is phosphorylated in the proline-rich region at Ser 157, and in the EVH2 region by Ser 239 and Thr 278. All three of these sites can be phosphorylated by both PKA and PKG. There is, however, some suggestion that phosphorylation at Ser 239 may be selective for NO/PKG[129]. VASP has additional known sites of phosphorylation, but they are not mediated by PKA or PKG.

Figure 1.5: VASP domains, interacting proteins, and PKA and PKG mediated phosphorylation sites.

VASP consists of the EVH1 domain, proline-rich domain and an EVH2 domain. The EVH1 domain interacts with the actin binding proteins vinculin and zyxin, the proline-rich domain with the actin binding protein profilin, and the EVH2 domain with F- and G-actin. Phosphorylation of Serine 157 (S157) occurs in the proline-rich domain, and Serine 239 (S239) and Threonine 278 (T278) in the EVH2 domain. All three sites can be phosphorylated by PKA or PKG[130] (Figure 1.5 adapted from[130]).



Phosphorylated VASP is not detected in basal unstimulated human platelets prior to stimulation with antiaggregatory prostanoids or nitric oxide donors[129]. PGE_1 has been demonstrated to increase cAMP formation accompanied by a rapid conversion of VASP from the dephosphorylated to the phosphorylated form consistent with the site Ser 157[131] detected by Western blotting. While PKA has been described as preferentially phosphorylating Ser 157, PKA stimulated via prostacyclin was also found to phosphorylate Ser 239[129]. PGE_1 was also able to induce a strong phosphorylation at Ser 239 detected by western blotting using the 16C2 monoclonal antibody[132], which is specific for this site alone.

1.5.2 Use of the 16C2 monoclonal antibody to detect Serine 239 VASP phosphorylation.

The 16C2 monoclonal antibody was generated against a phosphorylated peptide RKVS(239)KQE representing the VASP Ser 239 phosphorylation site. The creation of this antibody allowed for detection of VASP phosphorylation in intact cells, where previously it could only be detected using Western blotting. Subsequent experiments determined that detection of VASP phosphorylation in platelets using 16C2 antibody labelled flow cytometry techniques, demonstrated a close correlation with western blot analysis[133]. This close correlation for Ser 239 phosphorylation was found in response to PKA and PKG stimulation using 10μM PGE_1 and 100μM SNP respectively[133].

The availability of a flow cytometry method for the detection of platelet VASP phosphorylation lead to the development of the VASP platelet assay[134] (further described in detail in Chapters 1.9.2.1 and 2.2). ADP induced $P2Y_{12}$ receptor stimulation is associated with dephosphorylation of VASP[135] and inhibition of the $P2Y_{12}$ receptor with $P2Y_{12}$ antagonists is associated with VASP phosphorylation[134]. Data acquired using the VASP platelet assay has been widely published in the literature and furthermore has been used to determine patients that are at risk of further cardiac events through "high on treatment platelet reactivity". (discussed further in Chapter 1.10 "What is the evidence that efficacy is sometimes impaired").

1.6 Disease states with abnormal PGI_2 /cAMP platelet responses.

A rare heterozygous Gs-deficient patient was found to have diminished cAMP release in response to prostacyclin which resulted in hyperreactive platelets and a prothrombotic state[136], with gain of function of Gs associated with increased cAMP levels and a bleeding phenotype[136]. Additionally in human subjects, congenital absence of the $P2Y_{12}$ platelet receptor is associated with an inability of ADP to suppress the prostaglandin E_1-induced increase in platelet cAMP and with defective mobilisation of cytoplasmic Ca^{2+} and a moderately severe bleeding disorder[137].

Furthermore, diseases associated with high incidences of atherothrombotic events such as ischaemic heart disease, diabetes and obesity have been associated with abnormalities in platelet response to PGE_1 and platelet production of cAMP. Patients with stable angina pectoris undergoing PCI when compared with healthy subjects, were demonstrated to require higher doses of PGE_1 to achieve a 50% reversal of platelet aggregation also producing significantly less cAMP when stimulated with the same dose of PGE_1 [138]. Thus, both biochemical and physiological responses to PGE_1, are impaired. Type 2 diabetics were also found to have platelets that were less sensitive to PGE_1 [139] compared with healthy controls and cAMP generation in response to PGE_1 was also less [140]. The platelets of obese subjects were also found to be less sensitive to iloprost and the ability of iloprost to increase platelet cAMP was reduced compared with healthy controls[141] with further studies demonstrating that this loss of response can be restored by weight loss[142]. These findings are consistent with impairment of the cAMP/PGI_2-signalling pathway in these diseases.

1.7 Disease states associated with abnormal NO/cGMP platelet responses.

NO acts on platelets via effects mediated by cGMP after activation of soluble guanylate cyclase. There is considerable evidence that decreased platelet sensitivity to NO and reduced cGMP production is associated with coronary disease. Chirkov et al[13,143] demonstrated that patients with stable angina pectoris (SAP) were hyporesponsive to the anti-aggregatory effects of NO when

compared with normal subjects and produced less cGMP than normal subjects when exposed to the same dose. This was more markedly impaired in patients with ACS when compared with SAP[143] . Type 2 diabetics have also been shown to have decreased platelet NO sensitivity[144] as have subjects with obesity[142,145] and PCOS[9,10]. In diabetics, this NO resistance is modulated by hyperglycaemia[146]. Platelet sensitivity to NO was restored as was blunted cGMP response to NO in a group of obese subjects who successfully lost weight[142].

These abnormalities have been demonstrated in these diseases, which are also associated with premature cardiac aging and endothelial dysfunction, prior to the development of thrombotic cardiac disease. Furthermore, they have also been associated with platelet hyperaggregability, and it has been shown that NO resistance is a separate, additional characteristic of platelet function[147].

Table 1.2 - Pharmacology of commonly used anti-aggregatory anti-platelet drugs

Predominant action	**Drug**	**Mechanism of action**	**Accessory actions**	**Determinants of effect**
$P2Y_{12}$ inhibition	Clopidogrel	Irreversibly binds to ADP binding site of $P2Y_{12}$ receptor		Bioactivation: CYP2C19-dependent
	Prasugrel	Irreversibly binds to ADP binding site of $P2Y_{12}$ receptor		Bioactivation
	Ticagrelor	Reversibly binds to $P2Y_{12}$ receptor blocking signalling of ADP.	Blocks adenosine re-uptake, ENT-1 inhibitor	Twice daily dosing
	Cangrelor	Reversible $P2Y_{12}$ binding		Intravenous only
Cox inhibition	Aspirin	Cyclooxygenase inhibitor		
Phosphodiesterase (PDE) inhibition	Dipyridamole	Inhibits PDE 5 and PDE 3	Blocks adenosine re-uptake, ENT-1 inhibitor	
	Cilostazol	Inhibits PDE3		

Table 1 outlines the mechanisms of effect of the most commonly used agents that are discussed within this thesis.

ENT-1 Equilibrative Nucleotide Transporter-1

1.8 Mechanism of action of P2Y12 receptor antagonist drugs: thienopyridines and directly acting inhibitors.

Thienopyridines

Thienopyridines are pro-drugs that require in vivo hepatic CYP P450 based metabolism to produce their irreversibly binding thiol-containing functional active metabolites. While their mechanisms of action are virtually identical, their metabolism and drug interactions and side effects differ[148]. Ticlopidine and clopidogrel were developed and were in wide clinical use, prior to the discovery of the $P2Y_{12}$ receptor to which they bind[91].

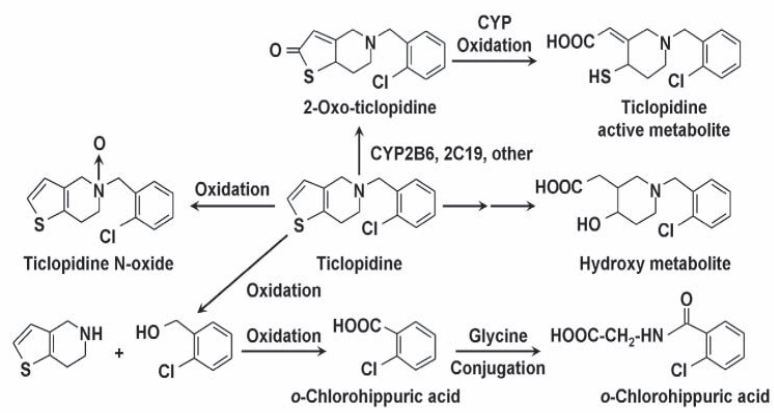
Ticlopidine:- first generation thienopyridine.

Ticlopidine was previously frequently used in the prevention of atherothrombosis. However, in particular, its haematological side effect profile, including bone marrow suppression and thrombotic thrombocytopenic purpura (TTP), lead to the development of clopidogrel and the relative discontinuation of its use.

The CYPs involved in the formation of the ticlopidine active metabolite are unknown[148] and its active metabolite structure was not identified until 2004[149]. The results provided by Yoneda suggested the ticlopidine active metabolite was one-tenth the potency of the other thienopyridines. Ticlopidine irreversibly inhibited ADP effects on PGE_1 mediated cAMP production, with no effect on $P2Y_1$ function[150]. Studies both support[151] [152] and fail to support the finding that

ticlopidine potentiates PGE_1 responses[150]. The metabolic pathways involved in ticlopidine active metabolite formation are outlined in Figure 1.6.

Figure 1.6: Metabolic pathways of ticlopidine[148]



Clopidogrel:- "second generation" thienopyridine

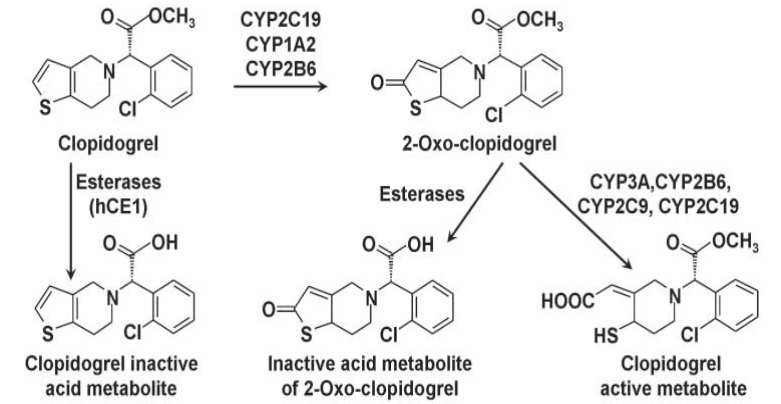
Clopidogrel is absorbed in the intestine where it is transported by ABCB1[153], and 85% is transformed by esterases into inactive metabolites. Clopidogrel is a pro drug and its activation path involves 2 steps (1) cytochrome P450 based oxidation to generate 2-oxo clopidogrel and (2) cytochrome P450 based activation to generate a thiol-based active metabolite that prevents ADP from binding to the $P2Y_{12}$ receptor. These two oxidative steps are both CYP2C19 dependent, with a total of 4 CYPs involved[148] to create the clopidogrel active metabolite.

Functional $P2Y_{12}$ receptors have been demonstrated to exist as oligomers in platelet lipid rafts[97]. These dimerise and become partitioned out of the lipid raft upon binding by the clopidogrel active metabolite. The G protein subunit Giα is also co-located in lipid rafts with $P2Y_{12}$-mediated inhibition of formation of cAMP significantly decreased upon platelet cholesterol depletion[154]. Clopidogrel's thiol derivative active metabolite binds directly to the ADP binding site of the $P2Y_{12}$ receptor, which leads to the prevention of binding by ADP. It binds covalently and irreversibly to $P2Y_{12}$ receptor cysteine residues, most likely cysteine 97[97].

Clopidogrel, via its active metabolite, specifically impairs $P2Y_{12}$ receptor-mediated ADP effects on PGE_1 mediated cAMP production and VASP-P[155], and results in platelets that are more sensitive to the inhibitory effects of PGE_1 [156].

The metabolic pathways involved in clopidogrel active metabolite formation are outlined in Figure 1.7.

Figure 1.7: Metabolic pathways for clopidogrel biotransformation including formation of active metabolite[148]



Prasugrel:- "third generation" thienopyridine

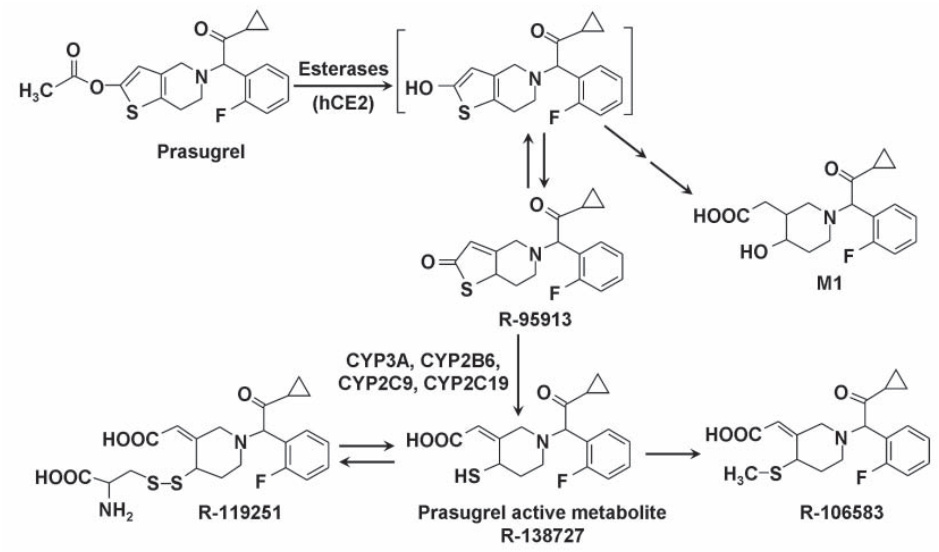
Prasugrel is absorbed in the intestine and undergoes hydrolysis mediated predominantly by the enzyme human carboxylesterase 2 (hCE2) to form the thiolactone R-95913[157]. The thiolactone derivative is then metabolised via a single CYP mediated oxidation step to the prasugrel active metabolite (P-AM)[148] with at least half of the prasugrel dose generating the P-AM, largely in the intestine and the liver with the hepatic CYP-dependent oxidation step in part CYP2C19 dependent.

Patients with moderate liver impairment formed similar amounts of active metabolite, to healthy subjects, suggesting that a significant proportion of active metabolite is formed prior to hepatic P450 oxidation[158]. While the active metabolites of clopidogrel and prasugrel are equipotent, prasugrel's metabolism by intestinal enzymes leads to formation of a greater proportion of active metabolite when compared with clopidogrel, and less dependence on CYP2C19 [148].

The P-AM has been demonstrated to be a specific inhibitor of the $P2Y_{12}$ receptor and to limit the inhibitory effect of ADP on cAMP generation by PGE_1 [159]. At high concentrations a small inhibitory effect on aggregation induced by low dose thrombin and collagen was detected[159]. Blockade of the $P2Y_{12}$ receptor with P-AM has been demonstrated to enhance the ability of PGE_1 to inhibit platelet aggregation[160] and also to greatly increase the anti-aggregatory effect of nitric

oxide[161]. The metabolic pathways involved in prasugrel active metabolite formation are outlined in Figure 1.8.

Figure 1.8: Metabolic pathways for prasugrel biotransformation, including formation of active metabolite [148].



Direct $P2Y_{12}$ inhibitors

Cangrelor

Cangrelor is an ATP analogue with a high affinity for, but reversible binding to, the $P2Y_{12}$ receptor which was developed to be resistant to breakdown by ectonucleotidases[162]. It is administered intravenously and its inhibitory effects are clinically detectable once it has been infused. It is described as a direct $P2Y_{12}$ inhibitor and does not require in vivo metabolism to produce an active drug.

It inhibits not only ADP-induced aggregation, but also that due to thrombin and thromboxane. Like the thienopyridines, it has been demonstrated to potentiate the anti-aggregatory effect of prostacyclin[156,160,163].

Ticagrelor

Ticagrelor is a cyclopentyl-triazolo-pyrimidine, which is a distinct class of anti-aggregatory agent from thienopyridines and ATP analogs[164]. It is not a pro-drug and is a reversible oral inhibitor of the $P2Y_{12}$ receptor. It binds to the $P2Y_{12}$ receptor at a distinct site from the thienopyridine ADP binding site[165]. Ticagrelor is hypothesised to bind to the $P2Y_{12}$ receptor inhibiting the conformational change and G protein activation usually initiated by ADP binding, “locking” the receptor in an inactive state despite ADP binding[165]. Once ticagrelor dissociates from the receptor, the receptor is again functional.

Ticagrelor inhibits the cellular uptake of adenosine via the inhibition of equilibrative nucleoside transporter 1 (ENT1) receptors found on erythrocytes[166]. Ticagrelor induced increases in adenosine levels have been demonstrated via whole blood aggregometry to contribute to ticagrelor-induced platelet inhibition via the A_{2A} receptor. This A_{2A} mediated platelet inhibition therefore represents an additional mechanism of action to that mediated via the $P2Y_{12}$ receptor[74].

However, like other $P2Y_{12}$ receptor antagonists, ticagrelor has also been demonstrated to promote the inhibitory effects of agents that increase cAMP[160] including PGE_1.

1.9. How is the efficacy of $P2Y_{12}$ receptor antagonists assessed?

The biochemical and physiological effects of $P2Y_{12}$ receptor antagonists have been tested largely using platelet aggregometry-based techniques to detect residual platelet aggregation and using VASP based biochemical testing whilst taking the drug. These results have also been placed into clinical context according to whether further atherothrombotic events have occurred at a time when the drug was supposed to still have been taken.

1.9.1 Platelet aggregometry

Platelet Rich Plasma Aggregometry

Light transmission aggregometry was devised by Born, using the platelet response to ADP, with the technique first published in 1962[167] using an aggregometer also devised by the author. Anticoagulated blood is spun to create platelet rich plasma (PRP), which is separated from platelet poor plasma. Using a platelet aggregometer, the PRP is placed into cuvettes and warmed to body temperature, whilst undergoing stirring. The platelet poor plasma is used as a control to represent no aggregation. The principle is that light cannot pass through the platelets when they are in suspended in PRP prior to the addition of an agonist and that once aggregates form light can then pass through and this change can be detected. The change in light transmission is recorded as a percentage. Historically the platelet count has been adjusted between 200 and 300 to enable standardisation. This has recently been contested[168] and it is thought that subjects with a platelet count in the normal range do not require this adjustment. There are variations of this technique including washing platelets prior to aggregometry testing[169]. PRP responses to agonists to can also be measured by impedance.

Whole Blood Aggregometry

A method for detecting platelet aggregation using whole blood was first published in 1980[170]. A new type of aggregometer was devised that measured changes in electrical impedance caused by platelet accretion. The authors describe the technique as follows “Two electrodes are inserted into the platelet

rich plasma or blood. During the initial contact with the platelet containing solution a monolayer of these cells is deposited onto the electrodes. In the presence of aggregating agents, platelets in the bulk phase aggregate onto this monolayer and thus coat the electrodes. This is detected because there is a fall in conductance between the two electrodes".

It has not been possible to standardise the differing platelet aggregometry techniques and methods available in different laboratories. This in part has led to the development of more easily standardised techniques, the most popular of which has been flow cytometry based.

1.9.2 Flow Cytometry based techniques

Flow cytometry is a method to evaluate cell surface or intracellular proteins, through the use of specific fluorescently labelled antibodies binding to cell antigens, which are detected using a flow cytometer. The fluorescently labelled cells are in a liquid suspension of individual cells which passes a laser beam. Capillary forces cause the cells to pass the flow cell and the labelled antibodies are stimulated by the laser light. Light detectors called photomultipliers detect the signal according to the wavelength they emit. Additionally the size and granularity of the cells can be determined by the laser and result in forward scatter which is dependent on cell volume and side scatter which is a result of cell granularity.

1.9.2.1The Platelet VASP Assay

The phosphorylation of VASP is dependent on the level of activation of the $P2Y_{12}$ receptor which in turn is targetted by clopidogrel and other $P2Y_{12}$ antagonists[134]. Dephosphorylation of VASP occurs following $P2Y_{12}$ receptor stimulation by ADP[135]. Conversely, inhibition of the $P2Y_{12}$ receptor by $P2Y_{12}$ antagonists has been shown to induce phosphorylation of VASP in a dose-dependent manner[134]. Hence levels of VASP phosphorylation/dephosphorylation potentially reflect $P2Y_{12}$ receptor inhibition/activation[134].

The Platelet VASP assay was devised to create a standardised flow cytometry assay[134], as previously Western blotting was required to detect VASP-phosphorylation (Chapter 1.5.). A citrated sample of whole blood undergoes incubation with PGE_1 and PGE_1 +ADP for 10 minutes before undergoing fixation with paraformaldehyde followed by permeabilisation with non-ionic detergent. The cells were labelled with a primary monoclonal antibody against serine-239 phosphorylated VASP and followed by a fluorescein isothiocyanate-conjugated polyclonal anti-mouse antibody PE labelled with CD61 to detect platelets. Analysis is then performed on a flow cytometer at a medium rate with the platelet population identified by forward and side scatter with 10 000 platelet events gated. A platelet reactivity index (PRI) was calculated from the median fluorescence intensity (MFI) of the samples incubated with PGE_1 or PGE1+ADP according to the formula:

$$\mathbf{PRI=[(MFIc_{(PGE1)}-MFIc_{(PGE1+ADP)}/MFIc_{(PGE1)}]\ x\ 100}$$

The assay is described by the manufacturer to give stable results for up to 24 hours[134]. While the advantages of this test include being able to collect a small sample of blood which does not require manipulation, the assay itself requires a skilled technician, maintenance of a flow cytometer is expensive and turnaround time is up to 40 minutes.

1.9.2.2 VASP Fix

Very recently developed is VASP Fix which is iloprost rather than PGE_1-based and uses an antibody to detect VASP at Ser-157 rather than Ser-239[171]. The manufacturers state this eliminates the detection of EP3 based signalling, but at this stage the kit has not been linked to outcome data.

1.9.3 Verify*Now* $P2Y_{12}$ Test

The VerifyNow $P2Y_{12}$ Test was developed in an attempt to provide a standardised point-of care test for thienopyridine response. It is a stand-alone cartridge based unit with a mixing chamber which contains the platelet agonist ADP at a concentration of 20μmol/L, PGE_1 (22nmol/L) and lyophilised human fibrinogen coated beads. After citrated whole blood is added to the mixing chamber, the platelets become activated with the activated GP IIb/IIIa platelet receptors binding to the fibrinogen coated beads causing agglutination. The unit also contains a light source and measures the light transmission through the chamber, which increased as the platelets agglutinate to the beads and fall out of the solution. It also contains a second channel containing thrombin receptor

agonist to approximate baseline activity. The test is reported in PRU (P2Y12 Reaction Units), indicating the extent of ADP-mediated aggregation recorded for that sample. Base PRU is calculated using the aggregation response to thrombin with percent inhibition calculated from the PRU result and the BASE result.

1.9.4 Multiple Electrode Aggregometry

Whole blood aggregation can also be performed by the multiplate analyser, which has 5 channels for parallel tests using impedance sensors via electrode wires. The impedance change determined by each sensor is recorded independently with each result transformed into arbitrary aggregation units (AU) plotted against time. Approximately 8 AU correspond to 1 Ohm[172]. These impedance electrodes are linked to a computer and the assay is semi-automated with guided automatic pipetting. Platelet response to multiple agonists can be assessed simultaneously.

1.9.5 The PFA-100 system

The platelet function analyser (PFA) attempts to mimic the conditions in a breached vessel, by measuring the time taken for a platelet plug to close an aperture 150μM wide coated with collagen and ADP or collagen and adrenaline. Anticoagulated blood is aspirated through a capillary tube and the 150μM aperture. Standard PFA-100 has not been shown to be a reliable method for determining $P2Y_{12}$ receptor occupancy. A single study outlining a PFA-100 system which had iloprost added to it (0.5nM) was able to detect clopidogrel administration[173].

1.10 $P2Y_{12}$ antagonists: clinical utility studies: impact on stent thrombosis

1.10.1 Basis for dual anti-aggregatory therapy

There are very clear data for the superiority of dual-antiplatelet therapy (DAPT) in patients undergoing coronary artery stenting when compared with oral anticoagulation. DAPT (thienopyridine and aspirin) vs aspirin and anti-coagulants showed a clear benefit of DAPT not only for the prevention of stent thrombosis, but also a lower risk of myocardial infarction and a reduced need for repeated intervention[174,175]. These initial studies were performed using ticlopidine, with non-inferiority studies later performed with clopidogrel[176].

Additionally, the use of drug-eluting stents to reduce risk of in-stent stenosis resulted in a lack of stent endothelialisation and hence a finding emerging in 2006[177] of increased risk of stent thrombosis: this problem has been partially corrected with more recent types of drug eluting stents [178].

1.10.2 Stent thrombosis

The majority of stent thromboses occur early (0 to 30 days after PCI) although very late stent thrombosis can occur. In clinical practice this translates to an expected rate of early stent thrombosis of $\leq 1\%$ and beyond 30 days of 0.2 to 0.6% per year[179,180]. Acute stent thrombosis often presents as STEMI and emergency revascularisation is required. This is associated with significant mortality of 20-45%, with survivors at a significant risk of recurrence[181].

Currently after stent placement for acute coronary syndrome (ACS), aspirin is advised to be continued indefinitely and $P2Y_{12}$ antagonists and risk of stent thrombosis represents the main clinical basis for the experiments in this thesis. Thienopyridines such as clopidogrel were initially developed as potentially more efficacious than aspirin and indeed the CAPRIE trial[182] established this role. However, more recently, the main clinical role for this group of drugs has evolved to one of prevention of stent thrombosis; for example in the PCI-CURE trial[183].

Since it appears that the prevention of stent thrombosis is the major form of clinical efficacy of clopidogrel and related drugs, data regarding its efficacy or failure of effect then tend to emphasise stent thrombosis as an end-point. This is potentially a problem as regards the utility of clopidogrel in other circumstances (e.g. atrial fibrillation, coronary events in the absence of PCI).

1.11 Clinical evidence for the use of $P2Y_{12}$ antagonists in cardiovascular disease

Clopidogrel

Less clear, is the role of clopidogrel outside of the acute interventional setting. Clopidogrel was first trialled in 1996 with the CAPRIE study (clopidogrel vs aspirin in patients at risk of ischaemic events) which which compared clopidogrel (plus placebo) with 325mg aspirin (plus placebo)[182]. Patients with a previous ischaemic stroke, myocardial infarction or known peripheral vascular disease were eligible. The primary outcome measurements were ischaemic stroke,

myocardial infarction, or vascular death. Clopidogrel was found to be as effective as aspirin in reducing thrombotic events in patients presenting with MI or stroke. However, the group with the greatest benefit from clopidogrel were those with PVD.

The CURE study in 2001 investigated the addition of clopidogrel to aspirin versus placebo in patients with acute coronary syndromes (ACS) and non-ST elevation[184]. The primary outcome was a composite of death from cardiovascular disease, non fatal MI and stroke. In this setting, clopidogrel had clear benefits with endpoints occurring in 9.3% for clopidogrel +aspirin and 11.4% with aspirin alone. The rate of in hospital refractory or severe ischaemia, heart failure and revascularisation was also less with clopidogrel and aspirin than aspirin alone. PCI-CURE investigated patients that had undergone PCI enrolled in the CURE study and found that long-term clopidogrel use was beneficial in reducing major cardiovascular events[183].

In CREDO (2002)[185], patients were assigned a 300mg loading dose of clopidogrel pre PCI than received a 75mg maintenance dose post procedure with an aspirin dose of 325mg. Pre-treating with clopidogrel was not found to have a significant effect, however 1 year of dual anti-platelet therapy was superior to 4 weeks, regarding the subsequent frequency of ischaemic events.

Clarity TIMI (28), in 2005, investigated patients who had presented within 12 hours of the onset of STEMI. Clopidogrel was received as a 300mg loading dose followed by a 75mg maintenance dose. Addition of clopidogrel improved the patency rate of the infarct-related artery and reduced ischaemic complications[186].

The CHARISMA study (2006) investigated the effect of adding 75 mg of clopidogrel to 75-162mg of aspirin alone in patients considered high risk which was defined as documented coronary artery disease, PVD or cerebrovascular disease[187]. Those with documented cardiovascular disease were designated "symptomatic" and those with multiple atherothrombotic risk factors but no evidence of cardiac disease were considered "asymptomatic". Overall, this study was negative[188] although heterogeneity of outcomes within the groups was claimed by the authors.

ACTIVE-A investigated patients with atrial fibrillation at an increased risk for stroke for whom warfarin was considered unsuitable[189]. They were assigned clopidogrel or placebo as well as aspirin (dose 75 to 100mg per day). Fewer major vascular events occurred in those taking clopidogrel and aspirin with fewer strokes and MI, but at the expense of substantially increased major bleeding.

Prasugrel

The TRITON (TIMI 38) study compared prasugrel (60mg loading dose) followed by 10mg per day) versus clopidogrel (300mg loading dose followed by 75 mg per

day) and found evidence of improved outcomes with prasugrel, albeit at the cost of increased major bleeding[190]. In a specific analysis of patients receiving coronary stents (n=12844) within TRITON, prasugrel reduced the frequency of stent thrombosis both for bare metal stents and for drug eluting stents[191].

Ticagrelor

The only substantial comparison between clopidogrel and ticagrelor reported to date is the PLATO study, where patients with acute coronary syndromes were randomised to receive either clopidogrel (loading dose 300-600mg, maintenance dose 75mg daily) or ticagrelor (loading dose 180 mg maintenance dose 90mg twice a day)[192]. There was evidence of improved outcomes with ticagrelor and notably mortality rates were significantly lower than with clopidogrel. Unlike TRITON, bleeding rates were only marginally increased with ticagrelor; the only common adverse effect with ticagrelor was development of dyspnoea a side-effect probably resulting from adenosine accumulation[74].

1.12 What is the evidence that efficacy of anti-aggregatory drugs (e.g. clopidogrel) is sometimes reduced?

There are many studies linking high on-treatment platelet reactivity to post-PCI adverse clinical event occurrences[193]. Furthermore, studies began to emerge using ROC curve analysis to define a threshold at which on treatment platelet reactivity was associated with fewer thrombotic events in patients undergoing PCI[193]. Studies which detected a prognostic value using VASP phosphorylation analysis (VASP PRI)[194-197 198] found an optimal cut-off value of PRI between 48-53%,

irrespective of whether the primary end point was stent thrombosis alone or an agglomerate of thrombotic-type events[193]

Similarly, a cut-off value above 235 and 240 PRU appears to predict increased risk of MACE and stent thrombosis[199 200,201, 202] when using the VerifyNow $P2Y_{12}$ assay. Multiplate (multiple electrode aggregometry) has also been demonstrated using ROC to have a measurable threshold where stent thrombosis is more likely to occur[203].

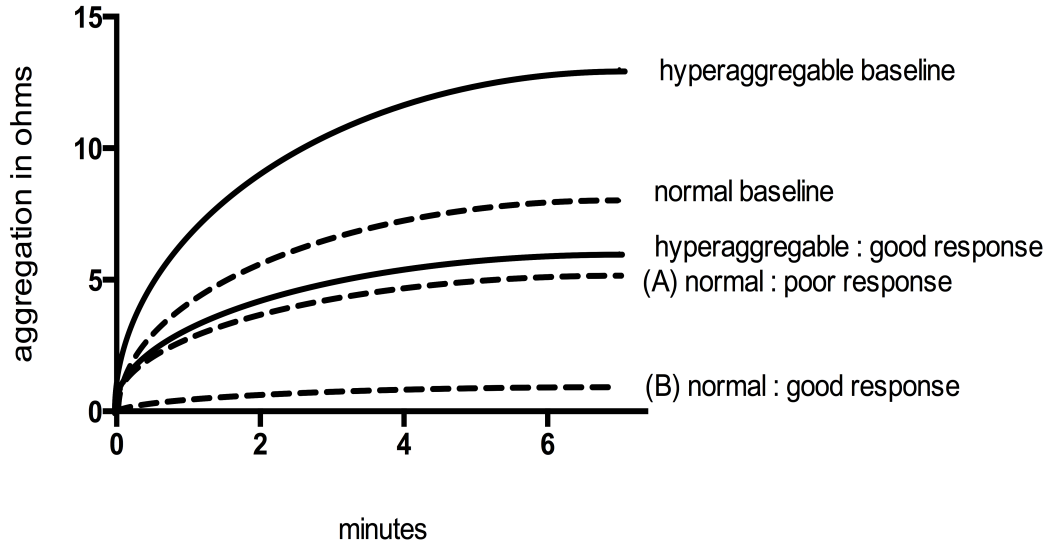
While individual studies involving platelet aggregometry have been linked to post PCI event outcomes[195,199,204-215] the lack of standardisation between laboratories regarding the methodology used, including techniques in platelet preparation and dose of the agonist ADP, means a definitive cut off recommendation cannot be made at present.

1.13 High on treatment Platelet Reactivity

The recent medical literature has delineated "high on treatment platelet reactivity" (HTPR) as a factor predicting increased risk of thrombotic events in patients receiving clopidogrel or analogous anti-aggregating agents[193]. This applies irrespective of whether platelet aggregometry or measures of VASP phosphorylation are utilised, although the precise definitions of HTPR vary (see Chapter 1.12)

What must be appreciated is that HTPR reflects the nett impact of (a) pre-treatment (hyper) aggregability and (b) (presumably defective) anti-aggregatory effects, as depicted schematically in Figure 1.9. Thus HTPR accurately defines clinical risk, but is not a pure increase related to pharmacological effect.

Figure 1.9: Potential causes of "high on treatment platelet aggregation".



Solid lines: Aggregation profile of hyperaggregable blood before and after normal response to treatment.

Dotted lines: Aggregation profile of individual with normal aggregation pre-clopidogrel.

(A) = poor response to clopidogrel

(B) = good response to clopidogrel

Both the normal poor responder and the hyperaggregable good responder have similar on treatment responses (HTPR), but have differing pharmacological responses to treatment.

1.14 What are the postulated mechanisms of high on treatment platelet reactivity? Genomic vs non-genomic mechanisms.

1.14.1 Genomic mechanisms

1.14.1.1 Pharmacogenomics

1.14.1.1.1 Role of CYP2C19 genotype

Considerable interest has surrounded whether the drug's metabolism is a major contributor to high on treatment platelet reactivity. Both activation steps involve CYP2C19 and this genotype has emerged as a cause of clopidogrel "resistance" in patients who had undergone PCI, with those who had loss-of-function alleles found to have rates of stent thrombosis 2 to 3 times that of those without these alleles[216]. Healthy subjects were also found have higher VASP PRI and more platelet aggregation when taking clopidogrel if they had loss-of-function alleles[217]. In healthy subjects, the loss-of-function allele CYP2C19*2 was associated with diminished platelet response to ADP and these subjects were twice as likely to have a cardiovascular ischaemic event or death[218]. Conversely, the CYP2C19*17 allele is associated with an "ultrametaboliser" phenotype, resulting in an increased risk of bleeding and significantly lower ADP-induced platelet aggregation compared with the wild type[219]. However further investigations[220,221] did not support such a pivotal role for CYP2C19 and questioned other causes of thrombotic events whilst taking clopidogrel.

Moreover, high on treatment platelet reactivity is detected in a greater proportion patients with ACS than healthy subjects[222]. However, the results from a number of studies have repeatedly observed that the CYP2C19 genotype distribution in subjects suffering from ischaemic heart disease were not seen to deviate from Hardy-Weinberg equilibrium[153,223,224]. Predominantly the subjects of these trials were of European descent and the distribution of CYP2C19 genotypes were consistent with those reported for white populations.

1.14.1.1.2 Role of ABCB1

Clopidogrel resistance may be based on impaired absorption from the gastro intestinal tract. The ABCB1 gene (previously known as P-glycoprotein) encodes an ATP-dependent drug efflux pump with a broad substrate specificity for xeno-compounds such as drugs. It is extensively distributed and expressed, including in the intestinal epithelium, acting as a physiologic barrier against the absorption of clopidogrel. Patients taking clopidogrel with the ABCB1 TT and CT genotypes had worse outcomes for the variables death, acute MI or stroke compared with the ABCB1 CC alleles[153]. When combined with two loss-of-function genes there was a rate of events five times greater compared with wild type CYP2C19[153].

1.14.1.3 Drug Interactions involving bioactivation.

A number of medications when taken with clopidogrel have been found to result in a decreased inhibitory effect, generally on the basis of inhibition of clopidogrel

bioactivation. However, whether this leads to a change in clinical outcomes is less clear.

1.14.1.3.1 Drug interactions involving CYP2C19

1.14.1.3.1.1 Proton Pump Inhibitors (PPIs).

PPIs are associated with varying degrees of CYP2C19 inhibition. In healthy subjects, a metabolic drug-drug interaction was detected between clopidogrel and omeprazole but not clopidogrel and pantoprazole[225], consistent with omeprazole being the most potent of the PPIs for CYP2C19 inhibition and pantoprazole the least[226].

The Omeprazole Clopidogrel Aspirin (OCLA) study evaluated patients undergoing coronary artery stenting who were to receive dual anti-platelet therapy with aspirin and clopidogrel. These patients were randomised to receive omeprazole for 7 days or placebo. VASP PRI was measured on the day of procedure and on day 7[227]. Baseline mean VASP PRI did not differ between the two groups (83.2% placebo; 83.9% omeprazole), but testing on day 7 found a significant difference with the placebo group having a mean PRI of 39.8% and the omeprazole group 51.4%. Additional data suggest that esomeprazole also induces significant inhibition of clopidogrel response[228]. However, further studies have failed to demonstrate an increased number of cardiac events for patients with ischaemic heart disease taking PPIs when compared with placebo[229].

1.14.1.3.2 Drug interactions Involving CYP3A4

While the importance of CYP3A4 remains uncertain, CYP3A4 activity clearly correlates with bioactivation and with the extent of clopidogrel-induced inhibition of platelet aggregation[230,231]. Primarily the relevance of CYP3A4 status is regarding potential drug interactions.

1.14.1.3.2.1 Calcium Channel Blockers (CCBs)

CCBs are known inhibitors of CYP3A4. In one study, patients with coronary artery disease undergoing PCI taking clopidogrel were compared with a similar group receiving CCBs, with both groups undergoing laboratory testing with ADP induced platelet aggregometry and VASP PRI. The PRI was higher in patients receiving CCBs compared with patients not taking these drugs and decreased platelet inhibition was twice as common in the patients taking CCBs[232]. Thus CCBs may potentially reduce the efficacy of clopidogrel, although outcome data are lacking.

1.14.1.3.2.2 Statins

A number of statins, including simvastatin and atorvastatin are metabolised by CYP3A4 and act as weak inhibitors of the enzyme[233]. A laboratory based investigation demonstrated that atorvastatin impaired platelet responses to clopidogrel[234]. However, clinically conflicting results regarding a significant drug interaction between clopidogrel and CYP3A4 dependent statins has been reported:- patients enrolled in the CHARISMA study underwent secondary analysis reviewing cardiovascular outcomes in patients taking CYP3A4

dependent (CYP3A4-MET) or independent (non CYP3A4-MET) statins[235]. The CYP3A4MET group had greater BMI, and a higher prevalence of hypercholesterolaemia, PVD, prior stroke, carotid endarterectomy and coronary artery bypass grafting. Despite this, no significant difference was found between patients taking CYP3A4-MET and non-MET statins.

1.14.1.4 Mutations of the $P2Y_{12}$ receptor

The $P2Y_{12}$ receptor has 5 common polymorphisms, 4 in complete linkage disequilibrium and these 4 polymorphisms determine two haplotypes H1 and H2[98]. In healthy subjects, the H2 haplotype (T744C) has been associated with higher maximal aggregation in response to ADP, and platelets incubated with iloprost generated less cAMP than in subjects with the H1 haplotype, with all ADP concentrations tested[98]. However, subsequent studies in CAD patients did not find modulation of platelet response to clopidogrel in early or long term phases of treatment[236].

1.14.1.5 Platelet Hyperreactivity.

Platelet hyperreactivity has been detected in healthy subjects, where a small proportion of individuals have been demonstrated to have unusually robust responses to submaximal levels of agonists[237]. These effects were found to be both reproducible and have prolonged duration. Furthermore, the Framingham offspring study demonstrated significant heritability of platelet response to the agonists ADP, adrenaline and collagen, in sibling pairs at low risk for coronary

heart disease[238], which suggests platelet phenotype may have an inherited component.

The platelet responses of asymptomatic family members of subjects with premature coronary heart disease, exhibited moderate to strong heritability of platelet response to the platelet agonists adrenaline and ADP[239] with a significant proportion of these subjects persistently hyperresponsive. This gives further support to the likely existence of genes that modify platelet reactivity. However at this time, knowledge regarding such genes is incomplete.

1.14.2 Non-genomic mechanisms

1.14.2.1 Platelet Hyperreactivity

There is also evidence linking platelet hyperreactivity to atherothrombotic disease including myocardial infarction[240]. A study of survivors of myocardial infarction were assessed for spontaneous platelet aggregation (SPA) three months after myocardial infarction, and then reviewed over a period of 5 years with those positive for SPA having three times the rates of death or further myocardial infarction when compared with those negative for SPA[240]. Additionally, as discussed in Chapter 1.1, platelet hyperreactivity has been associated with ischaemic heart disease[7], Type 2 diabetes[8] and PCOS[9] compared with healthy subjects.

1.14.2.2 Insulin resistance

Insulin resistance, commonly associated with obesity, is also associated with increased cardiovascular risk, even in the absence of the metabolic syndrome[241]. Insulin receptors are found on platelets[242], and insulin has a modulatory role on platelet aggregation[243]. In healthy subjects, platelets pre-incubated with insulin led to reduced ADP-induced Ca^{2+} mobilisation[244] and was able to decrease ADP-induced aggregation responses[245]. Furthermore, insulin attenuated the fall in cAMP caused by thrombin, with this effect occurring through Insulin Receptor Substrate (IRS)-1 and the inhibitory protein of adenylate cyclase, Gi[244]. In contrast, the platelets of subjects with Type 2 diabetes exhibit attenuation of these responses to insulin[245].

The homeostatic model assessment of β-cell function and insulin resistance (HOMA-IR) has been widely utilized in clinical studies since its development in 1985[246]. It is a method for assessing pancreatic β cell function and insulin responsiveness from basal (fasting) glucose and insulin or C-peptide concentration. It was originally calculated using the equation Fasting Plasma Insulin (FPI mU/L) x Fasting Plasma Glucose (FPG mmol/L)/22.5 i.e. HOMA-IR =FPI x FPG/22.5. Low HOMA-IR scores indicate high insulin sensitivity and high scores insulin resistance. Insulin secretion is pulsatile, and repeated measurements over 5 minutes have been incorporated into the model. However a single sample has also been shown to be acceptable if a population estimate is sought[247].

Increasing HOMA-IR has repeatedly been shown to be associated with increased cardiovascular risk[241,248], with or without the presence of the metabolic syndrome and in subjects with Type 2 diabetes mellitus[249]. It has also been associated with an increased mortality risk both overall and from cardiovascular disease[250]. Moreover, in patients with central obesity, previous platelet insensitivity to iloprost and SNP and correspondingly reduced cAMP and cGMP production was significantly improved with weight loss, and these changes in platelet function were statistically correlated with HOMA-IR[142].

In a group of clopidogrel-treated patients post minor ischaemic stroke or TIA, ADP stimulated platelet aggregation in whole blood using multiplate testing and metabolic variables including HOMA-IR were correlated one month after presentation. “Non responders” were found to be significantly more insulin-resistant using HOMA-IR with median HOMA-IR 4.5 and 2.1 in the non-responder vs responder groups respectively[251]. Furthermore, a study of obese subjects treated with clopidogrel or prasugrel suggested that the associated presence of metabolic syndrome predicted lack of attainment of therapeutic VASP PRI: specific comparison with HOMA-IR was not performed[252].

Therefore, there is equivocal evidence linking insulin resistance with poor platelet responsiveness to thienopyridines. However, these data are entirely univariate analyses and no data are available regarding the underlying biochemical mechanisms responsible for this putative association.

1.14.2.3 Diabetes

Diabetes is a disease with a high rate of atherothrombotic complications[253] and well documented platelet hyperreactivity[8]. Furthermore, diabetes has been associated with increased rates of high on treatment platelet reactivity[254 255]. Platelets from Type 2 diabetic subjects exhibit impaired responsiveness to insulin and increased responses to ADP[245]. In platelets from normal subjects ADP dose-dependently reduced iloprost-induced cAMP production and the addition of insulin partly attenuated this effect. In subjects with Type 2 diabetes, the decline of iloprost-induced cAMP formation was significantly greater at the same concentration of ADP and the attenuating effect of insulin was lost[245]. Furthermore, diabetic platelets had decreased sensitivity to the $P2Y_{12}$ antagonist AR-C69931MX (cangrelor). $P2Y_{12}$ activation was therefore described as increased in the platelets of Type 2 diabetic subjects and insulin no longer modulated this signalling pathway. In a separate study, insulin was found to enhance the cAMP responses to PGI_2 in platelets of lean non-diabetic subjects but not in obese insulin resistant subjects[256]. Platelets from diabetic subjects have been found to be less sensitive to the anti-aggregatory effects of PGI_2 [257] and PGE_1[258]. Additionally, the degree of stimulation achieved by the adenylate cyclase activator forskolin in type 2 diabetic patients was lower when compared with healthy subjects[259] . These findings support that the platelet responses of diabetic subjects are impaired with respect to $P2Y_{12}$ signalling, adenylate cyclase stimulation and prostanoid induced cAMP formation.

A number of additional mechanisms have been demonstrated to contribute to the platelet hyperreactivity detected in diabetic subjects. Glycation of platelet surface proteins increases platelet adhesion, with decreased membrane fluidity increasing the tendency of platelets to activate, leading to increased incorporation into thrombi[260,261] . Induction of hyperglycaemia and hyperinsulinaemia in healthy subjects increases platelet reactivity[262]. The platelets of diabetic subjects are less responsive to the anti-aggregatory effects of NO[263]. Genetic variations of Insulin Receptor Substrate 1 (IRS-1), found downstream of the insulin receptor, are associated with different platelet function profiles and can identify Type 2 diabetes mellitus patients with a hyperreactive platelet phenotype[264]. Insulin resistance leads to an increased intracellular calcium concentration and enhanced platelet degranulation and aggregation[265]. Platelets from diabetic subjects have also been demonstrated to have an increased production of thromboxane A2[266]. There is also some evidence that diabetes may be associated with impaired bioactivation of clopidogrel. Erlinge et al, found that patients with diabetes were significantly overrepresented in the clopidogrel and prasugrel "poor responder" groups, and had significantly lower levels of active metabolite then patients without diabetes[254].

1.14.2.4 Cigarette Smoking.

Polyhydrocarbons in cigarette smoke are known inducers of cytochrome P450s including CYP1A2 which is involved in the first oxidative step in the generation of clopidogrel active metabolite. Post-hoc analyses of numerous studies have

shown clinical benefits in a greater proportion of smokers than non-smokers[267] and it has been demonstrated that smokers do generate larger amounts of CYP1A2 dependent active metabolite than non-smokers[268] . However, this beneficial effect has also been demonstrated in patients taking prasugrel and ticagrelor, which are not dependant on CYP1A2 to create active metabolites[267,269].

The PARADOX study[270] also identified a greater effect of clopidogrel in smokers, however it had various limitations. The BMI of non-smokers was significantly less then for the non-smokers with the same dose of clopidogrel given to each group. Furthermore device reported inhibition of platelet aggregation trended lower in smokers compared with non-smokers but this did not reach statistical significance. While a greater production of clopidogrel active metabolite was demonstrated for smokers compared with non-smokers, this was also the case with prasugrel active metabolite. Of additional interest, this study did not find significant differences in the production of active metabolite between patients with or without loss of function mutations of the CYP2C19 gene implying that smoking activates pathways independent of CYP2C19.

1.14.2.5 Increased BMI/body weight

Increased BMI has been associated with greater platelet reactivity in subjects taking clopidogrel[271-274] using ADP-based platelet aggregometry and VASP PRI. However, in these studies, clopidogrel was not dose adjusted according to weight, leaving it unclear as to whether this attenuated clopidogrel effect is a result of

inadequate dose for body weight as a result of fixed dose regimens. Bonello-Palot et al were able to successfully reverse high on treatment platelet reactivity measured via VASP-PRI of >50%, by administering additional loading doses of clopidogrel in subjects with and without loss-of-function CYP2C19 genes. Subjects with a raised BMI were unusual in that they did not respond to this loading dose adjustment[275]. This study supports that additional factors apart from clopidogrel underdosing are involved in the lack of clopidogrel effect seen in those with increased BMI.

Loss of integrity of the cAMP and cGMP platelet pathways occurs in obesity. Platelets of obese subjects have been demonstrated to be less sensitive to the inhibitory effects of prostacyclin and NO donors and to produce less cAMP and cGMP nucleotides in response to these anti-aggregatory autocoids[141]. Additionally, obese subjects had impaired platelet PKA and PKG signalling that was associated with decreased phosphorylation of VASP at Ser 157 and Ser 239 in response to cAMP and cGMP analogs when compared with healthy controls[276]. Weight loss in subjects with central obesity has furthermore been demonstrated to reverse platelet insensitivity to prostacyclin and NO donors and to restore previously blunted platelet cAMP and cGMP production[142].

There is an absence of prospective trials assessing the effect of dose-adjusted clopidogrel on clinical outcomes. A retrospective trial[277], has recently demonstrated that body size is a determinant of active metabolite exposure and residual platelet reactivity for both prasugrel and clopidogrel. Body size was

analysed as body weight, BMI and body surface area (BSA). Platelet reactivity was measured by ADP stimulated platelet aggregometry, PRU and VASP PRI with body weight and BSA demonstrating marginally stronger correlations than for BMI. VASP-PRI demonstrated a stronger correlation with body size than ADP based aggregometry and PRU. Irrespective of which of the body indexes were used, "there was a trend towards a decrease in area under the curve (AUC) as body size increased[277]". This relationship was more marked for prasugrel than clopidogrel. These data support the concept that thienopyridine dosing should include consideration of body size.

1.15 Scope of the present study

We wished to investigate the putative role of post-$P2Y_{12}$ receptor mechanisms of clopidogrel resistance. To do this we attempted to investigate the potential contribution of the following platelet pathways that are known to be downstream of the $P2Y_{12}$ receptor:-

1. PGI_2/ PKA/cAMP pathway, using platelet response to PGE_1 and adenylate cyclase blockade using SQ22536.
2. PI3-kinase, using platelet response to wortmannin.
3. Thrombospondin-1 signalling as a potential means of inhibiting adenylate cyclase signalling.
4. Furthermore given the additive effects of cAMP and cGMP in stimulating the effector pathway of VASP phosphorylation, we also evaluated the possible contribution of integrity of the NO/sGC/cGMP pathway, utilising the NO donor sodium nitroprusside (SNP).

Additionally we wished to investigate

(i) the impact of insulin resistance on clopidogrel response and

(ii) the effect of obesity on clopidogrel response.

Primary objectives of the experiments summarized in this thesis were :

1. To evaluate whether the integrity of the platelet cAMP pathway correlated with clopidogrel effect.

2. To evaluate whether integrity of the platelet cGMP pathway correlated with clopidogrel effect.

Chapter 2: outlines the experimental methodologies used and within this thesis.

Chapter 3: This describes an investigation of the potential role of PI3-kinase signalling as a modulator of clopidogrel responsiveness. Dose response curves for platelet PGE_1 and wortmannin inhibition in healthy subjects were evaluated in order to determine the concentrations that would be applied to the study subjects.

Chapter 4 : This describes an evaluation of determinants of response to clopidogrel after 7 days treatment. Specifically we sought to evaluate:-

i. The extent of influence of pre-clopidogrel platelet sensitivity to PGE_1 and NO donors, compared with variability in CYP2C19 status.
ii. Potential interactions of symptomatic coronary disease and variable BMI with this process.
iii. The effect of a weight-adjusted dosing regimen on clopidogrel response.
iv. Impact of PI3-kinase inhibition with wortmannin as a predictor of clopidogrel response.
v. The impact of insulin resistance as measured by HOMA-IR.
vi. The effect of obesity.

Chapter 5:

This was an evaluation of the potential influence of :-

a) Adenylate cyclase modulation using SQ22536 and its effects on PGE_1 and wortmannin inhibited platelet aggregation and

b) Increased extracellular thrombospondin-1 concentrations in healthy subjects to modulate effects of PGE_1 using ADP based receptor signalling.

Chapter 6

Conclusions and future perspectives.

CHAPTER 2: EXPERIMENTAL METHODOLOGIES

Three main techniques were used to gather data during experiments.

1. Platelet aggregometry including:-
    a. whole blood aggregometry,
    b. platelet rich plasma optical (PRP-optical, also known as Light Transmission Aggregometry [LTA]) and
    c. platelet rich plasma impedance (PRP-impedance)
2. VASP phosphorylation quantification using a flow cytometry based method to determine VASP PRI.
3. PCR to determine patient CYP2C19 loss-of-function genotypes.

2.1 Platelet aggregometry

2.1.1.Specimen Collection

Venous blood was collected from subjects after resting or being supine for at least 15 minutes. 19 gauge venepuncture butterfly needles were used to collect blood from healthy subjects, with blood collection from cardiac patients via a 7 French femoral or 5 French brachial arterial line. Blood was collected from subjects slowly via a syringe and was transferred gently from the syringe against the wall of plastic tubes containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate); with acidified

citrate used to minimise deterioration of platelet function during experiments. The tubes were then gently inverted 5 times to mix the blood and the anticoagulant. Blood samples rested on the laboratory bench for 20 minutes before use for whole blood aggregometry or underwent immediate centrifugation for the preparation of PRP for further use in optical or impedance aggregometry.

2.1.2 Platelet aggregometry methods

Platelet aggregometry was performed in a Chronolog Model 560 dual channel impedance aggregometer or a Chronolog Model 700 four-channel aggregometer (Havertown, PA), for all types of platelet aggregometry methods

Whole blood aggregometry

For whole blood aggregometry, cuvettes with 500µL of physiological saline were prewarmed prior to the addition of 450µL of whole blood. After incubation for 5 minutes, stir bars and electrodes were inserted and calibration performed. Experiments were performed at 37°C with a stirring speed of 900rpm. Baseline responses to 5µM and 2.5µM ADP were obtained as duplicates. Data regarding measurement of platelet aggregation was recorded in ohms (Ω) using the computer programme Aggrolink 8.

Platelet Rich Plasma aggregometry (PRP)- Optical (Light Transmission Aggregometry)

This method requires the collection of platelet rich plasma (PRP) and platelet poor plasma (PPP). Blood samples were centrifuged twice for 10 minutes with the brake off at 800g and platelet rich plasma was collected with a Pasteur pipette and transferred to a new polypropylene tube. The resultant platelet rich plasma was measured to obtain a platelet count using a Sysmex XE-1000, and was adjusted to a platelet count of 250 x 10^9L using platelet poor plasma (PPP). The remaining blood sample then underwent a 3000g spin for 20 minutes to obtain the platelet poor plasma. Optical aggregometry cuvettes were used with PPP used to represent 100% light transmission. Experiments were performed at 37°C with a stirring speed of 900 rpm and data collected using the Aggrolink programme.

Platelet Rich Plasma aggregometry-Impedance.

PRP was collected as described as per the PRP-optical protocol. The resultant PRP was not adjusted for platelet count and underwent the same protocol as per whole blood aggregometry, beginning with 450µL of PRP being added to 500µL of prewarmed saline.

Recording of platelet aggregometry measurements.

Data regarding measurement of platelet aggregation were recorded using the computer programme Aggrolink 8 and were recorded in ohms (Ω) (whole blood aggregometry and PRP impedance) or percentage change (%) (Platelet Rich Plasma optical) with data collected for 7 minutes. The Aggrolink computer interface connected to an IBM compatible computer. Continuous measurements

of the aggregation response allowed the automatic calculation of the maximal amplitude and slope of the aggregation curve.

2.1.3 Addition of reagents and potential modulators of platelet aggregation

All concentrations of prostaglandin E_1 (PGE_1); sodium nitroprusside (SNP), wortmannin and DMSO were incubated for 1 minute before the addition of ADP as the platelet agonist. SQ22536 was added 30 seconds prior to ADP or PGE_1. Thrombospondin- 1 (TSP-1) underwent 15 minute incubations prior to the addition of ADP.

Quantitation of inhibition of ADP-induced aggregation utilised methodology as initially described by Chirkov et al[13]. Essentially, this involves estimation of aggregation with ADP alone in comparison with ADP together with a putative inhibitor of aggregation (see schematic Figure 2.1).

Figure 2.1 Method for determination of inhibition of platelet aggregation using PGE_1.

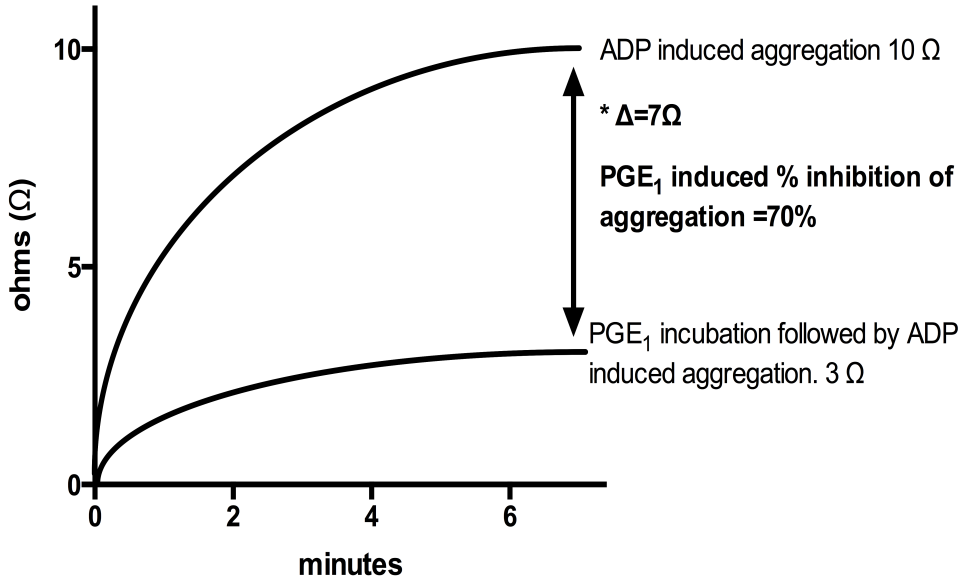


Figure 2.1: To determine inhibition of aggregation, baseline response to ADP is obtained (10Ω). After a 1 minute incubation with PGE_1, the same concentration of ADP induced a 3Ω response. Inhibition of aggregation is calculated as the percent difference between baseline aggregation and aggregation after incubation with PGE_1 (70%).

2.2 VASP-Phosphorylation (VASP-P) evaluation to determine VASP-PRI.

2.2.1 Establishing flow cytometric analysis of VASP phosphorylation

The VASP-P PRI assay was introduced into the Basil Hetzel Institute, The Queen Elizabeth Hospital for the purposes of this thesis. The VASP-P PRI assay is described by its manufacturers as producing stable results if performed within 24 hours of blood sample collection[134]. Studies quoted tested baseline VASP-PRI in healthy subjects and patients with ischaemic heart disease. VASP-PRI was re-estimated in the patients with ischaemic heart disease when taking clopidogrel. Furthermore, Bonello obtained reproducible VASP-PRI results within 24 hours of sample and determined an intraassay coefficient of variation of <5% and interassay coefficient of variation <8%[197]. Studies were performed currently to demonstrate the validity of these coefficients of variation.

Samples from 3 healthy subjects were taken before and after receiving a loading dose of 300mg and a subsequent maintenance dose of 75 mg clopidogrel for one week. These were serially tested in our laboratory in triplicates over a 24 hour period for intraassay assessment and over a 48 hour period for interassay assessment using the VASP-Biocytex kit. The subsequent experiments were performed using the Basil Hetzel Institute Becton-Dickson FACSCanto II analyser over a 24 to 48 hour period both at baseline and after 1 week of taking

clopidogrel. Samples were found to have intraassay (Table 2.1) and interassay (Table 2.2) coefficient of variations consistent with the published literature. These data therefore suggest that the assay is highly reproducible for up to 24 hours post sample collection. In Tables 2.1 and 2.2 for all three subjects, mean values (and CV) are provided for each time point.

Table 2.1: Intraassay coefficients of variability (CV) for VASP-PRI at baseline and after 7 days of clopidogrel (1) within 4 hours and (2) at 24 hours post sampling.

Subject Age/gender	VASP PRI (%)			
	Baseline <4 hours	Baseline 24 hours	Day 7 <4 hours	Day 7 24 hours
26M	79 (3.2%*)	82 (3.1%)	30 (5%)	31 (4.9%)
42M	99 (3%)	93 (5.1%)	58 (3.5%)	54 (4.69%)
62M	81 (3.3%)	87 (4.92%)	61 (4.34%)	62 (4%)

*CV figures (%) are indicated in brackets.

Table 2.2 Interassay coefficients of variability (CV) for VASP-PRI at baseline and after 7 days of clopidogrel (1) within 4 hours of blood sampling, (2) at 24 hours post sampling and (3) 48 hours post sampling.

Subject Age/gender	VASP PRI (%)					
	Baseline <4 hours	Baseline 24 hours	Baseline 48 hours	Day 7 <4 hours	Day 7 24 hours	Day 7 48 hrs.
26M	79 (5%*)	80 (6%)	75 (11%)	29 (7%)	30 (6%)	33 (10%)
42M	96 (7%)	99 (8%)	102 (10%)	54 (9%)	53 (8.6%)	65 (11.4%)
62M	79 (6%)	86 (7%)	84 (10%)	63 (8%)	62 (8%)	70 (9.5%)

*CV figures (%) are indicated in brackets.

2.2.2 Platelet VASP kit reagents.

Platelet VASP kits (Platelet VASP, Diagnostica Stago (Biocytex), Asnieres, France) were stored below 5°C in our laboratory coldroom and used within one month of opening as advised by the manufacturer. The kits contained:-

(i) Reagent 1: 60ml vial of diluent

(ii) Reagent 2a: 1 vial of PGE_1, which was reconstituted, with 400µL of distilled water.

(iii) Reagent 2b: 1 vial of PGE_1 and ADP which also required reconstitution with 400µL of distilled water.

(iv) Reagent 3: 300µL vial of paraformaldehyde fixative

(v) Reagent 4a: 200uL vial of anti VASP-P mouse monoclonal antibody plus permeabilisation agent.

(vi) Reagent 4b: 100µL vial, negative isotypic control (mouse monoclonal antibody) + permeabilisation agent.

(vii) Reagent 5: 300µL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC + platelet counter-staining reagent-PE (anti CD 61-PE) plus permeabilisation agent.

2.2.3 Method -VASP-P Protocol

Whole blood was collected into 3.5 ml 3.2% sodium citrated tubes and a previously standardised flow cytometry assay was applied. All reagent incubations were performed on the laboratory bench at room temperature. After

each step, blood and reagents were homogenised using a vortex set at low speed for 1 to 2 seconds.

1. Ten microlitres of whole blood was pipetted into 3 flow cytometry tubes and incubated with PGE_1 (tube one with the addition of Reagent 2a) or with PGE_1 and ADP for 10 minutes (tube 2 with the addition of Reagent 2b) with the third tube representing a control and also incubated with reagent 2b.
2. All 3 tubes containing whole blood were then fixed with paraformaldehyde (Reagent 3) for 5 minutes, after which they were permeabilised with non-ionic detergent.
3. The whole blood sample was then labelled with a primary monoclonal antibody against serine 239-phosphorylated VASP (16C2): tubes 1 and 2 with Reagent 4a, with tube 3 undergoing incubation with reagent 4b as an isotype control, for a further 5 minutes.
4. In the last step of the antibody staining protocol, a secondary fluorescein isothiocyanate-conjugated polyclonal anti-mouse antibody (Reagent 5) that included a PE labelled anti CD61 antibody, allowing for the detection of platelets, was incubated for 5 minutes at room temperature.
5. Lastly, 2 mls of reagent 1 diluent was then added and the contents of the tubes could then be stored at 2 to 8C for 2 hours before being analysed by flow cytometry if immediate analysis could not be performed.

2.2.4 Instrument set-up and data acquisition.

Training was undertaken for operating the flow cytometer through the Basil Hetzel Institute prior to subject recruitment. Subject analyses were performed on a Becton Dickinson (Plymouth, UK) FACS Canto II flow cytometer (2 lasers, 6 colour configuration) with data acquired and analysed using FACSDiva 6.1.2 computer software program (Becton Dickinson).

To perform the protocol, two cytograms were required, the first using forward scatter log vs side scatter log and the second FL1 log vs FL2 log. Using a medium flow rate, tube 1 was run using forward scatter and side scatter to locate the platelets within the cellular cloud and exclude the lymphocytes from analysis. This represented the "A" region. The FL1 log vs FL2 log cytogram was then gated using the "A" region (Figure 2.2 – Figure 1). The photomultiplier for FL2 was adjusted to get the FL2 cloud at the beginning of 10^3. The FL1 photomultiplier was then adjusted to position the FL1/FL2 cloud at the beginning of 10^2. On this cytogram a "B" region was created to exclude cellular debris (Figure 2.2 – Figure 2). Data was then collected from Tube 1 with at least 5000 events collected from the "B" region. Without any changes to the voltage settings, data was then collected from tubes 2 and 3 (Figure 2.2 - Figures 3 and 4).

Tube 3 acted as a negative control with the fluorescence recorded subtracted from the mean fluorescence intensity (MFI) results obtained by tubes 1 and 2. After

cytometric analysis, the corrected MFI (MFIc) is obtained after subtraction of the MFI value obtained for the negative control to the MFI values obtained for monoclonal antibody staining with anti-VASP-P (Tubes 1 and 2).

MFIc(PGE_1) =MFIc (T1)=MFI (Tube 1)-MFI (Tube 3). This then gives a T1 or MFIc (PGE_1) value with the background fluorescence removed.
MFIc (PGE_1 +ADP)=MFIc (T2) =MFIc (Tube 2) –MFI (Tube 3) This then gives a T2 or MFIc (PGE1 +ADP) value with the background fluorescence removed.

The Platelet Reactivity Index (PRI) is then calculated as a percentage from the median fluorescence intensity (MFI) of the samples incubated with PGE_1 alone or PGE_1 and ADP according to the formula[134]

$$PRI = \frac{MFIc\ (PGE_1) - MFIc\ (PGE_1 + ADP)}{MFIc\ (PGE_1)} \times 100$$

Figure 2.2: Examples of scatter plot settings to detect VASP-P using a Becton Dickinson FACS Canto II flow cytometer (compatible with the FACSCalibur).

Figures obtained on Becton Dickinson instrument, type FACSCalibur :

Fig. 1 : Setting of the "A" region on tube T1

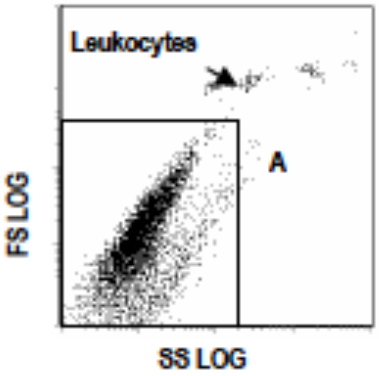


Fig. 2 : Setting of the "B" region on tube T1
(MAb VASP-P, PGE1 condition)

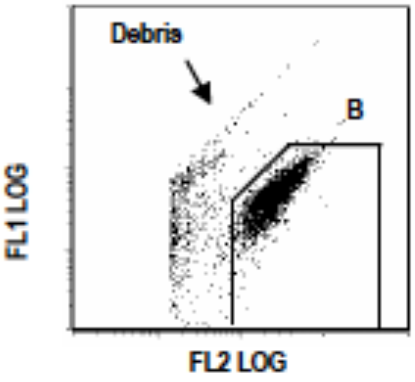


Fig. 3 : Tube T2 analysis
(MAb VASP-P, PGE1+ADP condition)

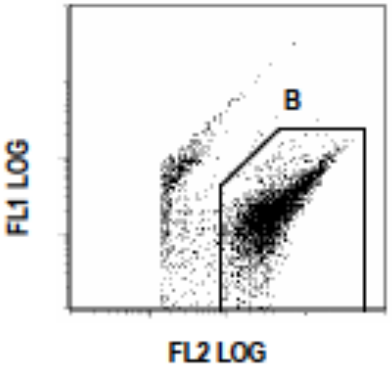
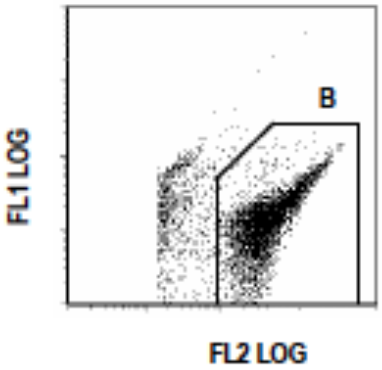


Fig. 4 : Tube T3 analysis
(Negative control, PGE1+ADP condition)



Note : the setting of fluorescence compensation is not required due to the use of the negative isotypic control.
However the setting of fluorescence compensation does not modify the test result.

2.3 Genotyping for CYP 2C19

All information kindly supplied by Healthscope Laboratories.

Sample collection and DNA extraction

Buccal smears were collected from patients with coronary heart disease and healthy subjects. Two swabs were twirled in turn against the lining of each cheek for at least 15 seconds before air drying. The swabs were then kept in a refrigerator until they were transported to a Healthscope laboratory. These swabs then underwent manual deoxyribonucleic acid (DNA) extraction at Healthscope Molecular, Clayton, Melbourne using the QIAmp DNA Blood Mini Kit (QIAGEN N.V., Venlo, the Netherlands). Genotyping was then performed at this site. The CYP2C19 alleles that underwent detection and their reference sample numbers are outlined in Table 2.2.

Table 2.3: CYP2C19 gene single nucleotide polymorphisms (SNPs) that underwent detection and their reference sample numbers.

Allele	RS number	Amino acid change
*2	4244285	681G>A
*3	4986893	636G>A
*17	11188072	-3402C>T
*17	12248560	-806C>T

Massarray Amplification PCR

This step describes the amplification of target loci by multiplex PCR. The amplicons are then purified and used as templates for primer extension. PCR amplification was performed for CYP2C19*2; *3; & *17 from a master mix created from distilled RNAase free water, buffer, $MgCl_2$, dNTPs, Taq polymerase and primer mix which was pipetted into a 384 well PCR plate. Stock CYP2C19 DNA was then added to the master mix. The plate is then sealed and centrifuged. The plate was then placed in a 384 well thermal cycler and underwent the following PCR programme

94°C 15 minutes 94°C-20 seconds

55°C-30 seconds x 45 cycles

72°C -60 seconds 72°C 3 minutes 15°C - ∞

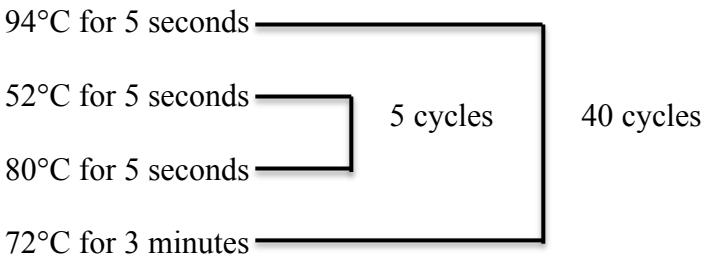
SAP reaction

SAP treatment is performed in order to remove unincorporated dNTPs from amplification products. A SAP mix was prepared composed at distilled RNAase free water, SAP buffer and SAP enzyme. SAP mix was added to each well before sealing the plate and centrifuging. The plate was again placed in a 384 well thermal cycler and run using a 37°C for 40 minutes, 85°C for 5 minutes and 4°C forever PCR program.

Typlex reaction

The primer extension or Typlex (iPLEX) reaction is a universal method for the detection of single-base polymorphisms or small deletions/insertions in amplified DNA. During the iPLEX reaction the primer is extended by one-mass modified nucleotide. A typlex mix composed of distilled RNAase free water, buffer, termination mix, UE primers and Thermosequenase is prepared and then pipetted into each well. The following PCR programme was performed

94°C for 30 seconds



4°C forever

Extended reaction products Cleanup

This clean up step is performed to optimise the spectrometry analysis of the extended products. A cationic resin, SpectroCLEAN, is used to remove ions such as Na^+, K^+ and Mg^{2+} which can result in high background noise when using mass spectrometry. Clean resin is spread into a 384mg dimple plate using a spreader and is then allowed to dry for 20 minutes at room temperature. The 384 well reaction plate has purified water added to each well and is then centrifuged. The resin is transferred to the reaction plate and then centrifuged to pellet the resin.

Spotting Primer Extension Products on Spectro Chips followed by Matrix-assisted laser desorption/Ionisation – time of flight mass spectrometry (MALDI-TOF)

The analytes are arrayed onto a 384-sample Spectrochip using a Sequenom Nanodispenser. The sequenom's SpectroChip is coated with a matrix that allows crystallisation of the PCR product on its surface. The MALDI-TOF principle is that a laser is fired at the crystal which ionises the molecules. These ions travel through a vacuum tube to an ion detector based on their mass. Time of flight measures the difference in time, different molecules hit the detector, and software is used to calculate the mass of the fragments, which correspond to different genotypes.

2.4 Additional Baseline testing: Complete Blood Examination, fasting glucose and fasting insulin levels.

Blood was collected into:

(1) 4 ml K3 EDTA tubes for a complete blood examination (CBE) to determine whether subjects were anaemic or thrombocytopenic prior to aggregometry or clopidogrel administration;

(2) 2ml sodium fluoride/K3EDTA for glucose measurement and

(3) 10 ml serum gel separator tubes for determination of fasting insulin levels.

These bloods arrived at the laboratory within 1 hour of being taken to

ensure that the samples were spun within the advised time to ensure the greatest accuracy.

Testing was performed in the Queen Elizabeth Hospital by the SA Pathology departments of Haematology (CBE) and Biochemistry (glucose) and at the Royal Adelaide Hospital department of Immunology (insulin). The CBE was performed using a Sysmex XE-1000, glucose level was determined using an Olympus 2700 and the insulin level was measured with an ADVIA Centaur XP Immunoassay system.

2.5 Evaluation of platelet responsiveness to inhibitors of aggregation

Determination of IC_{50} values for PGE_1

Concentration-response curves for platelet response to PGE_1 in healthy subjects were constructed using whole blood aggregometry (WBA) and platelet rich plasma (PRP) optical. IC_{50} values were determined by interpolation. These were found to be 3 $x10^{-8}$M for WBA and 2 x 10^{-8}M for PRP. Concentration response curve data are described in full in Chapter 3 (Figures 3.2 and 3.3).

Determination of wortmannin concentrations for use in whole blood aggregometry.

Experiments were undertaken to determine the inhibitory effect of wortmannin on ADP-based aggregation. Serial dilutions of wortmannin with final concentrations 10^{-5}M to 10^{-8} M were pre-incubated for 1 minute prior to the induction of ADP 5μM stimulated platelet aggregation using whole blood aggregometry and platelet

rich plasma impedance and optical. Using whole blood aggregometry, a final concentration of 100nM of wortmannin induced 61% ±25% standard deviation inhibition of platelet aggregation. A concentration of 100nM wortmannin was then used to further investigate subjects in the clinical study. The wortmannin experiments are discussed in full in Chapter 3 in the section regarding mechanistic evaluations.

SNP experiments

No formal determination of SNP responsiveness was undertaken given this has been the subject of previous experiments[13,143]. In the event, $1x10^{-5}$M SNP was utilised in all cases, consistent with these previous findings.

CHAPTER 3: EVALUATION OF PATHWAYS INVOLVED IN PLATELET cAMP AND PI3-KINASE SIGNALLING.

3.1 Introduction and Objectives.

Platelet pathways downstream from the $P2Y_{12}$ receptor are adenylate cyclase mediated via Giα signalling and PI3-kinase mediated via Gβγ signalling (Figure 1.4 and Chapter 1.4.1). We wished to be able to characterise the functionality of these downstream effectors. Adenylate cyclase integrity was investigated using an activator of the adenylate cyclase/cAMP pathway through the use of PGE_1. Attempts were also made to use inhibitors of adenylate cyclase and experiments outlining the use of SQ22536 will be discussed in Chapter 5. In order to investigate the PI3-kinase pathway, the pan PI3-kinase inhibitor wortmannin was selected as a "subtraction" option, largely as there is no convenient activator of this pathway at this time.

Figure 3.1 Known effects of PGE_1 and wortmannin in human platelets. Schematic of the postulated downstream signalling pathways in which we sought to investigate inter-individual pathway integrity.

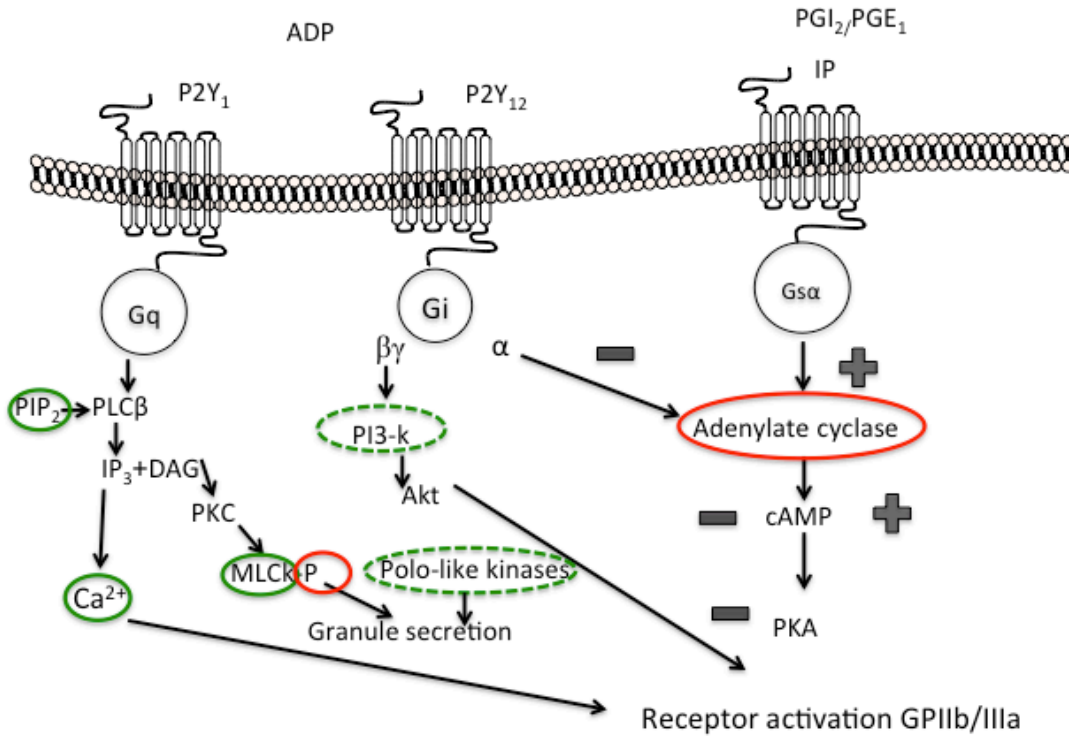


Figure 3.1: Known effects of PGE_1 and wortmannin in human platelets. PGE_1 stimulates adenylate cyclase via the Gsα protein coupled IP receptor indicated by the solid red highlighting and inhibits myosin light chain kinase phosphorylation (MLCk-P) as indicated by the hatched red highlighting. Wortmannin in micromolar (μM) concentrations is known to inhibit (1) myosin light chain kinase, (2) phosphatidylinositol-4,5-bisphosphate (PIP_2) synthesis and (3) attenuate the rise in [Ca^{2+}] caused by calcium entry, as indicated with solid green highlighting. Wortmannin in nanomolar (nM) concentrations is a pan-inhibitor of PI3-kinases and polo-like kinases as indicated with hatched green highlighting. (See Table 3.1)

3.2 Prostaglandin E_1

Prostaglandin E_1 (PGE_1) is a stable vasodilator prostanoid with an inhibitory effect on platelet aggregation via IP receptor/Gsα protein stimulation of cAMP/PKA signalling via adenylate cyclase. It has been widely used in the investigation of human platelets. PGE_1 mediated inhibition of cAMP/PKA signalling has been specifically applied to the investigation of human platelet TSP-1 signalling[278]; myosin light chain kinase regulation[279], ADP-induced intracellular Ca^{2+} responses[156], PKC phosphorylation[280] and congenital absence of the $P2Y_{12}$ receptor[281]. Effects of PGE_1 are similar to those of the more unstable prostanoid prostacyclin, but PGI_2 is substantially more potent[282], and may stimulate adenylate cyclase via slightly different cell surface receptors[283].

3.2.1 Methodological Experiments.

PGE_1 was dissolved in ethanol and frozen at -20C until ready for use where it was diluted into normal saline. Concentration response curves for five healthy controls not on any regular medications who had not taken anti-platelet medications in the previous fortnight were evaluated using whole blood aggregometry and platelet rich plasma (PRP) optical and platelet rich plasma impedance. Baseline platelet aggregation responses to 5μM ADP were determined. Varying concentrations of PGE_1 were then incubated for 1 minute prior to the addition of 5μM ADP. Only 4 subjects had completed dose response curves for PRP as one subject failed to obtain enough PRP to complete the experiment.

3.2.2 Results

Using these platelet aggregometry methods, PGE_1 IC_{50s} in response to 5μM ADP induced aggregation were determined to be 3×10^{-8}M (1.8×10^{-8}M SD) using whole blood aggregometry (Figure 3.2) and 2×10^{-8}M (9×10^{-9}M SD) using PRP (Figure 3.3). These results were in keeping with the IC_{50s} determined in previous studies for whole blood aggregometry[284] and PRP[285].

Figure 3.2: Whole blood aggregometry PGE_1 concentration response curves. Average IC_{50} $3x10^{-8}M$.

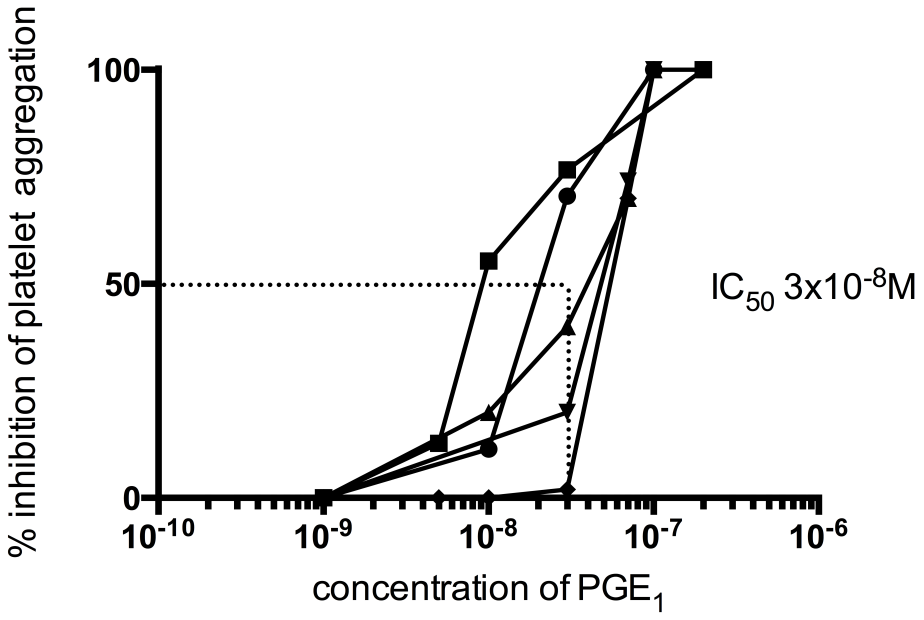
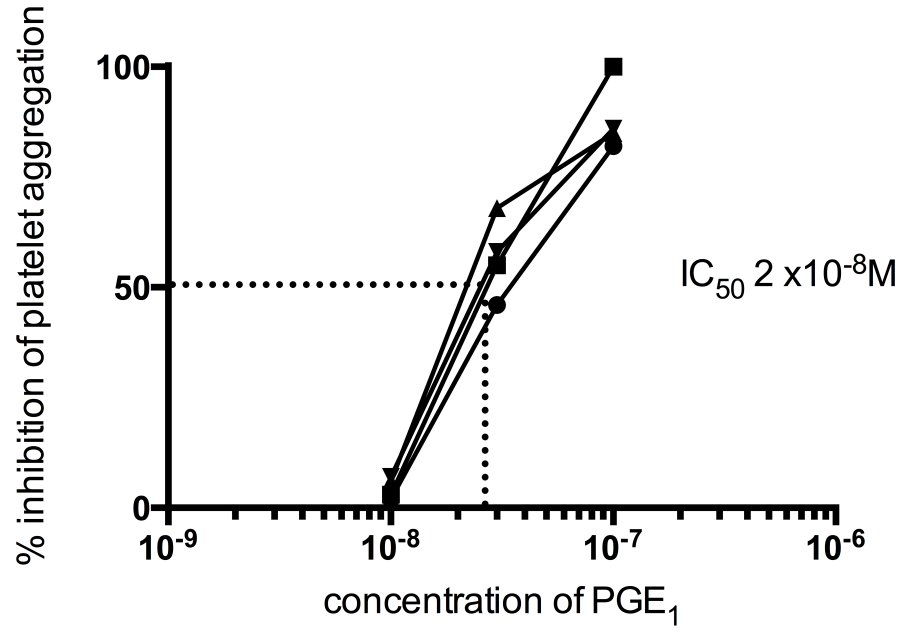


Figure 3.3: Light Transmission Aggregometry (platelet rich plasma based) aggregometry PGE_1 concentration response curves for 4 healthy subjects. Average IC_{50} $2x10^{-8}M$.



3.3 PI3-kinase signalling in platelets

3.3.1 Introduction

Platelet PI3-kinase signalling - role of class 1 PI3-kinase isoforms in platelets.

Phosphoinositide (PI) 3-kinase stimulation represents another downstream effector of $P2Y_{12}$ receptor activation in platelets and its continuous activation is required for maintenance of GPIIb/IIIa in their high affinity state for sustained platelet aggregation and for ongoing clot stability[108]. All Class I PI3-kinases are found in human platelets (Class IA p110α, p110β, p110δ Class IB p110γ) and all form the lipid second messenger (PtdIns(3,4,5)P_3). It has been demonstrated that the PI3-kinase p110β is primarily responsible for Gi-dependent phosphatidylinositol 3,4 bisphosphate (PI(3,4)P2) production in ADP-stimulated platelets[286] with the PI3-kinase p100γ having a co-operative protein scaffolding role[286]. This provides a mechanism for the recruitment of pleckstrin homology domain-containing proteins such as Akt in platelets (reviewed[287]). Regulation of PLC and Ca^{2+} mobilisation by PI3Kβ has also been demonstrated[288]. Additionally, a cooperative role for PI3Kα has been demonstrated with PI3Kβ signalling[289] and it may also be implicated in Akt activation[289]. Given the potential role of multiple isomers of platelet PI3-kinase, the pan-PI3k inhibitor wortmannin was used to investigate ADP stimulated platelet aggregation.

3.3.2 Wortmannin as a platelet kinase inhibitor.

Wortmannin was originally isolated from penicillin wortmanii[290] and is a mycotoxin produced by *Fusarium oxysporum*. It is a lipid and protein kinase

inhibitor and was not shown to be an inhibitor of PI3-kinase until the early 1990s. Its mechanism of action in this regard is to bind covalently with lysine 833 resulting in a large conformational change in the ATP binding site[291]. While its predominant effect PI3-kinase inhibition, it is by no means an inhibitor of this kinase alone[292] (Figure 3.1). The best-documented effects of wortmannin in human platelets are summarised in Table 3.1. It is not however an inhibitor of PKA or PKG[292].

Wortmannin has been demonstrated to inhibit multiple kinases found in human platelets. A concentration of 2 μM wortmannin, attenuated the rise in $[Ca^{2+}]i$ caused by calcium entry while having no effect on the mobilisation of Ca^{2+} from internal stores. It also reduced store-depletion-evoked protein tyrosine phosphorylation. At a lower concentrations (100nM) Ca^{2+} entry was not affected, nor was there an effect on the phosphotyrosine levels[293]. Micromolar concentrations of wortmannin also led to a 50% inhibition of PIP_2 synthesis and α granule release [294].

Polo-like kinases 1 and 3 are also inhibited by wortmannin[295]. Polo-like kinase (Plk) family members are serine/threonine kinases involved in the phosphorylation of proteins involved with cell cycle regulation. Plk-3 has recently been found in activated human platelets and Plk-3 knockout mice have a reduced ability to produce thromboxane in response to thrombin[296] consistent with a role in granular secretion.

Platelet myosin light chain kinase is inhibited by wortmannin at concentrations between 3-6μM[297] . Furthermore, cAMP signalling has been recently shown to regulate platelet myosin light chain phosphorylation, so the blockade of this kinase is certainly of interest to this thesis[279].

Table 3.1 Kinases and pro aggregant signalling blocked by differing concentrations of wortmannin in human platelets.

IC_{50} >1μM	**IC_{50} <1μM**
Myosin light chain kinase[297]	PI3-kinases
50% decrease phosphotidylinositol-4,5-bisphosphate (PIP_2) synthesis and α granule secretion[294]	Polo like kinases I and III[295]
Demonstrated 2 μM wortmannin attenuated store mediated calcium entry, no effect on mobilisation. Decreased tyrosine phosphorylation seen on western blotting[293].	

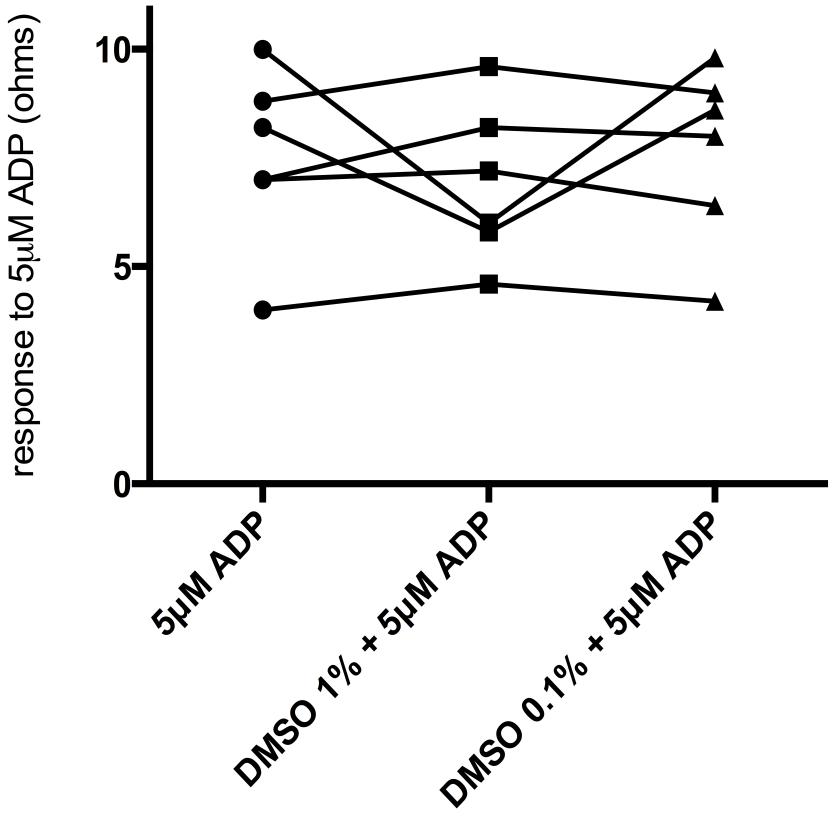
*IC50 wortmannin PI3k is approximately 7 nM[298].

3.3.3 Vehicle issues with wortmannin

Evaluation of Dimethyl sulfoxide (DMSO)

DMSO has been reported to have a variable inhibitory effect on platelet aggregation[299]. As wortmannin required solubilisation in DMSO prior to further dilutions, it was important to identify an acceptable concentration of DMSO that did not affect platelet function when used in whole blood aggregation. Final concentrations of DMSO of 1% and 0.1% were incubated for 1 minute in whole blood prior to platelet aggregation being initiated with 5 μM ADP in 6 healthy subjects. While there was an inhibitory effect of DMSO at a final concentration of 1% in 2 subjects, there was no inhibitory effect when a final concentration of 0.1% DMSO was utilised (Figure 3.4). This concentration was therefore utilised as the diluent in all wortmannin experiments.

Figure 3.4 : Effect of DMSO on 5µM ADP induced whole blood platelet aggregation at final concentrations of 1% and 0.1%.



3.4 Experiments to evaluate the contribution of PI3-kinase signalling on ADP induced platelet aggregation using the pan-PI3-kinase inhibitor wortmannin.

3.4.1 Methodological experiments in healthy subjects not taking clopidogrel using wortmannin

Using a stock solution of 1 mM wortmannin, which had been solubilised in DMSO and frozen at -20C, serial dilutions of wortmannin (final concentrations 10^{-5}M to 10^{-8}M where applicable) were prepared in normal saline after immediate thawing. Stock solution was not again frozen. Care was taken to shield the solutions from light both in the freezer and on the bench given wortmannin's known light sensitivity.

In 6 healthy subjects, experiments were undertaken to determine the inhibitory effect of wortmannin on ADP-induced aggregation. Wortmannin was pre-incubated for 1 minute prior to the induction of 5 μM ADP-stimulated platelet aggregation. Experiments were conducted in whole blood as well as PRP using impedance aggregometry in 3 healthy subjects and PRP-optical aggregometry in a further 3 subjects. Final concentrations of wortmannin ranged between $1x10^{-5}$ and $1x10^{-8}$M.

Wortmannin induced less inhibition of platelet aggregation in PRP using impedance compared with effects utilising whole blood aggregometry in 3

subjects. Similar experiments were performed in a further 3 subjects using whole blood aggregometry compared with PRP-optical aggregometry. Using this technique, there was a relatively minor difference between the inhibitory effects of wortmannin in whole blood and that in PRP-optical aggregation, although the effect in whole blood remained more extensive.

Further experiments using whole blood aggregometry were undertaken, as platelet experiments in the clinical study were to be conducted using this technique. Twenty healthy subjects not taking anti-platelet agents were selected via a human research ethics committee approved email that was distributed using the SA health network. Participant demographics are outlined in Table 3.2

Table 3.2: Participant Demographics: Healthy subjects participating in the wortmannin pilot study.

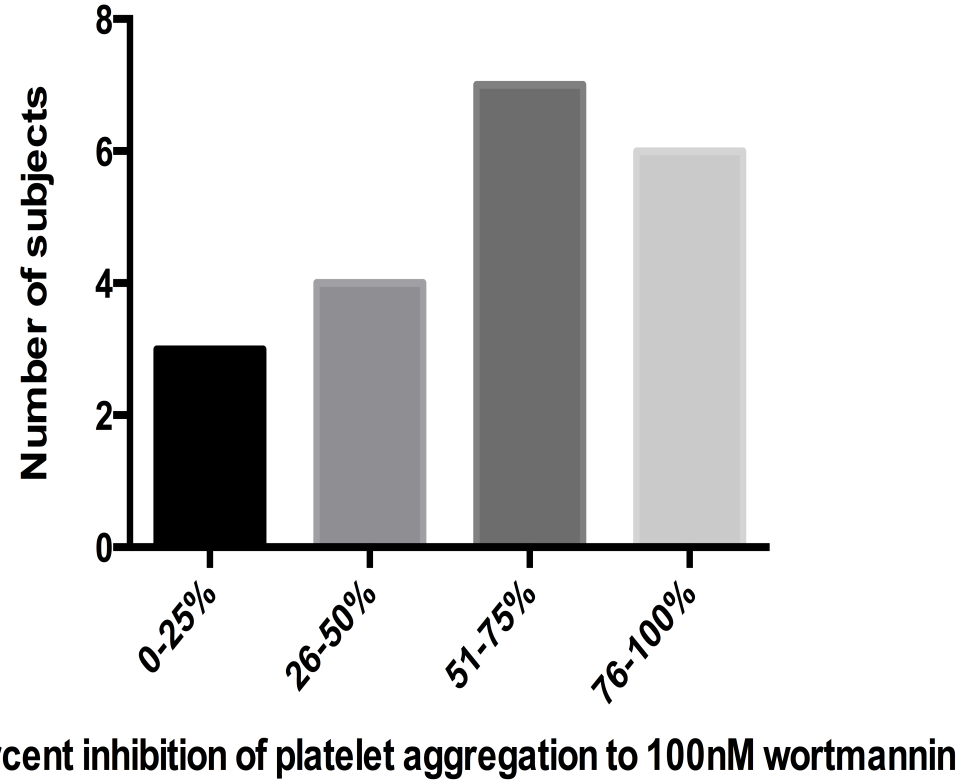
Number of subjects	**20**
Gender	9 male : 11 female
Average age	49.7 years
Age range	29-73 years

3.5 Results

In 20 healthy subjects, a final concentration of 1000 nM wortmannin, had a non-Gaussian distribution with a median of 100% inhibition of 5 μM ADP-induced aggregation, with a range of 68%-100% inhibition. In these same subjects a final concentration of 100 nM wortmannin induced 61%±25% standard deviation

inhibition of platelet aggregation, demonstrating marked heterogeneity between individuals at this concentration (Figure 3.5). In these subjects, a final concentration of 10 nM wortmannin induced a median of 0% inhibition with a range between 0-63% inhibition. Thus, these results were non-Gaussian and a significant number of subjects no longer had evidence of inhibition of platelet aggregation in response to ADP. Therefore the potency of wortmannin in inhibiting ADP-induced aggregation varied markedly between normal individuals, not only on the basis of data at 100 nM, but also as evidenced by markedly differing thresholds for inhibitory effects.

Figure 3.5: Heterogeneity of inhibition of ADP-induced aggregation by wortmannin (100nM). Quartiles of response in the presence of 5μM ADP in 20 healthy subjects shown.



3.6 Discussion:

These experiments were performed to characterise potential heterogeneity in platelet responsiveness among healthy subjects in two effector pathways downstream from the $P2Y_{12}$ receptor using ADP-induced platelet responses. There was indeed considerable heterogeneity between individuals to both PGE_1 and to wortmannin.

The majority of previously published studies regarding inhibitory concentrations of wortmannin in human platelets have been performed in PRP or washed platelets using optical aggregometry, so establishing inhibitory concentrations for use in whole blood aggregometry was of considerable importance. While there is a more substantial literature for PGE_1 IC_{50s} derived from PRP compared with whole blood aggregometry, our results were consistent with previous work using whole blood aggregometry[284].

There are some limitations to this approach. The use of PGE_1 to inhibit ADP induced aggregation assesses the integrity of the IP/adenylate cyclase/cAMP/PKA signalling pathway in its entirety and cannot determine which component of the pathway, is the major source of the variability. It may have been of interest to consider the inhibitory responses of ADP-induced platelet aggregation to forskolin which is a direct activator of adenylate cyclase[300].

Furthermore, as previously summarised (Table 3.1) wortmannin is also not a specific probe, being a pan-inhibitor of PI3-kinase as well as of other kinases found in human platelets. However, the specific contributions of individual platelet PI3-kinase isoforms in platelet aggregation have not been entirely

elucidated, so performing initial investigations potentially targetting all PI3-kinases was not without merit.

It can be concluded that (i) these data demonstrate that the modulation of either cyclic AMP based or PI3-kinase based signalling potentially may exert marked effects on platelet responsiveness to ADP, and that (ii) even among normal subjects, such responsiveness exhibits marked inter-individual heterogeneity. These data therefore provide an impetus for further evaluation of the impact of these signal effector pathways in the presence of cardiovascular disease states.

CHAPTER 4: POST-P2Y$_{12}$ RECEPTOR DETERMINANTS OF CLOPIDOGREL EFFECT

Primary hypothesis: Impact of variable PGE$_1$/cAMP and NO/sGC-based signalling.

Secondary objectives: Investigate impact of variable BMI, symptomatic cardiac disease, PI3-kinase inhibition, insulin resistance and obesity.

4.1 Introduction.

Impairment of the PGI$_2$/cAMP and NO/cGMP pathways is well documented to be associated with ischaemic heart disease[12,13], obesity[145,276] and diabetes[245,263]. Loss of integrity of these pathways represents a potential biochemical mechanism for lack of clopidogrel response, as they represent downstream effectors of the P2Y$_{12}$ receptor. Furthermore, the PGE$_1$/cAMP and NO/cGMP pathways provide crucial inhibition of platelet aggregation. We also wished to investigate the potential role of the PI3-kinase pathway, which is activated via P2Y$_{12}$ signalling.

When measuring clopidogrel effect, studies in patients with ischaemic heart disease, often only measure the response that is detectable when the patient is on treatment with clopidogrel. Thus "high on treatment platelet reactivity (HTPR)[193]" is often used as a surrogate term to imply clopidogrel resistance. This

approach does not take into account the potential heterogeneity in pre-treatment platelet aggregability, nor does this approach distinguish platelet hyperaggregability from impaired response to clopidogrel. We wished to compare baseline and on treatment responses to clopidogrel and thus derive change in platelet response to ADP and in VASP-phosphorylation in order to detect the pharmacological shift present as a result of taking the drug. We then sought to identify correlates of inter-individual variability in response: these would be evaluated by univariate followed by multivariate analyses. The central hypothesis to be tested was that integrity of the post-receptor signalling pathways represented an independent determinant of clopidogrel response, thus we evaluated: (i) PGE_1/adenylate cyclase

(ii) NO/soluble guanylate cyclase

signalling in patients awaiting initiation of clopidogrel treatment.

Furthermore, body weight (and BMI) have also been proposed as influencing clopidogrel response. Rates of clopidogrel resistance in patients with stable angina have been noted to increase with increasing BMI[272], with body weight greater than 80kg also found as an independent correlate of poor clopidogrel response in diabetic patients undergoing stent insertion[301]. In healthy subjects taking clopidogrel, subjects with normal BMI (average 21.9 kg/m^2) VASP PRI >50% was detected in 23% of subjects,[135] whereas another overweight cohort (BMI 27.2 kg/m^2) taking the same loading dose detected VASP PRI >50% in 63%[302].

While these data argue for obesity/increased BMI to represent a state of clopidogrel resistance, in none of the previous studies was clopidogrel dosing weight-adjusted: it therefore remains possible that diminished response to clopidogrel represents primarily or entirely a reduction in concentration of the drug at its site of activation. On the other hand, there are theoretical reasons why obesity might be associated with clopidogrel resistance. Central obesity has been demonstrated to have decreased platelet sensitivity in both the PGI_2/cAMP and NO/cGMP pathways, which was restored by weight loss[142].

There have been very few clinical assessments of clopidogrel response in healthy subjects along the optimal lines of a pre and post treatment comparison as discussed above. Those that have been performed vary between studies for subject weight/body mass index; dosing regimen of clopidogrel and methods chosen to measure clopidogrel response[135,217,302] making comparisons difficult. None of these studies have attempted to adjust clopidogrel dose for variability in body weight. In healthy subjects $P2Y_{12}$ antagonist effect is enhanced in the presence of prostacyclin[163]. These data support the idea that PGI_2 effects may modulate clopidogrel response and begs the question of the role of this pathway in modulating heterogeneity of response.

Given the paucity of studies adjusting clopidogrel dose for weight, assessing clopidogrel effect with pre and post clopidogrel testing and investigating healthy subjects as well as those with ischaemic heart disease, we wished to design a

clinical study that would incorporate these factors. To do so required recruiting a range of subjects with different body weights and heterogeneous platelet responses to PGE_1 and SNP. In view of the known association of obesity[145], angina pectoris[12,14] and advanced age [303] with variable combinations of impairment of PGE_1 and NO-based signalling, we sought to evaluate individuals with and without symptomatic ischaemic heart disease and with variable BMI.

4.2 Objectives of the study

We wished to perform a clinical study to investigate the following **primary hypotheses**

1. That pre-clopidogrel integrity of the platelet PGE_1/cAMP pathway is a genotype-independent correlate of clopidogrel effect
2. That pre-clopidogrel integrity of the platelet SNP/NO/cGMP pathway is a genotype-independent correlate of clopidogrel effect.

Secondary objectives of the study were to investigate

i. the impact of variable BMI, utilising weight adjusted dosing.
ii. symptomatic cardiac disease
iii. The impact of PI3-kinase inhibition with wortmannin as a predictor of clopidogrel response.
iv. The impact of insulin resistance as measured by HOMA-IR
v. Effect of obesity on response to clopidogrel.

4.3 Study design.

4.3.1 Participant selection and recruitment.

In view of the need to maximise heterogeneity of post-receptor signalling, the study was performed in both healthy subjects and patients with known ischaemic heart disease. Patient selection criteria were:

1. Known symptomatic ischaemic heart disease as confirmed by a coronary angiogram.
2. Intention to perform Percutaneous Coronary Intervention (PCI) with associated coronary artery stent insertion of at least one vessel and to initiate clopidogrel therapy.

Recruitment of Participants

Healthy subjects were recruited by local advertisement. Selection criteria were: age >35 years (to correspond with the anticipated age of the patients); and the absence of known ischaemic heart disease. In order to expedite the standardisation of clopidogrel dose per unit while utilising daily doses of either 75mg or 150mg, participants with extremes of weight were excluded: all participants had weights in the range 45kg to 160 kg. Healthy subjects attended the Basil Hetzel Institute opposite the Queen Elizabeth Hospital to undergo venepuncture and have their weight and height obtained to determine the dose of clopidogrel they then received for the following 7 days. They were also consented during this first visit.

With the exception of one patient recruited through the Lyell McEwin Hospital, patients with symptomatic ischaemic heart disease were interviewed, consented and then weighed in the Queen Elizabeth Hospital cardiology catheter laboratory with blood for analysis obtained immediately after the coronary angiogram had been performed. Cardiology patients were followed up the next day in coronary care prior to discharge to ensure they received the correct maintenance dose of clopidogrel on the day of discharge and for the following 7 days. The majority of participants underwent day 7 (on treatment) blood testing at the Basil Hetzel Institute, with a small number of patients with ischaemic heart disease undergoing blood taking in their homes.

For all participants the exclusion criteria were:

1. Currently taking or prior adverse reaction to any $P2Y_{12}$ antagonist.
2. Currently taking oral anticoagulation
3. Current regular treatment with nonsteroidal anti-inflammatory drugs excluding aspirin.
4. Known inherited or acquired bleeding tendency, as determined at pre-enrolment interview.

Protocol Consents: Human Research Ethics Committee (HREC) Approvals and Therapeutic Goods Association (TGA) Approval.

The Lyell McEwin and Queen Elizabeth HRECs approved the protocol. A written informed consent was obtained from each subject for the study with a separate

consent obtained for genotyping, prior to study entry. The study was registered as a clinical trial with the TGA and was given the Clinical Trial Registration Number (CTN) 2011/0404.

4.4 Study Protocol

4.4.1 Overview

(1) Subjects were enrolled after obtaining separate consents for undergoing

i. collection of buccal smears to allow genotyping for CYP2C19 and
ii. venepuncture or blood collection for platelet experiments, fasting glucose and insulin levels for calculation of HOMA-IR.

(2) Subjects' weight in kilograms and their height in centimetres was obtained to allow calculations for body mass index. Weight was used to determine the maintenance dose of clopidogrel administered as per the dose adjusted for weight regimen.

(3) Pre-clopidogrel (baseline) and on treatment platelet experiments were performed "blinded" to the results of CYP2C19 genotyping .

(4) Post-clopidogrel (on treatment) platelet experiments were undertaken after repeat venepuncture after 7 days of clopidogrel therapy adjusted as per the dose adjusted for weight regimen.

4.4.2 Components

CYP2C19 genotyping

In order to identify individuals with loss-of-function mutations, buccal smears were obtained at entry. Results were not known at the time of performing the platelet aggregometry experiments or flow cytometry analysis. The CYP2C19 genotype protocol used specific polymerase chain reactions (PCR) followed by single base extension assays that was read on a MALDI-TOF mass spectrometer (Sequenom MassARRAY San Diego, CA). The following alleles were identified: CYP2C19*1, *2, *3 and *17. Analysis of genotype was performed with extensive and ultrarapid genotypes considered "good metabolisers" of clopidogrel (*1/*1; 1/17; *17/*17); with all other combinations containing loss-of-function genes excluded from the "good metaboliser" group (*1/*2 and *2/17). DNA extraction and genotype identification via PCR was performed by Healthscope Laboratories, Melbourne, Australia.

Pre-clopidogrel assessment of platelet physiological status

Prior to initiation of clopidogrel therapy and potential coronary artery stent insertion, blood was obtained from fasting patients/subjects via a femoral venous line or an antecubital vein. All subjects had been resting or supine for the previous 15 minutes prior to blood collection, as described in Chapter 2.1.1. Blood was utilised for the following investigations:

(i) determination of 2.5μM and 5μM ADP induced aggregation;

(ii) inhibition of 5μM ADP induced aggregation by 30nM PGE_1, and 100nM wortmannin, and 2.5μM ADP induced aggregation by $1x10^{-5}$M sodium nitroprusside (SNP);

(iii) quantitation of VASP phosphorylation (VASP-P).

Furthermore, complete blood examination, blood glucose level and plasma insulin level were routinely obtained from these fasting patients/subjects at baseline. These fasting baseline glucose and insulin levels were used to calculate the HOMA-IR.

Clopidogrel dosing regimen

Clopidogrel hydrogen sulfate was supplied by the Queen Elizabeth Hospital Pharmacy. All of the clopidogrel tablets supplied belonged to the same batch. After completing baseline investigations, clopidogrel therapy was initiated according to a weight-based regimen. All participants weighing 45-80 kg received a loading dose of 600 mg clopidogrel followed by 75 mg daily, while patients weighing >80 kg received the same loading dose of 600mg, but a maintenance dose of 150 mg. This regimen was continued for 7 days, after which ongoing clopidogrel dosage in patients post coronary stenting was at the discretion of the treating cardiologist.

By dose adjusting for weight we were attempting to create a more standardised dose per unit weight regimen. However we were limited by the available formulations of clopidogrel tablets. Using the weight-adjusted regimen, the upper limit of 160kg corresponds to a dose per unit weight of 0.94 which is equivalent

to an 80 kg patient receiving 75mg (dose per unit weight also 0.94). Likewise, administration of 75 mg per day in a 40 kg patient results in a dose per unit weight of 1.85 which corresponds to an 81kg subject receiving 150mg. Therefore the "weight-adjusted dosing regimen" actually permitted two fold variability in dose per unit weight, but this variability was still less than had occurred in many previous studies. Furthermore, given this heterogeneity, we prospectively sought to evaluate the dose per unit weight: response relationship.

Assessment of clopidogrel effect

Follow-up evaluation was performed 7 days post-initiation of clopidogrel therapy. Platelet aggregometry and evaluation of VASP-P were repeated. Clopidogrel effect was expressed as:

a) ΔADP-induced aggregation, i.e., percent inhibition of ADP-induced aggregation when taking clopidogrel compared with baseline ADP-induced aggregation, to determine the pharmacological shift in ADP response.
b) ΔVASP-P, i.e., percent difference between pre-clopidogrel VASP-PRI and VASP-PRI on clopidogrel, to determine the pharmacological shift in VASP-P from baseline.

Platelet experiments.

Whole Blood Platelet aggregometry

Whole blood aggregometry was performed utilising a Chrono-log model 700 CA (Chronolog, Havertown, PA, USA) as previously described (Chapter 2.1).

Baseline and on treatment aggregation responses to both 2.5μM and 5.0 μM ADP were determined. Inhibition of 5μM ADP induced platelet aggregation by 30nM PGE_1 and 100nM wortmannin and 2.5μM ADP induced platelet aggregation by 10^{-5}M SNP were assessed with the incubation of these potential inhibitors for 1 minute prior to ADP. Inhibition of platelet aggregation was calculated as percent reduction relative to the effects of ADP alone. Inhibition of aggregation by clopidogrel was expressed as percent reduction in ADP response after the subject had taken clopidogrel as per the protocol regimen.

Quantitation of VASP-P

VASP-P analysis was performed using a commercial kit (Biocytex, France), with flow cytometry performed using a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Platelet reactivity index (PRI) was calculated as previously described (Chapter 2.2). Testing was performed within 24 hours of blood collection, with the majority of samples processed within 4 hours. VASP phosphorylation was detected at Ser-239 using the 16C2 antibody.

4.4 STATISTICAL METHODS

1. Clopidogrel effect (measured as ΔADP response or ΔVASP phosphorylation) was correlated with the dose-per-unit weight via linear regression, while extent of response for subjects with and without loss-of-function mutations

was compared utilising non-paired Wilcoxon rank sum test for ΔADP and non-paired T-test for ΔVASP-P.

2. Evaluations of responses to PGE_1 and SNP at baseline were utilised to perform linear regression analyses comparing these responses and subsequent responses to clopidogrel therapy.

The power of the study was determined as regards the primary end-point (difference between good and poor responders to PGE_1). There was 84% power to detect >7% difference in ΔADP at $P<0.05$.

Secondary analyses included:

1. Comparisons of demographics and of patients with/without CYP2C19 mutations were performed via an unpaired t-test, unpaired Wilcoxon test or a Chi squared test, as appropriate.
2. Using SPSS software, multivariate analysis evaluated determinants of clopidogrel sensitivity by backwards stepwise multiple linear regression for ΔADP and ΔVASP-P. Parameters evaluated were age, presence or absence of IHD, genotype (good metaboliser vs loss-of-function metaboliser), pre-clopidogrel platelet response to PGE_1, pre-clopidogrel platelet response to SNP, BMI and baseline response to ADP in ohms.
3. Comparisons of clopidogrel effect in normal subjects and patients with IHD were performed via an unpaired t-test.

4. Evaluation of the impact of variable BMI on clopidogrel response was determined by linear regression. Additionally, categorical comparisons were undertaken between individuals ≥80kg in weight vs those weighing less than 80kg as well as obese vs non obese subjects, in order to determine whether obesity/increased BMI correlated with impaired PGE_1/NO platelet responses.
5. All statistical correlations of wortmannin effect with other parameters of platelet reactivity were performed utilising wortmannin concentrations of 100nM, thus avoiding correction for multiple comparisons. In particular correlations were sought between extent of inhibition of ADP response with wortmannin versus extent of PGE_1 and SNP-induced inhibition and clopidogrel effect.
6. Impact of insulin resistance. HOMA-IR was analysed via linear regression ΔADP, ΔVASP-P, pre-clopidogrel platelet response to PGE_1 and pre-clopidogrel response to SNP. Multivariate analysis via linear regression was performed using SPSS with ΔVASP-P as the dependent variable and pre-clopidogrel PGE_1 response, pre-clopidogrel SNP response, genotype and HOMA-IR as the independent variables.
7. Effect of obesity on response to clopidogrel. ANCOVA was performed for ΔADP and ΔVASP-P and pre-clopidogrel PGE_1. Unpaired T-testing was performed for HOMA-IR in non-obese vs obese subjects.
8. Tests of proportions were performed utilising Chi test with Yates continuity correction or Fisher's exact test as appropriate.

All data were expressed as mean ± SD, unless otherwise stated. All comparisons were 2-sided. Statistical analyses were performed using SPSS and GRAPH PAD PRISM 6 softwares.

4.5 RESULTS

As the objectives of this study were multiple and overlapping, data for each component are presented separately, beginning with the primary hypothesis (related to the impacts of adenylate cyclase/soluble guanylate cyclase signalling) and then preceding to the secondary hypotheses of:

(1) impact of variable BMI
(2) impact of symptomatic IHD
(3) potential impact of variable PI3-Kinase signalling (a substudy of participants from the clinical study).
(4) Impact of insulin resistance as measured by HOMA-IR
(5) Effect of obesity on response to clopidogrel

Throughout the graphs in chapter 4, (except for Figures 4.4A and B which represents all participants of the study) healthy subjects are denoted with red data points and patients with IHD black data points.

Participant demographics

Patients with IHD (n=22) undergoing non-emergent PCI and healthy subjects (n=31) were studied. Participant demographics are outlined in **Table 4.1**. Notably, none of the healthy subjects and all of the patients were receiving regular low dose aspirin therapy. Loss-of-function genotypes were identified in 13/31 healthy subjects and 7/22 patients with IHD. Ninety-eight percent of participants were of European ancestry.

Table 4.1: Participant Demographics – the clinical study

	HEALTHY SUBJECTS		**IHD**	
Genotypes	**All** (n=31)	**Good metaboliser** (n=18)	**All** (n=22)	**Good metaboliser** (n=15)
Median age (years)	49	44	64	64
Age range (years)	37-74	40-63	48-85	48-83
Gender (M: F)	17:14	12:6	13:9	8:7
Received double dose clopidogrel (n)	15	8	7	4
BMI median (kg/m^2)	27.5	24.1	26.6	25.7
BMI range (kg/m^2)	17.4-43.3	17.4-33.3	18.7-49.4	18.7-49.4
Potentially interacting pharmacotherapy				
PPIs	3%	0%	23%	26%
Fat soluble statins	3%	0%	27%	13%
CCBs	3%	0%	55%	53%
Current smoker	3%	0%	18%	13%
T2DM	3%	0%	9%	20%
BMI>30 (kg/m^2)	26%	11%	32%	33%

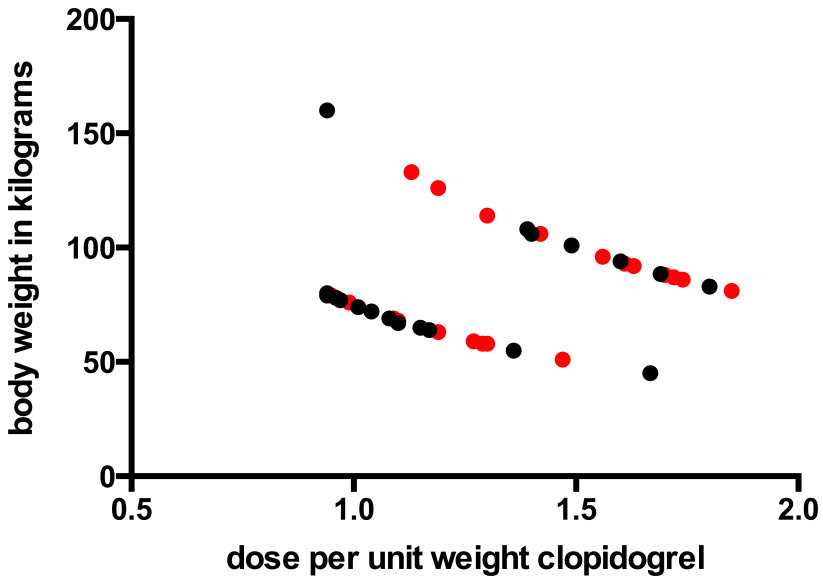
PPI = Proton pump inhibitor
CCB = calcium channel blocker
T2DM =Type 2 diabetes mellitus
BMI=Body Mass Index

4.5.1 Impact of dose-adjustment strategy:

Note heterogeneity of dose per unit weight relationship.

Twenty two subjects of the 53 enrolled subjects received 150mg or "double dose" maintenance clopidogrel as per the dosing by weight protocol for subjects with a weight >80kg. The use of a weight adjusted dosage regimen sought to minimise the "dilutional" effect of increased body weight on clopidogrel response (Figure 4.1).

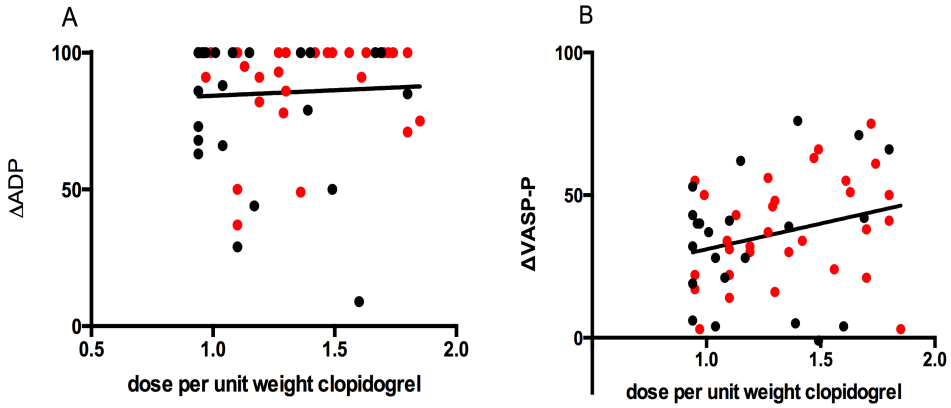
Figure 4.1: Subject clopidogrel dose per unit weight, according to body weight in kilograms.



Relationship of clopidogrel dosing with effects

Figure 4.2: Impact of clopidogrel dose per unit weight on measures of clopidogrel effect.

There was no significant impact of dose per unit weight on ΔADP (A). However, when response was measured as ΔVASP-P (B), there was a trend towards a dose response relationship (P=0.06).



4.5.2 Heterogeneity of platelet responsiveness to PGE_1

Platelet responsiveness to the inhibitory effects of 30nM PGE_1 assessed in vitro immediately prior to initiation of clopidogrel therapy, varied widely. Mean response was 70±28 (SD)% inhibition of aggregation, while the range of responses was 10% to 100%. Figure 4.3 illustrates examples of this variability, and corresponding responses to clopidogrel therapy. Both subjects were found to be homozygous for CYP2C19 *1/*1.

Figure 4.3: Heterogeneity of platelet responsiveness to PGE_1: basis for key evaluations.

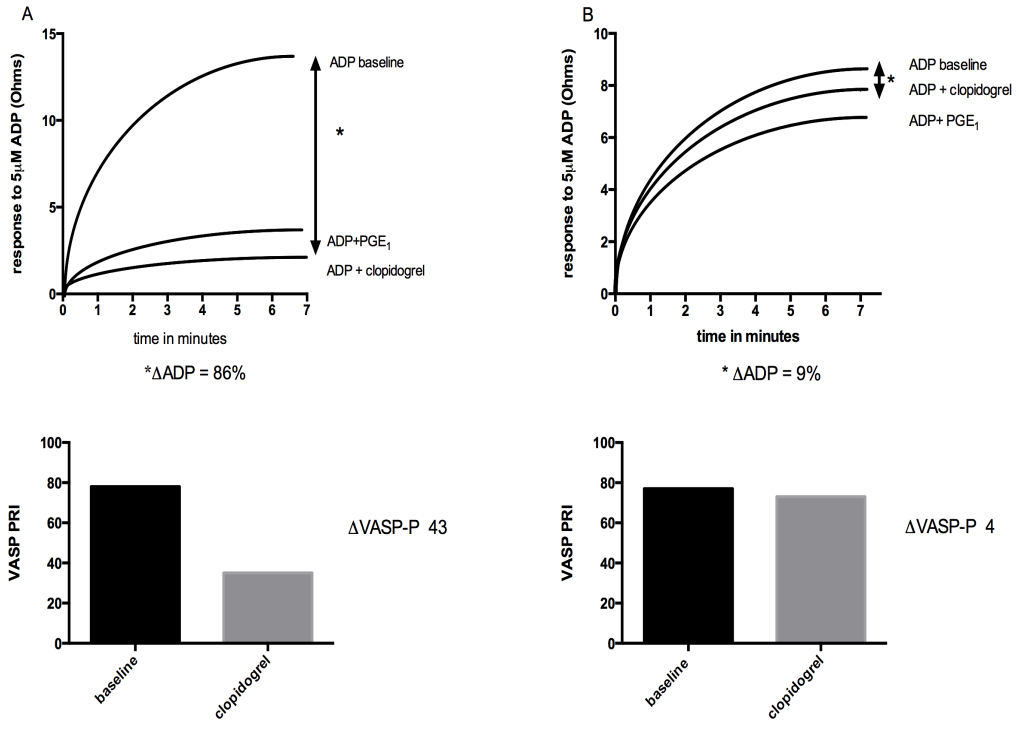
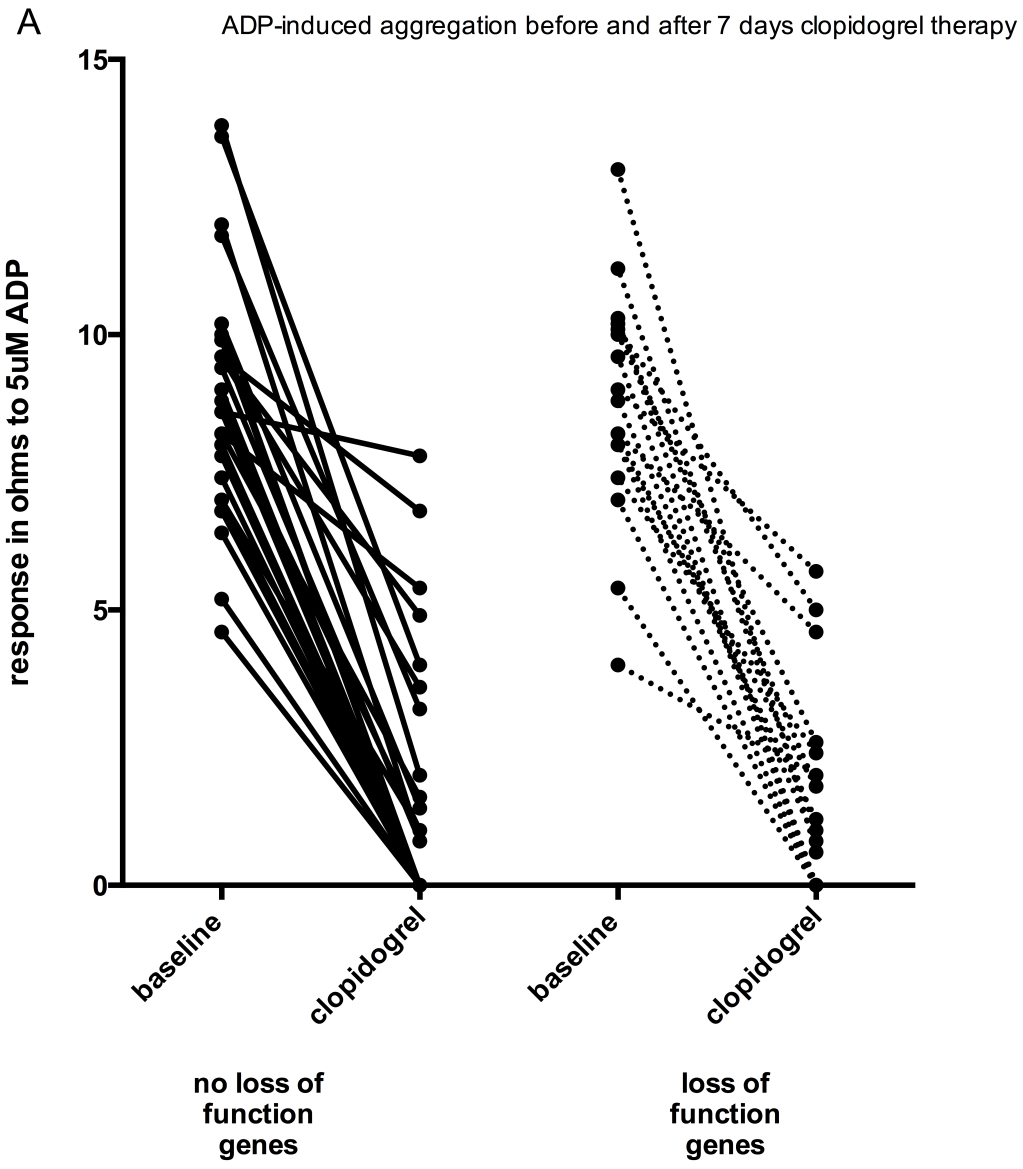


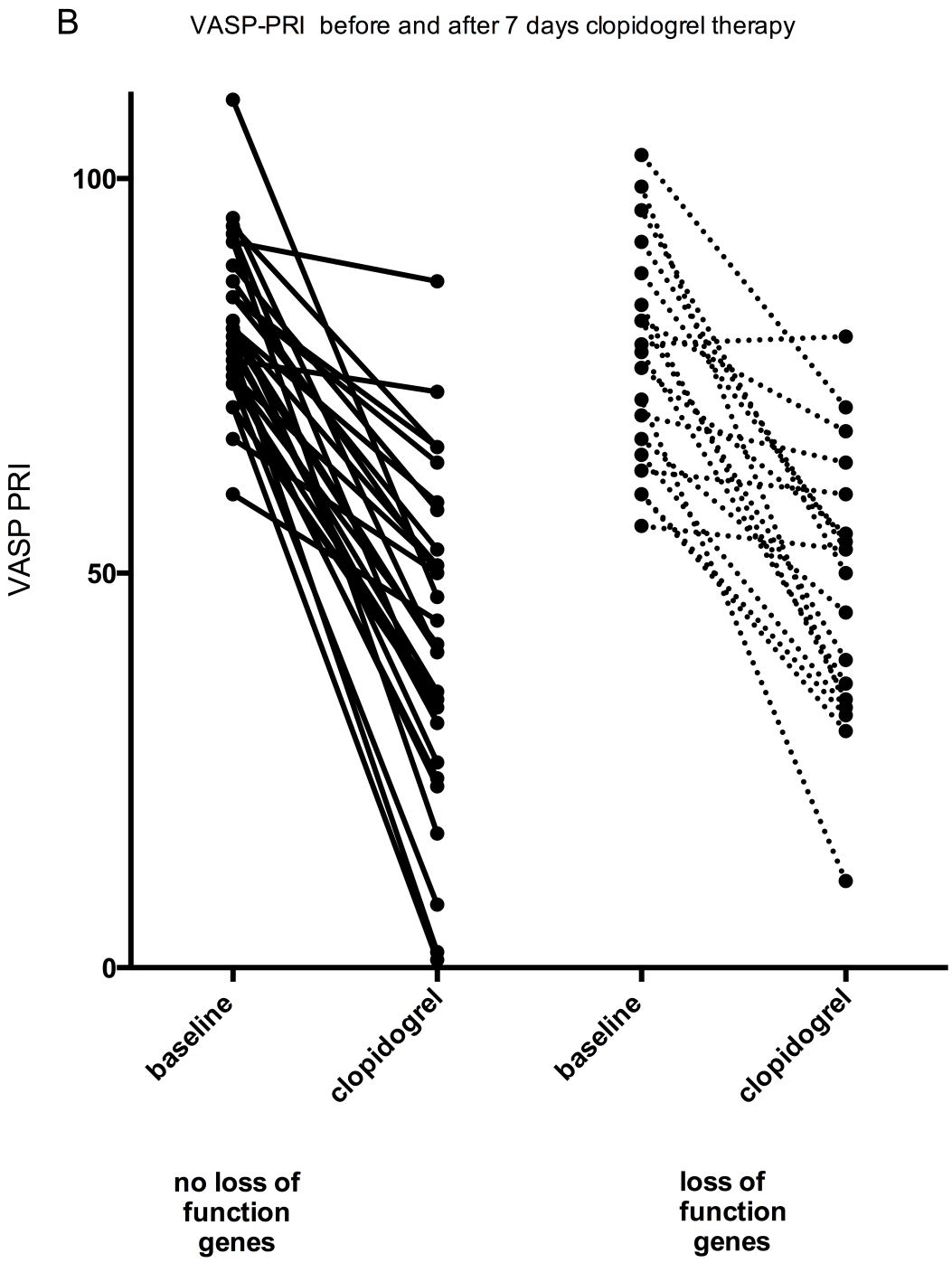
Figure 4.3: Examples of the effects of PGE_1 and clopidogrel on ADP-induced platelet aggregation (upper panels) and changes in PRI with clopidogrel (lower panel). Illustrative data for individuals with intact (A) and impaired (B) PGE_1 responsiveness are shown..

4.5.3 Effect of clopidogrel on ADP-induced aggregation and VASP-PRI in subjects with and without loss of function CYP 2C19 genotypes.

The effects of clopidogrel on ADP-induced aggregation and on VASP phosphorylation, as measured by PRI, are shown for individual subjects/patients, in Figure 4.4A and 4.4B. Median inhibition of ADP response was 100% while for PRI mean response was 37±20% SD. However, both parameters of response varied widely and 37% of individuals had on-treatment PRI >50%, a criterion of "clopidogrel resistance". This occurred in 44% of individuals with and 32% of individuals without loss-of-function mutations. On univariate comparisons, both ΔADP (Figure 4.4A) and ΔVASP-P (Figure 4.4B) tended to be greater for subjects without than those with loss-of-function mutations but this difference did not reach statistical significance.

Figure 4.4: Changes in (A) ADP-induced aggregation and (B) VASP PRI following 7 days clopidogrel therapy. Patients with loss of function mutations are indicated with dotted lines. Neither ΔADP nor Δ VASP PRI varied significantly between groups.




B
VASP-PRI before and after 7 days clopidogrel therapy
VASP PRI
100
50
0
baseline
clopidogrel
baseline
clopidogrel
no loss of function genes
loss of function genes

4.5.4 Primary hypotheses: Impact of platelet responses to PGE_1 and SNP and clopidogrel effect.

Baseline platelet response to PGE_1 predicts clopidogrel effect

There were significant correlations between pre-clopidogrel platelet sensitivity to PGE_1 and ΔADP and ΔVASP-P (Figure 4.5A and 4.5B), respectively. Importantly, these relationships remained significant for subsets with loss-of-function mutations (r=0.65; P=0.002 and r=0.58 P=0.008 for ΔADP and ΔVASP-P, respectively).

Baseline response to SNP and clopidogrel effect

When interactions between the pre-clopidogrel platelet sensitivity to SNP and subsequent effects of clopidogrel were considered (Figure 4.6A and 4.6B), there was no significant relationship with ΔADP (A), while for ΔVASP-P, there tended to be an inverse relationship (B).

Figure 4.5: Relationship between pre-clopidogrel platelet response to PGE_1 (measured as percent inhibition of aggregation) and response to clopidogrel, measured via ΔADP (A) and ΔVASP-P (B). There was a strong correlation between PGE_1 response and ΔADP (r=0.68, P<0.0001 in (A), but a weaker correlation with ΔVASP-P (r=0.39, P=0.005 in (B)).

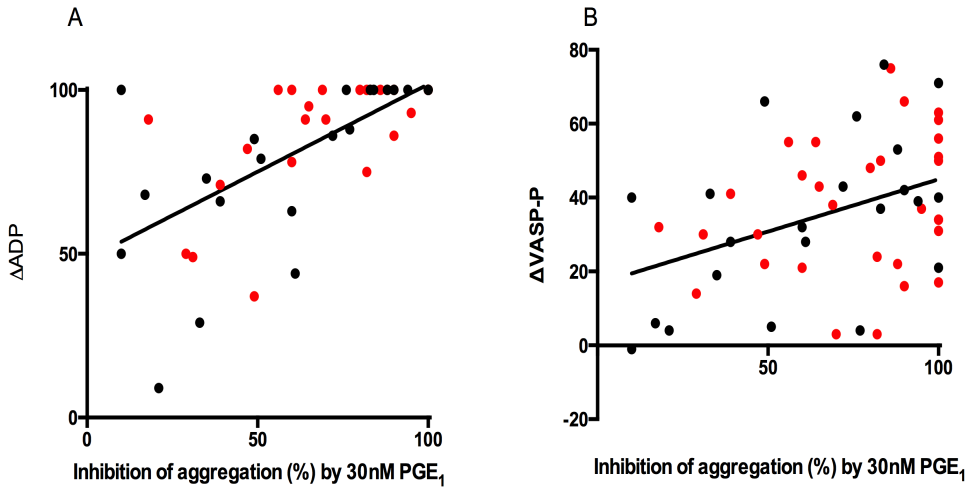
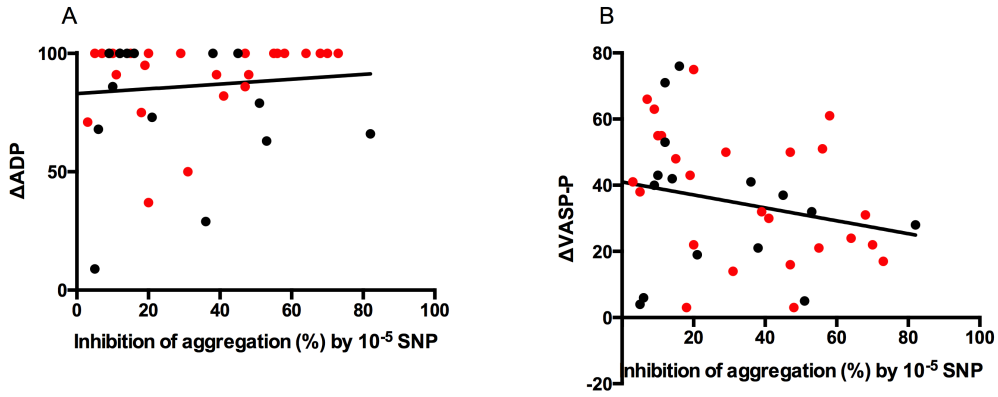


Figure 4.6: Relationship between pre-clopidogrel platelet response to SNP, measured as ΔADP (A) and ΔVASP-P (B). There was no significant univariate relationship between SNP response and ΔADP. However a borderline inverse relationship existed between SNP response and ΔVASP-P. (r=-0.23, P=0.09).



Multivariate analysis – Primary hypothesis.

On multivariate analysis (Table 4.2), PGE_1 response was a highly significant determinant of both ΔADP and ΔVASP-P (P<0.01). Furthermore, there was a significant and inverse association between ΔVASP-P and baseline SNP response. Genotype was not a significant independent correlate of either ΔADP or ΔVASP-P.

Table 4.2: Multivariate Analysis – Primary Hypothesis: Independent determinants of the two measures of clopidogrel response tested (ΔADP and ΔVASP-P).

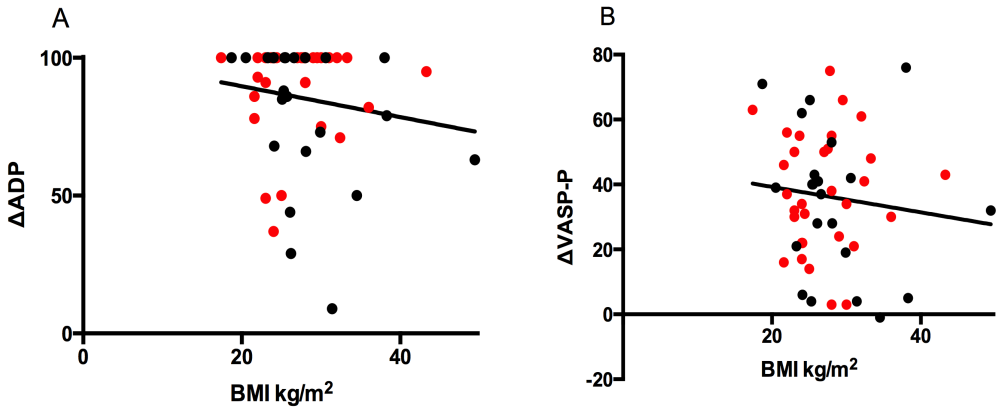
	Parameter	**β standardised coefficients**	**P value**
a. ΔADP	PGE_1 response	0.646	<0.0001
b. ΔVASP-P	PGE_1 response	0.411	<0.004
	SNP response	-0.321	0.021

Secondary hypotheses

4.5.6 BMI/body weight and clopidogrel effect

Linear regression analysis revealed no significant relationship (Figure 4.7) between BMI and (A) ΔADP or (B) ΔVASP-P. Although this was the method of analysis which had been selected prospectively, post-hoc analysis was also undertaken to determine whether categorical, rather than continuous, differences in BMI corresponded to variability in clopidogrel effect. For example, comparison of effect (both ΔADP [Figures 4.7C and 4.7D] and ΔVASP-P [Figures 4.7E and 4.7F]) for subjects with BMI <30 vs BMI ≥ 30 kg/m^2 revealed no major differences (for example, ΔADP 71.6 ±4.5 SD vs ΔADP 63.5 ±7.5 SD P=0.36).

Figure 4.7A and 4.7B: Impact of variable BMI on clopidogrel response measured via (A) ΔADP and (B) ΔVASP-P. In no case was there a significant relationship between BMI and response (P>0.2).



Figures 4.7C and 4.7D. Impact of BMI and body weight on clopidogrel response as measured by ΔADP in non-obese vs obese subjects (7C) and subjects ≤80kg and >80kg (7D). There was no significant difference between groups (P>0.35).

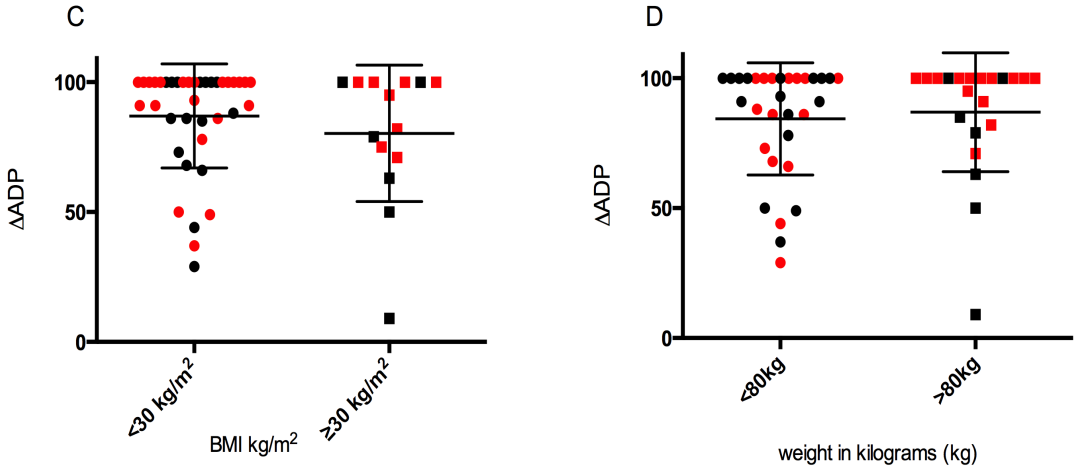
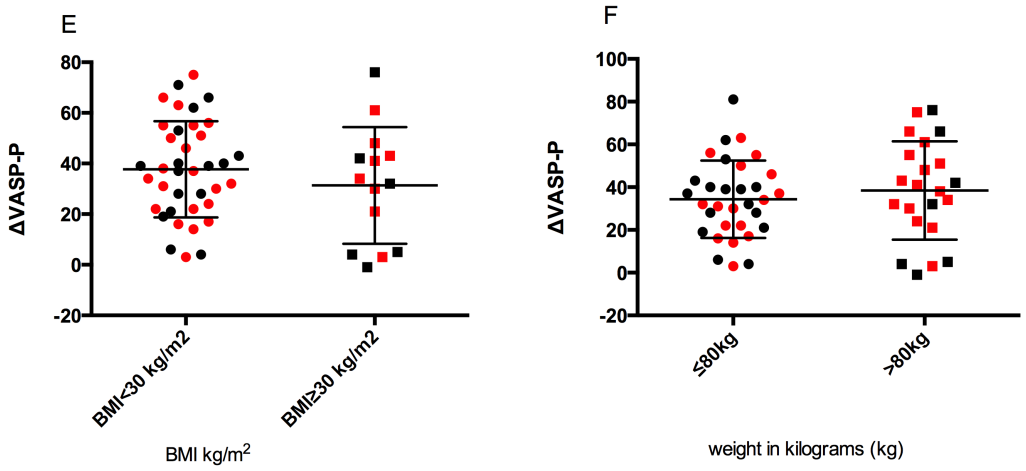


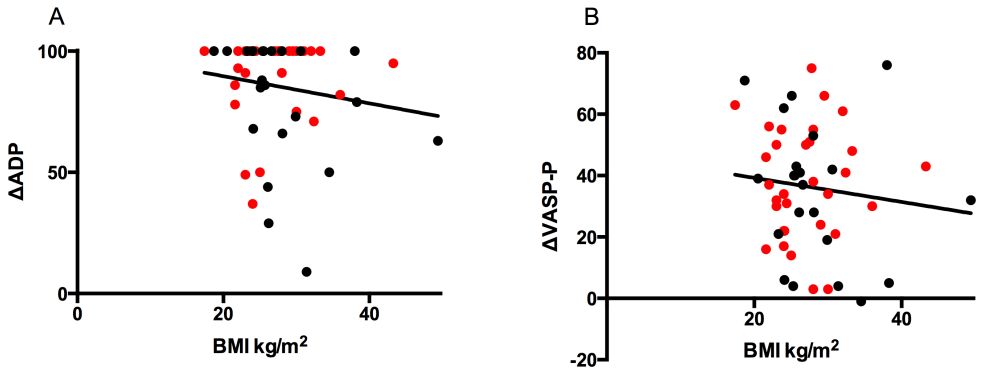
Figure 4.7E and 4.7F: Impact of BMI and bodyweight on clopidogrel response as measured by ΔVASP-P in non-obese and obese subjects (Figure 4.7E) and subjects ≤80kg and >80kg (Figure 4.7F). P values > 0.3.



Given that increased BMI was not associated with significant impairment of clopidogrel effect, the question arises as to whether it was in the current series, associated with platelet resistance to activators of adenylate cyclase and of soluble guanylate cyclase, as has previously been reported[142,263].

Utilising continuous analysis, as for clopidogrel response, we evaluated the relationship between response to PGE_1 (Figure 4.8A) or response to SNP and BMI (Figure 4.8B).

Figure 4.8A and B: Pre-clopidogrel platelet response to (A) PGE_1 and (B) SNP vs BMI. There was no significant relationship between via linear regression with either PGE_1 or SNP response and BMI (P>0.15).



We then explored the impact of

(a) Categorical rather than continuous analysis of impact of variable BMI (Figures 8C and D)

(b) Categorical evaluation of actual weight (Figures 8E and 8F) given that body weight and BMI are not precisely related to one another.

There were no significant variations between these categorical groups in any case (P values >0.3).

Figure 4.8C and D: Non-obese vs obese pre-clopidogrel platelet response to (C) PGE_1 and (D) SNP.

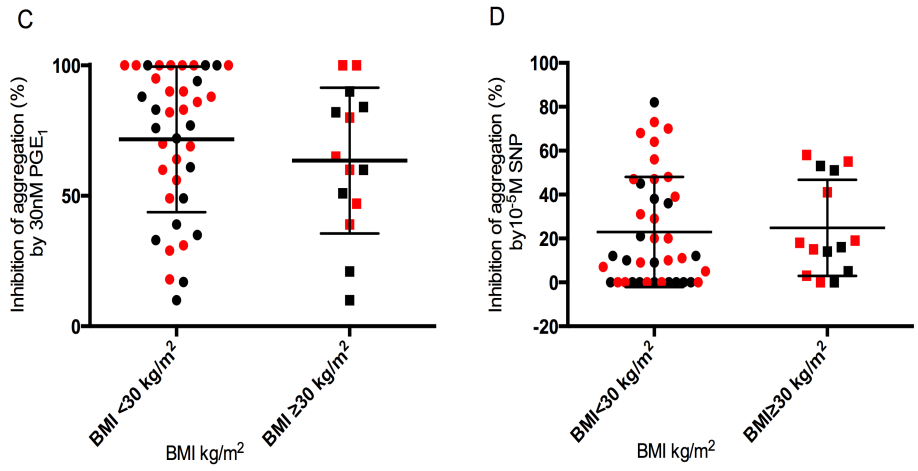
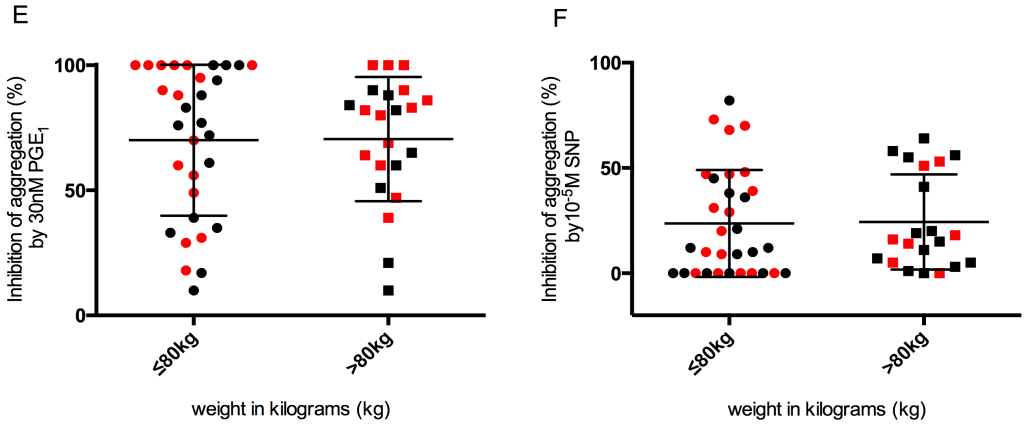


Figure 4.8E and F: Body weight ≤ 80kg and >80kg and pre-clopidogrel platelet response to (E) PGE_1 and (F) SNP.



4.5.7. Symptomatic ischaemic heart disease and clopidogrel effect.

Healthy subjects tended to develop more extensive changes in ΔADP (P=0.08) than patients with IHD (Figure 4.9A), but no similar trend was evident when ΔVASP-P was evaluated (P=0.6).

As regards the postulated biochemical basis for differential response to clopidogrel, pre clopidogrel platelet response to PGE_1 was 75%±25% SD in healthy subjects versus 61%±31% SD in patients with ischaemic heart disease. This difference did not however reach clinical significance. Median pre-clopidogrel platelet response to SNP was 20% inhibition in healthy subjects and only 10% in those with ischaemic heart disease (P=0.07) (Figure 4.10). Thus in this population presence of symptomatic IHD was associated only with non-significant trends towards

(a) Impaired platelet responsiveness to PGE_1

(b) Impaired platelet responsiveness to clopidogrel

Figure 4.9. Comparisons of healthy subjects (HS) and patients with ischaemic heart disease (IHD) measuring clopidogrel effect using (A) ΔADP (P=0.08) and (B) ΔVASP-P (P=0.6). Median values and interquartile ranges are shown for ΔADP and ΔVASP-P values.

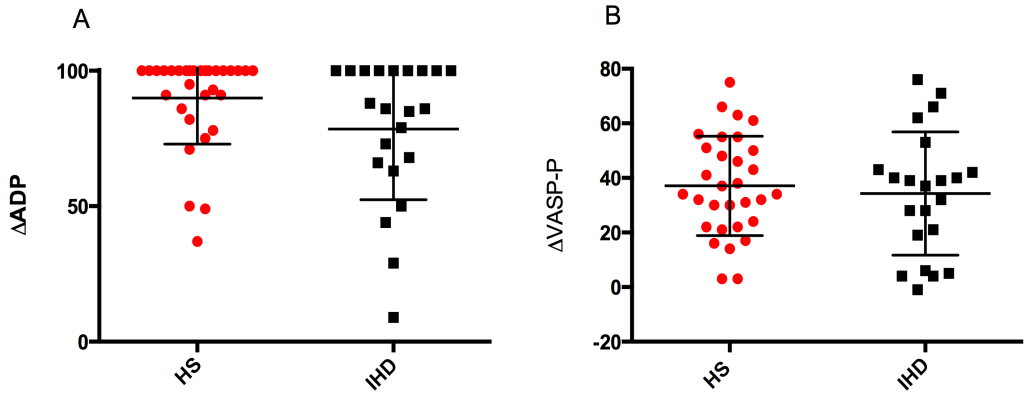
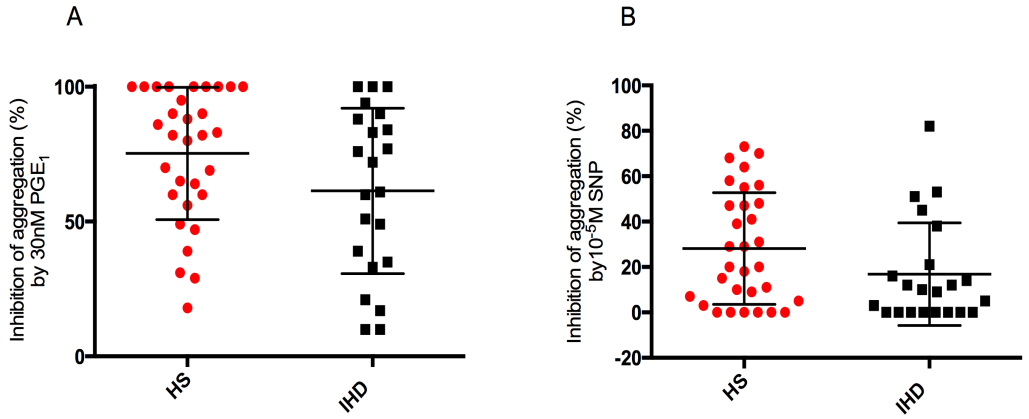


Figure 4.10 Comparisons of healthy subjects (HS) and patients with ischaemic heart disease (IHD) pre-clopidogrel platelet responses to (A) PGE_1 (P=0.1) and (B) SNP (P=0.07).



4.5.8 Investigation of ADP stimulated platelet aggregation using the pan-kinase inhibitor wortmannin.

Pilot studies were performed to determine the concentration-response curve characteristics of wortmannin-induced inhibition of ADP response (Chapter 3.4). A wortmannin concentration of $1x10^{-6}$M inhibited responses by 80% and a concentration of $1x10^{-8}$M inhibited only a third of subjects tested. However, with $1x10^{-7}$M wortmannin, mean inhibition of ADP response was 61 ± 25%. Therefore this concentration was utilised to evaluate bases for heterogeneity of wortmannin effect. Studies were performed in healthy subjects and patients with ischaemic heart disease in a subgroup of subjects from the primary hypothesis study.

Subject characteristics.

25 healthy subjects and 17 patients with ischaemic heart disease were investigated. (Table 4.3).

Table 4.3: Participant demographics –wortmannin substudy: Investigation of ADP stimulated platelet aggregation using the pan-kinase inhibitor wortmannin.

	HS	**IHD**
Subjects (n)	n=25	n=17
Median age (years)	45.5	64
Age range (years)	39-66	48-84
Gender (M:F)	16:9	11:6
BMI kg/m^2 median	25	26
BMI kg/m^2 range	17.4-36	18.7-49.4
T2DM (n)	Nil	2
BMI>30kg/m^2 (n)	4	3

HS Healthy subjects
BMI Body Mass Index
IHD Ischaemic heart disease
T2DM Type 2 diabetes mellitus

Does wortmannin response correlate with autocoid-induced inhibition of ADP response?

There was a statistically significant correlation between pre-clopidogrel platelet response to PGE_1 and inhibition of ADP induced platelet aggregation with 100nM wortmannin (Figure 4.11A). This was not found for pre-clopidogrel platelet response to SNP (Figure 4.11B).

Does wortmannin response predict clopidogrel response?

For all subjects there was a statistically significant correlation between ΔADP and 100nM wortmannin (Figure 4.12A) and ΔVASP-P (Figure 4.12 B).

Figure 4.11: Responses of all subjects to 100nM wortmannin correlated with (A) pre-clopidogrel platelet response to (A) PGE_1 ($p<0.001$, $r=0.62$) and (B) SNP ($P=0.14$).

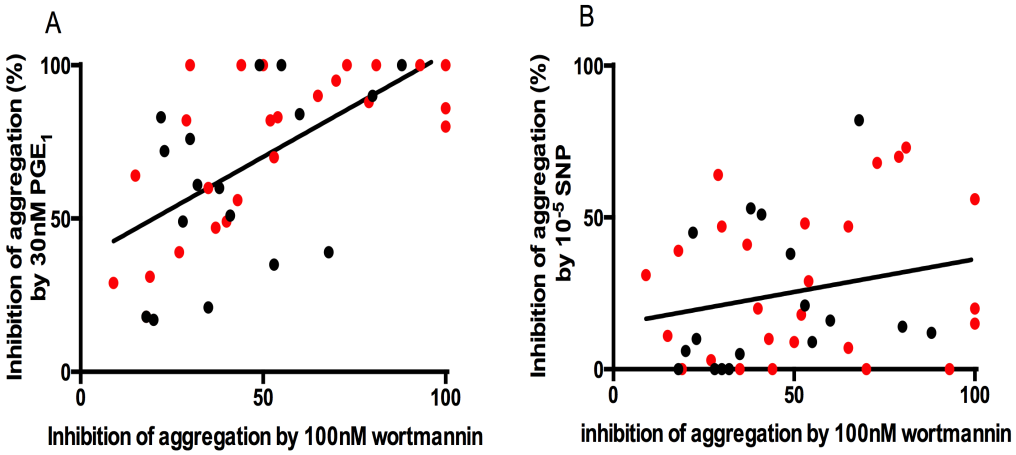
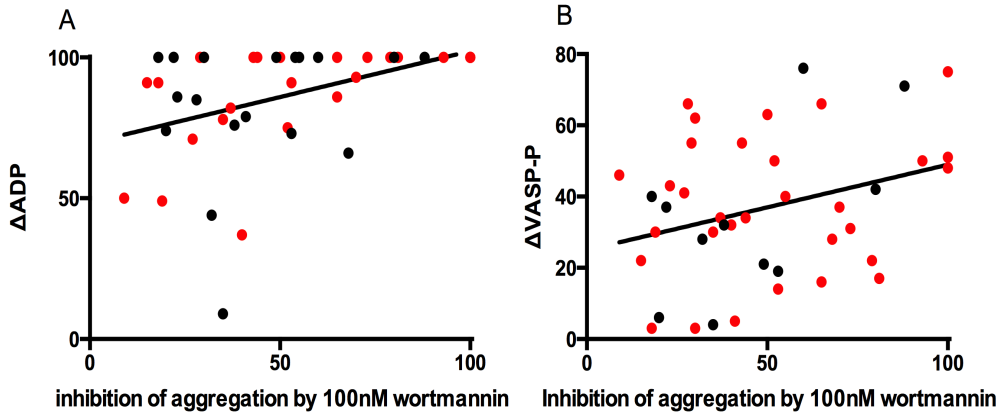


Figure 4.12: Responses of all subjects to 100nM wortmannin correlated with (A) ΔADP ($P=0.009$, $r=0.4$) and (B) ΔVASP-P ($P=0.05$).



4.5.9 Impact of Insulin Resistance

Insulin resistance as determined by Homeostasis Model Assessment (HOMA-IR) has been associated with markedly increased cardiovascular risk[241]. We defined Insulin Resistance on the basis of the simplified HOMA-IR equation (fasting plasma glucose (mmol/L) [FPG] x fasting plasma insulin (mU/L) [FPI] /22.5). All subjects enrolled in the study had a single episode fasting glucose and insulin taken as part of baseline blood sampling. Diabetic subjects and subjects with a fasting glucose of greater than 6 mmol/L with a HbA1c greater than 6% who were thus likely to be diabetic, irrespective of a formal diagnosis, were excluded from analysis. In one subject the insulin level was sample was mislaid by the laboratory. These exclusions left 48 subjects available for analysis.

HOMA-IR analysis was performed as a continuous variable to determine whether insulin resistance (expressed on a relative rather than an absolute basis) was associated with variability in clopidogrel effect. Analysis via linear regression found no significant relationship between HOMA-IR and ΔADP. However HOMA-IR vs VASP-P were statistically correlated (r= -0.31, P= 0.03). Both correlations are shown in Figures 4.13A and 4.13B.

Correlations were then sought between HOMA-IR and pre-clopidogrel platelet responsiveness to PGE_1 (Figure 14A) and to SNP (figure 14B). Neither of these correlations were statistically significant.

Figure 4.13: HOMA-IR vs (A) ΔADP and (B) ΔVASP-P respectively. There was no significant correlation between HOMA-IR and ΔADP (P=0.4), but the correlation with ΔVASP-P was inverse and significant (r=-0.31, P=0.03).

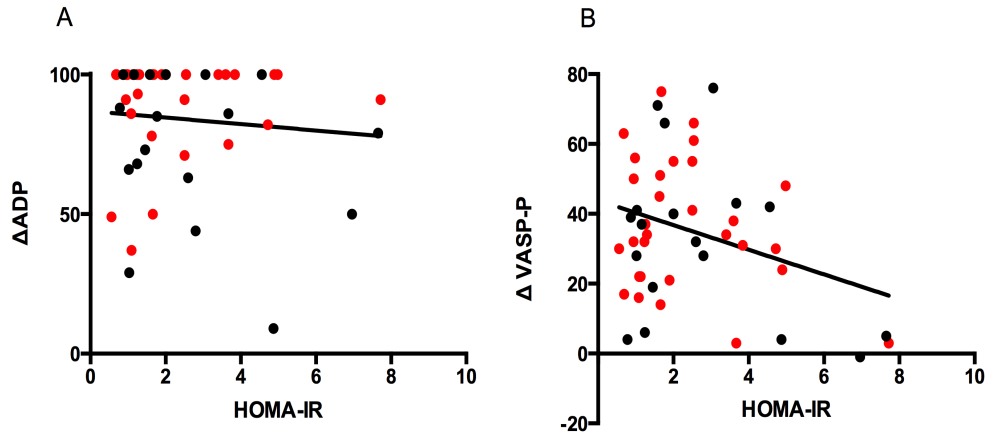
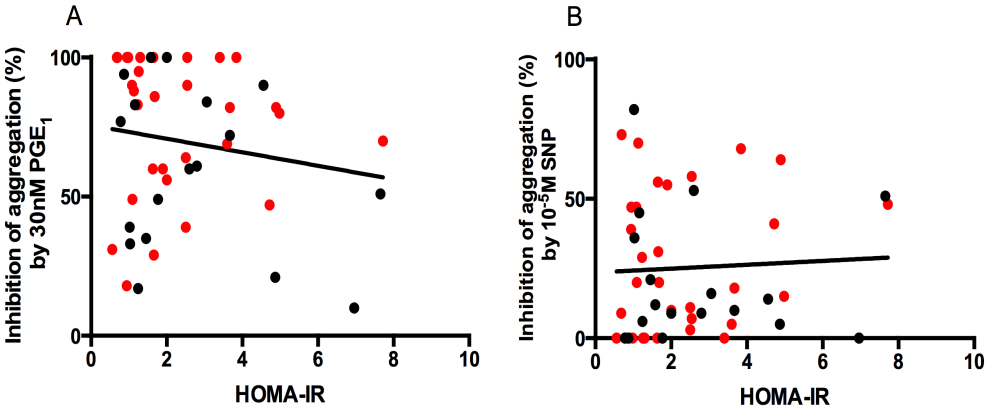


Figure 4.14: Correlations between pre clopidogrel platelet response to (A) PGE_1 or (B) SNP and HOMA-IR. Neither correlation was statistically significant analysed via linear regression (P>0.2 for both).



Multivariate Analysis and HOMA-IR

Multivariate analysis was performed via linear regression with ΔVASP as the dependent variable and pre clopidogrel platelet PGE_1 response, pre-clopidogrel platelet SNP response, genotype and HOMA-IR as independent variables (Table 4.4). PGE_1 was again found to be an independent predictor of ΔVASP with a P value of <0.01. None of the other parameters evaluated were independent predictors, although (as previously), SNP response tended to be an inverse predictor. As regards HOMA-IR, the trend for this to reflect ΔVASP-P was relatively weak on multivariate analysis.

Table 4.4. Multivariate analysis: correlates of ΔVASP-P. Only PGE_1 response was found to be an independent correlate of ΔVASP-P.

Parameter	Beta coefficient	P value
PGE_1 response	0.402	0.004
SNP response	-0.242	0.064
HOMA_IR	-0.219	0.095
Genotype	-.129	0.324

4.5.10 Effect of obesity on response to clopidogrel.

Participant demographics

Of the 48 subjects who underwent HOMA-IR analysis, 13 were obese with a BMI of ≥ 30 kg/m^2 (Table 4.5). All obese subjects had received dose adjusted clopidogrel for weight (weight range 81 to 160kg with median 103kg) with a maintenance dose of 150mg as per the protocol. Non obese subjects had only 23% of subjects receiving dose adjusted clopidogrel for weight (weight range of 45 to 101kg with median 74kg). Subject characteristics are outlined in Table 4.5.

Table 4.5: Participant demographics of subjects undergoing analysis for (1) HOMA-IR vs (i) pre-clopidogrel platelet response to PGE_1 and SNP and (ii) clopidogrel effect (Chapter 4.5.9) (2) effect of obesity in response to clopidogrel (Chapter 4.5.10).

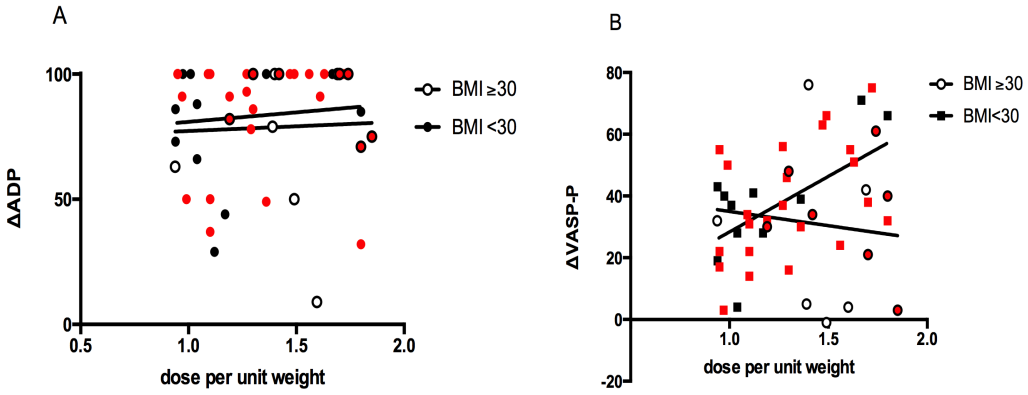
	Non-obese (BMI <30kg/m^2)	**Obese (BMI ≥30 kg/m^2)**
Subjects (n)	35	13
Subjects taking 150mg MD clopidogrel	23%	100%
BMI (kg/m^2) range	17.4-29.5	30-49.4
BMI (kg/m^2) median	25	32.4
Dose per unit weight range	0.95 - 1.72	0.94 - 1.85
Dose per unit weight median	1.18	1.49
Known ischaemic heart disease	(12) 34%	(6) 46%
HOMA-IR (median)	1.3	4.1

MD maintenance dose

There was no significant difference on ANCOVA between non obese and obese subjects and response to ΔADP (Figure 4.15A). On ANCOVA (Figure 4.15B) the ΔVASP-P: clopidogrel relationship varied significantly according to BMI. There was a significant difference in intercept of calculated relationships (F = 4.07, P=0.049) and the slopes of the two relationships also tended to vary from one another (F=3.85, P=0.06). These data therefore suggested that increases in clopidogrel dose per unit weight were associated with increased clopidogrel effect (as measured by ΔVASP-P) mainly in non-obese subjects.

However the ANCOVA for PGE_1 response:ΔVASP-P relationship (Figure 4.16) revealed no significant differences according to obesity, suggesting that the effects of obesity are not primarily based on impairment of PGE_1 response.

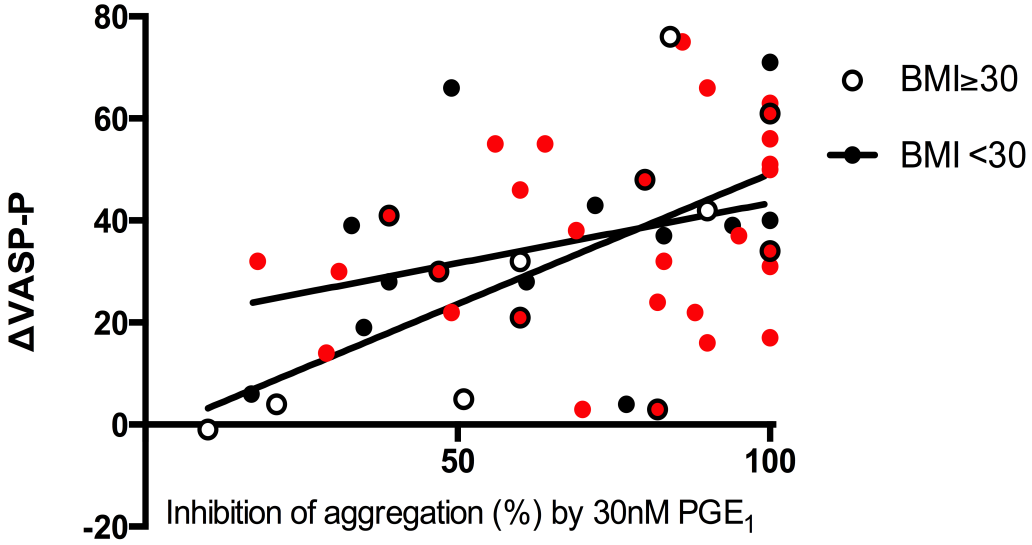
Figures 4.15 A and B. Effect of dose per unit weight clopidogrel on (A) ΔADP and (B) ΔVASP-P in non obese versus obese subjects.



Effect of obesity on relationship between pre-clopidogrel platelet response to PGE_1 and ΔVASP-P

There was no significant difference found between PGE_1 response and ΔVASP-P in non obese and obese subjects. (Figure 4.16). ANCOVA intercept (F=0.36 P=0.55) and slope F=1.59 P=0.21).

Figure 4.16: Effect of obesity on pre-clopidogrel platelet response to PGE_1 and ΔVASP-P.



Effect of obesity on HOMA-IR

As expected, there was a highly statistically significant difference between HOMA-IR in non obese vs obese subjects (P= <0.001). Median HOMA-IR was 4.1 for obese and 1.3 for non obese subjects.

4.6 DISCUSSION

Impact of variable PGE_1/cAMP and NO/sGC-based signalling.

The clinical study was designed to test the hypotheses that clopidogrel effect after 7 days' treatment is determined by pre-treatment platelet sensitivity to PGE_1. Furthermore, we wished to evaluate the potential impact of NO-based signalling on clopidogrel effect.

We primarily found that pre-clopidogrel response to PGE_1 was a strong and independent predictor of subsequent clopidogrel effect, irrespective of the method of measurement (either ΔADP or ΔVASP-P). The results related to PGE_1 were indeed in agreement with the primary hypothesis of this study. A number of previous investigations had provided data showing that clopidogrel effects might in theory be modulated by its interactions with adenylate cyclase/cAMP signalling[156,160,163].

On the other hand, the association between NO response (measured using SNP as an NO donor) and clopidogrel response was significant – but inverse – on multivariate analysis, but only evident on ΔVASP-P. This finding is both unexpected and intriguing, given that ΔVASP-P reflects the potential "input" from both adenylate cyclase and soluble guanylate cyclase activation. It is now appreciated that clopidogrel markedly potentiates NO/cGMP signalling [161]: thus the input of NO to ΔVASP-P is likely to increase variably during the initiation of clopidogrel therapy according to the degree of potentiation.

The utilisation of a weight-adjusted clopidogrel dosage regimen may have expedited the elucidation of these associations. Effects of clopidogrel on ΔVASP-P trended with dosage per unit weight (Figure 4.2B), so it is likely that minimising heterogeneity reduced the presence of another factor controlling variability of clopidogrel response.

The importance of genotype as a determinant of clopidogrel response has been shown both pharmacologically [217] and clinically [153,216,304] in previous studies. Indeed, as shown in Figure 4.4, response to clopidogrel on univariate comparisons tended to be greater in patients without than in those with loss-of-function mutations, without this difference reaching statistical significance. Similarly, on multivariate analysis there was no significant impact of genotype. These data emphasise that the impact of PGE_1 response is substantially more important than that of genotype as a modulator of clopidogrel effect. There are a number of potential reasons for this finding. First the extent of inhibition of clopidogrel bioactivation with particular loss-of-function mutations is highly variable [305]. Second, a number of investigators have demonstrated that, despite various reports associating stent thrombosis with impaired clopidogrel bioactivation, CYP2C19 genotype accounts for little [218] or no significant component [221,224] of the inter-individual variability in response to clopidogrel.

Our results show far stronger univariate correlations between PGE_1 responsiveness and ΔADP than ΔVASP-P, and this disparity is not specifically

explained by the data. At least two factors may be involved here. First, VASP-P reflects the impact of both cAMP and cGMP phosphorylation: if "cross-talk" were here to result in decreased soluble guanylate cyclase activity with adenylate cyclase activation, net effects on VASP-P would be diminished. Moreover, ΔADP reflects not only these processes, but also the interactions of cAMP with $P2Y_1$ receptor-related ADP signalling, and these effects may be synergistic [156,306].

Our finding that pre-clopidogrel platelet sensitivity predicts clopidogrel effect raises whether modulation of post $P2Y_{12}$ receptor signalling is also of relevance for other $P2Y_{12}$ antagonists. At this stage this has not been evaluated, although there is evidence that $P2Y_{12}$ inhibition using prasugrel active metabolite and ticagrelor can increase the potency of modulators of cAMP[160].

Impact of BMI and clopidogrel effect

The secondary hypothesis that clopidogrel responsiveness is related to BMI reflects to two categories of previous information:

(i) that in several previous studies, clopidogrel resistance appeared to be more prevalent among obese individuals[271,275]

(ii) that a number of investigators have reported impairment of both adenylate cyclase and soluble guanylate cyclase signalling in obese individuals[141,276]

Therefore, it would appear that if the primary hypothesis of this study were correct, a relationship between increased BMI and high on treatment platelet

reactivity would also be found. As it is apparent from the actual data, there was nothing approaching statistical significance in the BMI: clopidogrel response relationship, nor were subjects with increasing BMI significantly resistant to PGE_1 or SNP (Figure 4.8A and B). Therefore it is appropriate to attempt to explain this "paradoxical" finding.

An important consideration is the use of weight-adjusted clopidogrel dosing in the current study. Several previous studies which reported "high on treatment platelet aggregation" did not adjust clopidogrel dosage according to weight[271,274]. For these studies it can be assumed that clopidogrel "resistance" is effectively "dilutional" and these data can be disregarded as evidence of receptor/post-receptor impairment.

On the other hand, some clinical findings are more robust. For example, Bonello-Palot et al[275] have reported that changes in platelet reactivity with increases in clopidogrel dosing are less marked in obese than non-obese individuals. This finding suggests that obesity may indeed be associated with clopidogrel resistance. Furthermore, the claimed impairment of response to PGE_1 and SNP in obese individuals are clearly independent of clopidogrel dosing considerations.

The presumption that increased BMI (rather than inadequate weight-adjusted clopidogrel dose) would be associated with clopidogrel resistance reflected primarily on expecting that increased BMI would be associated with impaired

platelet response to PGE_1 and SNP[142,145]. PGE_1 response was somewhat impaired in the presence of increased BMI, but this did not reach statistical significance (P>0.15). However, these findings did not eventuate for SNP response. This is surprising: perhaps pharmacotherapy with ACE inhibitors might have potentiated SNP responses[307]. In any event, using weight adjusted dosing, neither increased BMI or body weight was associated with impaired clopidogrel effect. The overall conclusion to be drawn, therefore, should be that increased BMI is unlikely to represent a major cause of clopidogrel "resistance".

Impact of symptomatic cardiac disease and clopidogrel effect

No direct comparisons have been reported on the responsiveness to clopidogrel in healthy subjects versus patients with symptomatic myocardial ischaemia. Additionally, attempts to compare rates of high on treatment platelet reactivity across a spectrum of stable angina through to acute myocardial infarction requires the comparison of diverse patient populations and heterogeneous methodologies[222]. Nevertheless, attempts to compare rates of clopidogrel resistance across such a spectrum of healthy subjects through patients with stable angina and acute myocardial infarction does suggest an increased proportion of clopidogrel resistance in the presence of symptomatic cardiac ischaemia[308] [134,215,309,310].

In our study, the decision to evaluate the presence of symptomatic IHD was predicated on the assumption that there would be an association with significant impairment of PGE_1 and SNP response[13,138]. While the subjects with ischaemic

heart disease did have lower mean/median responses to PGE_1 and SNP respectively, in the event, these trends were non-significant. Given that the differences in responsiveness to PGE_1 between normal subjects and patients with IHD were small, this component of the study was underpowered to detect an associated impairment of clopidogrel responsiveness. The minimal impact of symptomatic IHD in the current study is not at odds with the central hypothesis tested; nor do the current results necessarily contradict previous findings of PGE_1 and SNP "resistance" associated with angina pectoris[12-15].

Conversely, the negative findings regarding this secondary hypothesis do not invalidate the concept that among patients with IHD, platelet resistance to PGE_1 occurs commonly[12] and is indeed associated with clopidogrel resistance in those circumstances.

Wortmannin effect on ADP stimulated platelet aggregation.

These experiments demonstrate that the anti-aggregatory effect of 100nM wortmannin is proportional to PGE_1 pathway activity. There are two possibilities arising from this data (a) "proportionality": activity of adenylate cyclase and PI3-kinase are mutually regulated (b) a non PI3-kinase "off target" effect of wortmannin regulating adenylate cyclase.

Wortmannin has an IC_{50} for PI3-kinase in the nanomolar range (Ki 7nM[298]) so the concentration used in the present study is approximately 2 orders of magnitude

greater. Given the paucity of data regarding the optimal concentration of wortmannin for PI3-kinase inhibition in whole blood aggregometry, we had selected the concentration that gave the greatest heterogeneity of response to ADP aggregation.

There is little information regarding interactions between wortmannin and adenylate cyclase signalling. However, 100 to 500 nM wortmannin also inhibited responses to the direct adenylate cyclase inhibitor forskolin, in the human colonic cell line T84, where interestingly 500nM wortmannin had been previously demonstrated by the same group not to inhibit cAMP formation[311]. While cAMP and PKA have been previously described as not being affected by wortmannin[292], from these studies and our own findings, it is apparent that it does indeed have effects on cAMP signalling and cannot be used at this concentration to elucidate the effect of PI3-kinase blockade alone.

A possible explanation for this relationship is an "off target" i.e. non-PI3-kinase effect of wortmannin. Wortmannin has been demonstrated to inhibit myosin light chain kinase (MLCk), found downstream of the $P2Y_1$ receptor, which when phosphorylated leads to platelet shape change. It has been recently demonstrated that myosin light chain kinase phosphorylation and platelet shape change are inhibited by PGE_1 mediated cAMP signalling through activation of myosin light chain phosphatase activity[279]. If wortmannin at a concentration of 100nM is

variably blocking myosin light chain kinase, it is consistent that inhibition of this kinase would correlate with PGE_1 effect on platelet aggregation.

This relationship is attractive to postulate because the generation of cyclic AMP, for example by PGE_1/adenylate cyclase, leads to decreased phosphorylation of myosin light chain kinase and inhibition of platelet aggregation[279]. This would imply that wortmannin is acting effectively as a blocker of the ADP stimulated effector pathway via inhibition of the $P2Y_1$ pathway required for co-stimulation with $P2Y_{12}$ for sustained platelet aggregation.

Impact of Insulin Resistance measured using HOMA-IR on clopidogrel response.

Insulin resistance, when measured using HOMA-IR, has been associated with increased cardiovascular risk, even in the absence of additional features of the metabolic syndrome. There was a significant inverse correlation between ΔVASP-P and HOMA-IR ($r=-0.29$, $P=0.044$) in a group of non diabetic subjects with and without ischaemic heart disease with variable BMIs. Multivariate analysis using linear regression was therefore undertaken to determine the contribution of HOMA-IR, PGE_1 response, SNP response and genotype to ΔVASP-P. This revealed that HOMA-IR was not an independent determinant of ΔVASP-P on the basis of the current data set. Therefore it is likely that the observed univariate relationship with HOMA-IR (Figure 4.13B) reflects at least in part the impact of differential insulin responsiveness on PGE_1 sensitivity.

The findings that ΔVASP-P is inversely correlated with HOMA-IR and that dose adjusting clopidogrel in obese subjects was not always as successful as for non obese subjects are intriguing. In obese subjects the average HOMA-IR was 4.1 compared with a median of 1.3 for the non-obese subjects which was significantly different via unpaired T-testing ($P<0.0001$). If insulin resistance is able to modulate clopidogrel effect, this has significant implications for the efficacy of this drug. Insulin has been demonstrated, in healthy volunteers, as attenuating platelet responses by interfering with cAMP suppression through IRS- and Gi[244] an effect which is lost in diabetics[245]. Diabetics have been demonstrated to generate less clopidogrel active metabolite than non-diabetic subjects although no subject body sizes or BMIs were given[254]. Whether this is the case for subjects who are insulin resistant remains unclear. In an obese Zucker rat model, insulin resistance reduced arterial prostacyclin synthase and eNOS activity but this has not been demonstrated in platelets[312]. However, given that HOMA-IR was not an independent determinant of ΔVASP-P (Table 4.4) it is inappropriate to speculate extensively about what is ultimately a less than clear-cut finding.

Impact of obesity on clopidogrel response

The relationship between PGE_1 response and ΔVASP-P did not vary significantly according to BMI on ANCOVA. Thus increasing BMI was again shown neither to represent a substantial cause of PGE_1 resistance, nor incrementally, to modulate response to clopidogrel in a PGE_1 independent manner. Subjects with BMI>30 kg/m^2 had markedly variable inter-individual responses to PGE_1, a

finding consistent with increased BMI not representing a major cause of clopidogrel "resistance" per se irrespective of PGE_1 response. ANCOVA of the relationship between clopidogrel dose per unit weight and ΔVASP-P revealed heterogeneity between subjects ≥30 BMI and those with BMI<30 (Figure 4.16). Essentially it appeared that in obese subjects there was a "flat " dose response relationship for clopidogrel. These findings correspond well with the reported finding from Bonello-Palot et al[275], that the obese individuals are uniquely unresponsive to increased clopidogrel dosing.

CHAPTER 5: MODULATION OF cAMP PATHWAY: TSP-1 AND SQ 22536

5.1 Thrombospondin-1 (TSP-1)

5.5.1 Introduction

TSP-1 is a homotrimeric, multidomain glycoprotein present in the extracellular matrix, first isolated from thrombin activated platelets[313,314]. Each monomer consists of an interchain disulfide bond, procollagen homology domain (PC) and Type I, II, III repeats[315]. A variety of normal cells, including endothelial cells, fibroblasts, adipocytes, smooth muscle cells, monocytes and macrophages secrete TSP-1[316]. TSP-1 has many binding sites and functional roles:- it functions at the cell surface to bring together membrane proteins and cytokines that regulate extracellular matrix structure and cellular phenotype. These complexes bring together various membrane proteins to form specialised regions for regulation of cell adhesion, migration and proliferation. The membrane proteins participating in these complexes include integrin-associated protein (CD47 or IAP)[317], CD36[318] and proteoglycans. Transforming growth factor β and platelet derived growth factor also bind to TSP-1.

TSP-1 has been proposed to have several platelet receptors, including CD36, CD47 and the glycoprotein Ib-V-IX complex. Furthermore it can indirectly influence platelet activity via binding to collagen, fibrinogen and von Willebrand factor and prevent the breakdown of von Willebrand Factor via cleavage by matrix metalloproteinases[319]. It circulates in low concentrations in plasma and is

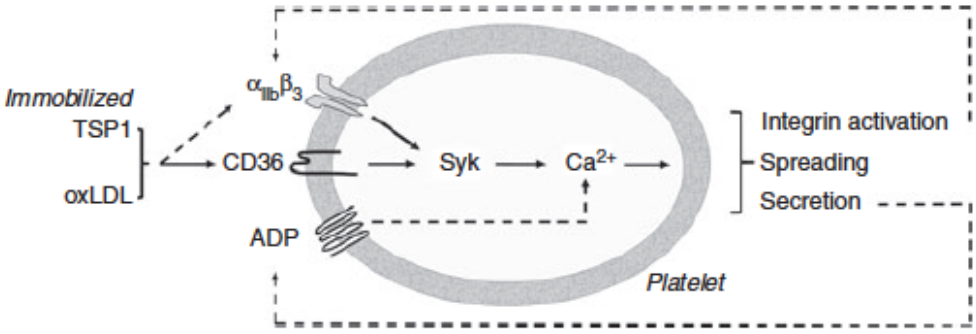
stored in platelet alpha granules where it is released on platelet activation increasing TSP-1 plasma concentrations by 100 fold [315].

5.2 Signalling pathways downstream of the CD36 receptor

5.2.1 Syk dependent CD36 signalling

The critical role receptor for TSP-1 from the point of view of the experiments in this thesis is CD36. CD36 is an abundant human platelet surface protein with 10 to 25 000 copies per cells, and is also known as Glycoprotein IV (GP IV)[320]. It is a double membrane-spanning protein with two short N- and C-terminal cytoplasmic domains and a large glycosylated extracellular domain with partly overlapping binding sites for thrombospondin-1, oxidised low density lipoproteins, oxidised forms of phospholipids and long-chain fatty acids[321-323]. The matrix of a (damaged) vessel wall contains surface immobilised forms of TSP-1 and oxLDL. It has been demonstrated using immobilised TSP-1 and oxLDL that CD36-dependant signalling leads to activation of Syk kinase and increased Ca2+ with resultant platelet adhesion and spreading[321]. CD36-dependent signalling responses of platelets in contact with immobilised TSP-1 or oxLDL, included platelet pseudopod formation, calcium rises, integrin IIb/IIIa activation, granule secretion producing autocrine mediators (ADP) and limited phosphatidyl serine exposure[321]. These responses were inhibited by CD36 receptor blockade.

Figure 5.1: Signalling pathway for immobilised TSP-1 and ox LDL via CD36[321].



5.2.2 TSP-1 modulation of cAMP/PKA is CD36-dependent.

CD36-dependent signalling also has a role in TSP-1 modulation of cAMP/PKA signalling. Pre-incubation of human platelets with TSP-1 prior to PGE_1 caused a time and concentration dependent reversal of platelet inhibition in response to the platelet agonist collagen[278]. PGE_1-activated cAMP rises were blunted when pre-incubated with TSP-1 which was further shown to be through a mechanism involving increased hydrolysis of cAMP by PDE3A. This was also CD36 dependent. A role in the regulation of the PKA based phosphorylation of VASP was also proposed as VASP phosphorylation at Ser^{157} was inhibited by TSP-1[278] independently of changes in cAMP. TSP-1 was also shown to induce phosphorylation of Src kinases, p38 and JNK. The ability of TSP-1 to modulate cAMP/PKA was lost when Src kinases were blocked, indicating that Src kinases play a role downstream from CD36.

Figure 5.2: Modulation of PGE_1 effect on platelet cyclic AMP generation and PKA by plasma TSP-1 via the CD36 receptor.

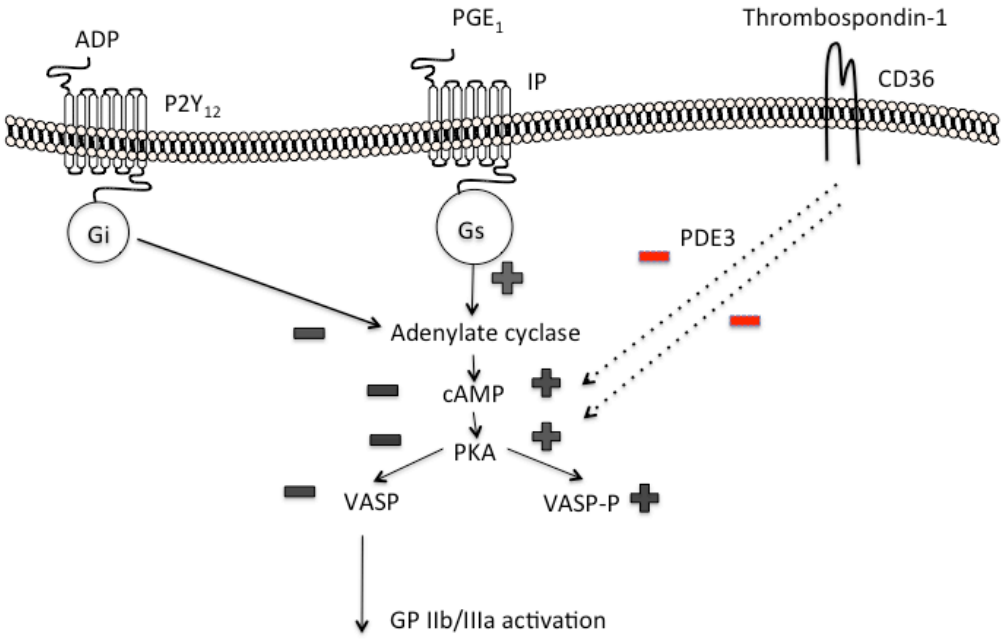


Figure 5.2: TSP-1 signalling via the CD36 receptor leads to attenuation of PGE_1-stimulated cAMP release and decreased PKA and VASP-phosphorylation, with increased GP IIb/IIIa activation.

CD47 dependent thrombospondin-1 signalling

TSP-1, acting via the CD47 receptor, has also been identified as a regulator of nitric oxide formation and effect in vascular smooth muscle cells[324] and in platelets[325]. TSP-1 was able to reverse the inhibitory effect of NO on thrombin-induced platelet aggregation under both high and low shear conditions[325]. Furthermore pre-treatment with TSP-1 prevented the phosphorylation of VASP mediated via PKG activators [325].

5.3 TSP-1 modulates ADP-stimulated PGE_1 responses.

We wished to determine whether exogenous TSP-1 could modulate the inhibitory effect of PGE_1 using ADP stimulated platelet aggregation. The current series of experiments was undertaken to explore the potential interactions, presumably via CD36 signalling of TSP-1 with platelet responsiveness to PGE_1. We chose to investigate only healthy individuals.

5.3.1 Methods

Four healthy subjects, aged between 30 and 40 years, who took no regular medications and had taken no anti platelet agents in the previous week, underwent blood sampling for platelet aggregometry (as per Chapter 2.1.1). All had weights in the healthy weight range and previous experiments had shown them to be sensitive to PGE_1 (90 to 100% inhibitory response to 30nM PGE_1 in response to 5μM ADP).

Preliminary experiments using whole blood aggregometry found the effect of TSP-1 to be difficult to reproduce, with erratic results occurring both intra and inter-individually. A decision was made therefore to use platelet rich plasma, while continuing to use impedance as the method to detect platelet aggregation, as for the whole blood aggregometry technique. The platelet count was not adjusted for these experiments and all subjects had CBEs, and importantly platelet counts, within the normal range.

5.3.2 Results

The interaction between TSP-1 and PGE_1 response was qualitatively similar in all four cases, but with varying concentration-response relationships. In all cases, a concentration of PGE_1 which inhibited ADP induced aggregation by >75% was chosen (as in Figure 5.3). TSP-1 produced partial reversal of this PGE_1 effect at a concentration around 2 $x10^{-10}$M, with loss of the inhibitory effect at higher concentrations (Figure 5.4). An example of an individual aggregogram is shown in Figure 5.3. Individual experiments to determine the concentrations of TSP-1 able to reverse the inhibitory effect of PGE_1 are shown in Figure 5.4. Concentrations of TSP-1 ranging from 2 nM to 2 x 10^{-13}M were also preincubated with PRP for 15 minutes prior to the addition of ADP to ensure there was no aggregatory effect of TSP-1. An inhibitory effect of TSP-1 was detected at 2nM but was not found for the concentrations between 10^{-10} and 10^{-13}M.

Figure 5.3: Platelet aggreogram. TSP-1 is able to partially reverse PGE_1 platelet response using ADP stimulation.

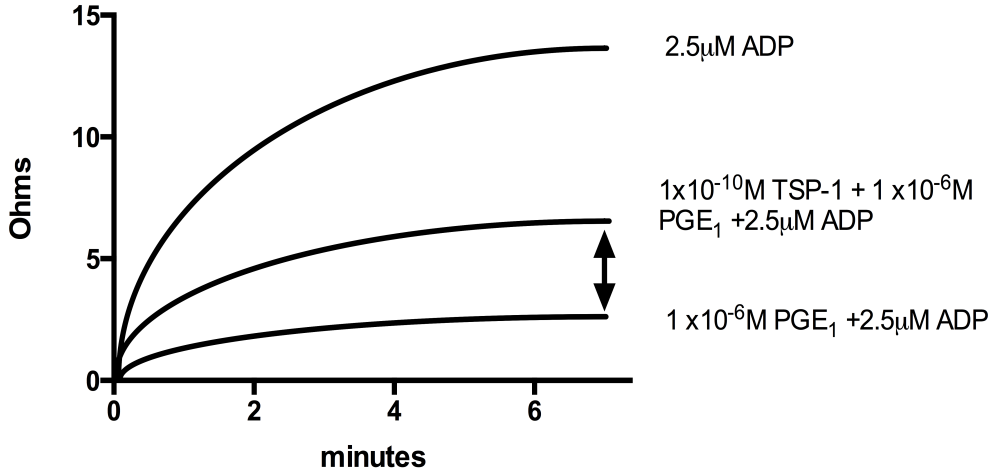
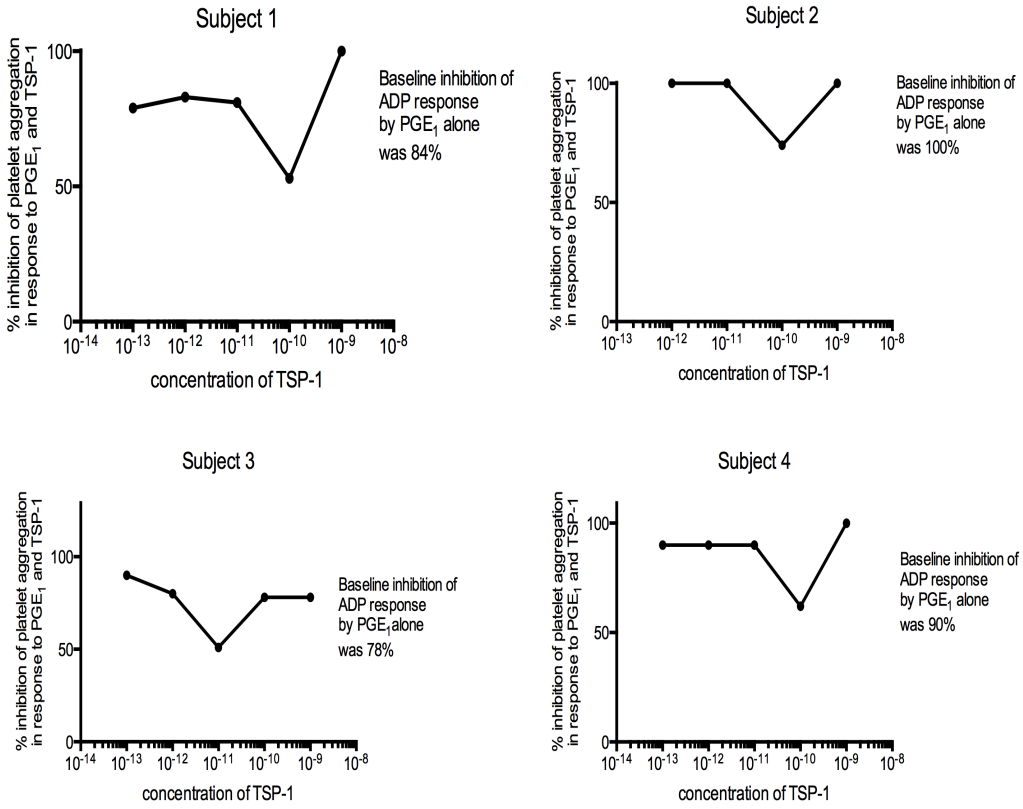


Figure 5.4: Individual subject examples of reversal of PGE_1 effect with varying concentrations of TSP-1.



5.3.3 Discussion

The central finding here is that under carefully controlled conditions, with aggregation in platelet rich plasma, TSP-1 partially reverses the anti-aggregatory effects of PGE_1. For each of the subjects tested, a concentration of TSP-1 could be identified (see Figure 5.4) which reversed the anti-aggregatory effect of PGE_1. The corresponding concentrations of TSP-1 ranged from 10^{-10} to 10^{-11}M. Previous platelet experiments with TSP-1 had used thrombin[325] and collagen[278] as agonists and given the focus of this thesis on $P2Y_{12}$ antagonists, demonstrating modulation of PGE_1 effect in response to ADP was of considerable interest.

Healthy subjects rather than cardiac patients were chosen for these investigations as their sensitivity to PGE_1 is greater, potentially increasing the likelihood of detecting an inhibitory effect on PGE_1 platelet response by TSP-1. Additionally serial experiments were required with some subjects. These would not be possible to perform in cardiac patients undergoing stenting as they would have commenced the ADP antagonist clopidogrel.

The key question which arises from these findings is whether TSP-1 might modulate the phenomenon of PGE_1 resistance, commonly seen in patients with symptomatic cardiac ischaemia[12]. Although this is an attractive hypothesis, proving it would require reversal of PGE_1 resistance by inhibitors of TSP-1 effect. To date, little work has been performed on inhibition of CD36-mediated signalling by TSP-1. However CD36 inhibition is emerging as an experimental strategy, and therefore it remains of great interest to perform such experiment. If

indeed PGE_1 resistance is largely a reflection of TSP-1 effect, a potential strategy for limitation of clopidogrel resistance might emerge thus.

5.4 Modulation of cAMP pathway using the adenylate cyclase inhibitor SQ22536.

5.4.1 Introduction

As foreshadowed in Chapter 3.1, we wished to evaluate whether there was heterogeneity of post $P2Y_{12}$ receptor signalling that could be identified by inhibiting adenylate cyclase: were some subjects more dependent on cAMP signalling for platelet aggregation and others PI3-kinase activation. To perform these experiments, SQ22536 was selected as an adenylate cyclase inhibitor. SQ22536 is (9-(tetrahydro-2-furanyl)-9H-purin-6-amine) an inhibitor of adenylate cyclase which can act on intact platelets. It is an adenosine derivative, with a 9-substituted adenine, able to inhibit adenylate cyclase but with no stimulatory effect[326]. It has an IC_{50} of 13μM for inhibition of prostaglandin E_1-stimulated increases in cAMP formation[326]. At concentrations greater than 100μM it has been demonstrated to reverse PGE_1 mediated inhibition of platelet aggregation and to suppress PGE_1-mediated cAMP formation[326]. We also wished to investigate whether SQ22536 could be used to inhibit the effect of tonic anti-aggregatory prostanoid activity in response to ADP.

5.4.2 Methods

Healthy subjects were approached via TQEH Human Research Ethics Committee approved SA Health email. Subjects that responded were interviewed to determine that they took no medications and had no known diseases or a bleeding tendency and that they were potentially available for 2 or 3 separate episodes of venepuncture. Fifteen subjects were recruited who ranged in age from 29 to 65 years. Baseline Complete Blood Examination was performed to exclude anaemia and thrombocytopenia, the presence of which can create unreliable platelet aggregometry results.

Platelet aggregation experiments were conducted in whole blood and platelet rich plasma using optical detection of aggregation (also known as Light Transmission Aggregometry or LTA). A series of pilot studies were performed to determine the effect of SQ22536:-

1. as a potential modulator of platelet response to PGE_1 and to determine the effect on baseline responses to ADP using whole blood aggregometry.
2. as a potential modulator of platelet response to PGE_1 and to determine the effect on baseline responses to ADP using platelet rich plasma aggregometry.
3. In modifying the effect of wortmannin on ADP-induced aggregation.

Whole blood and platelet rich plasma protocols were performed as described in Chapter 2.1. Platelet rich plasma platelet count was then obtained using the

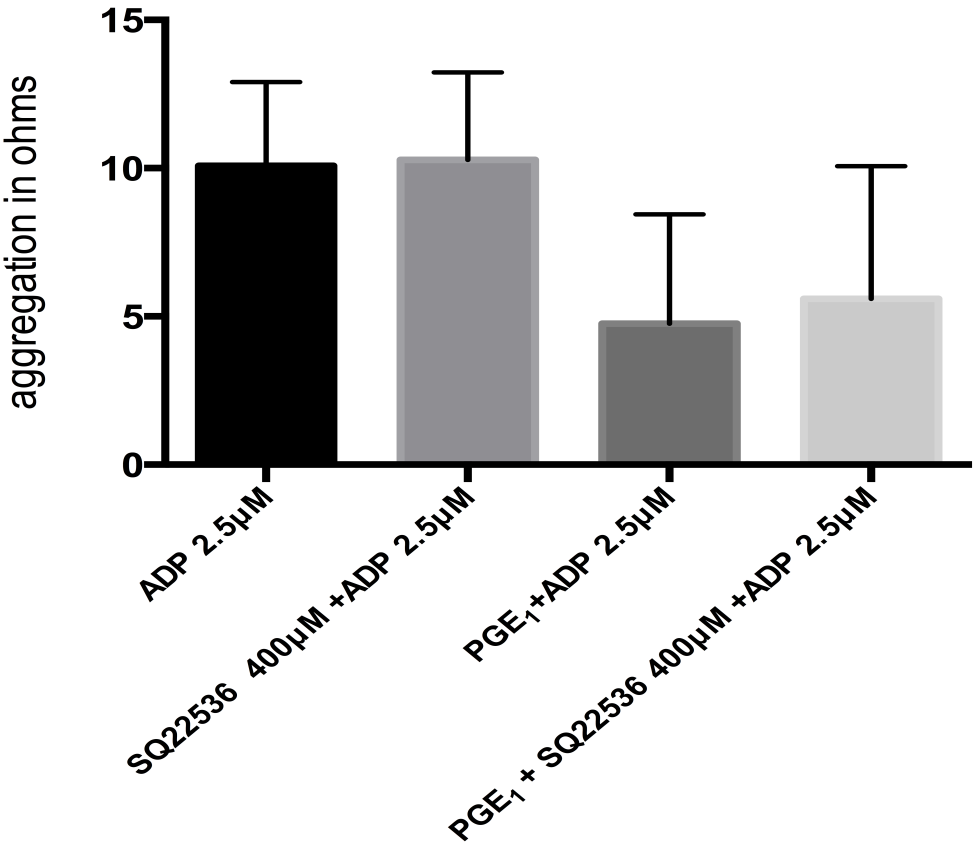
Queen Elizabeth Hospital Haematology laboratory Sysmex XE-1000 via a manual protocol, and then adjusted to 250 $x10^9/mm^3$ prior to performing optical aggregometry. PGE_1 and wortmannin of varying concentrations were preincubated with platelet samples for 1 minute before addition of ADP. SQ22536 30 seconds prior to addition of ADP, unless stated otherwise.

5.4.3 Results

5.4.3.1 SQ22536 as a modulator of PGE_1 response using whole blood aggregometry.

Three healthy subjects who were sensitive to PGE_1 (100% inhibition to 30nM PGE_1) were investigated. Baseline responses to ADP were not affected by 100μM SQ22536. However there was no effect of SQ22536 on PGE_1 mediated platelet response. Given that the concentrations of SQ22536 that will inhibit adenylate cyclase had been previously determined in platelet rich plasma only[326] we increased the concentration for use in whole blood aggregometry and extended the incubation time. In a number of subjects, a concentration of PGE_1 could not be found which suppressed PGE_1 by at least 40%. SQ22536 did not affect platelet aggregation at 400μM, nor was it able to reverse the effects of PGE_1 (Figure 5.5).

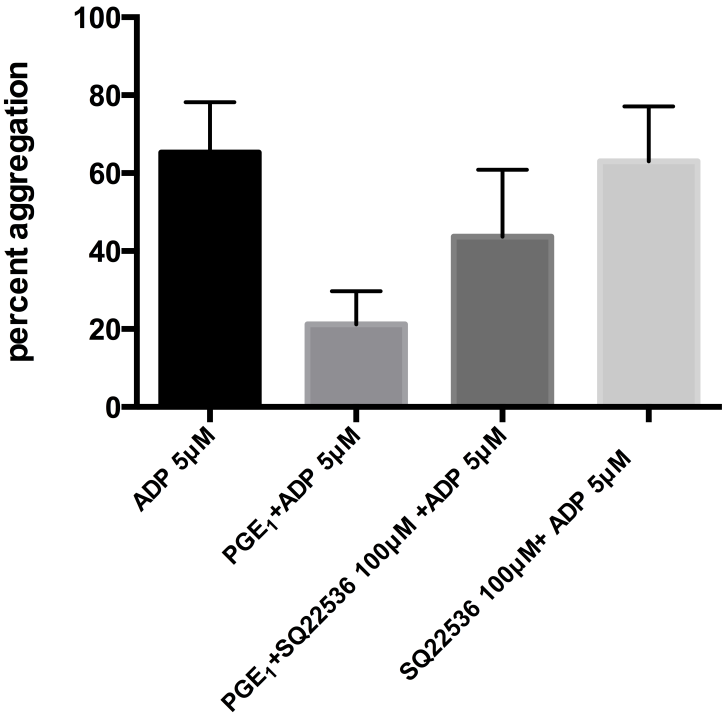
Figure 5.5: Whole blood aggregometry responses to ADP 2.5µM, effects of co-incubation with (i) SQ22536 400µM, (ii) PGE_1 to inhibit aggregation by >40% (varying concentrations) and (iii) PGE_1 and SQ22536 400µM. Mean data n=5.



5.4.3.2 SQ22536 as a modulator of PGE_1 response using platelet rich plasma optical aggregometry.

Five healthy subjects also sensitive to PGE_1 were investigated. Varying PGE_1 concentrations were used to inhibit platelet aggregation by between 50 and 80%. SQ22536 at a concentration of 100μM, while exerting no intrinsic effect on ADP-induced aggregation, inhibited platelet responsiveness to PGE_1 ($P<0.01$) as shown in Figure 5.6.

Figure 5.6: Platelet rich plasma optical aggregometry responses to ADP 5μM, and effects of co-incubation with (i) varying concentrations of PGE_1, (ii) PGE_1 + SQ22536 100μM and (iii) SQ 22536 100μM. In these experiments, PGE_1 concentrations were individualised in order to achieve 50-80% inhibition of platelet aggregation (median 67%).



5.4.3.3 SQ22536 and wortmannin effects on ADP based platelet aggregation.

In the current series of experiments, an interaction between the effects of 400μM SQ22536 in both whole blood and in platelet rich plasma with wortmannin at a concentration of 100nM was sought. Theoretically, it could be expected that the previously observed wortmannin effect on ADP-induced aggregation reflected blockade of myosin light chain kinase[297] then SQ22536 should tend to limit the anti-aggregatory effects of wortmannin (Figure 5.7).

Figure 5.7: Theoretical effect of SQ22536 on ADP-induced platelet aggregation responses to wortmannin.

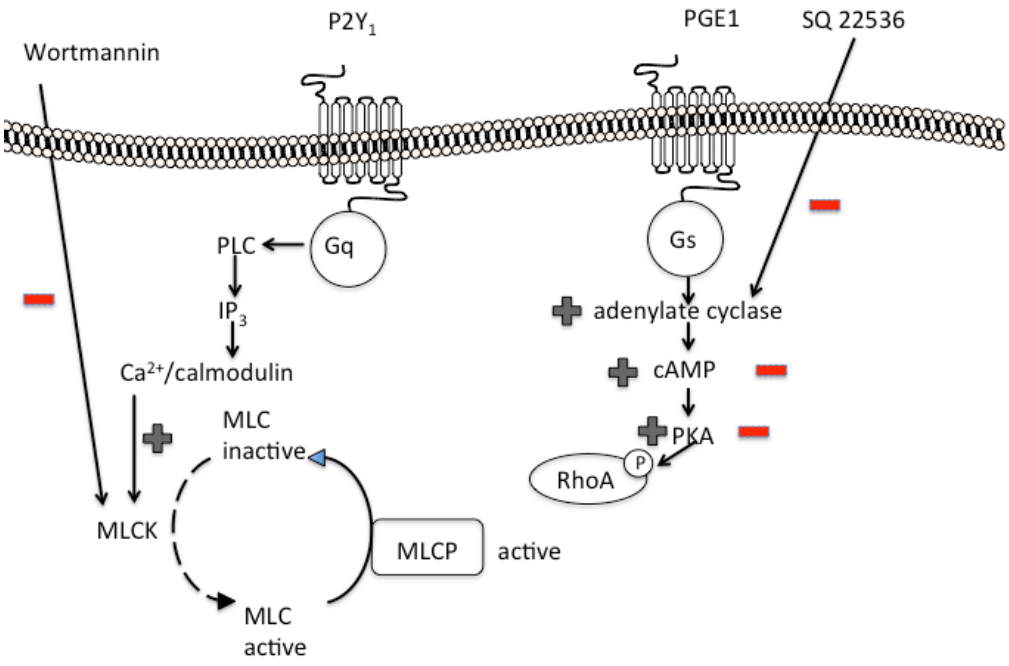
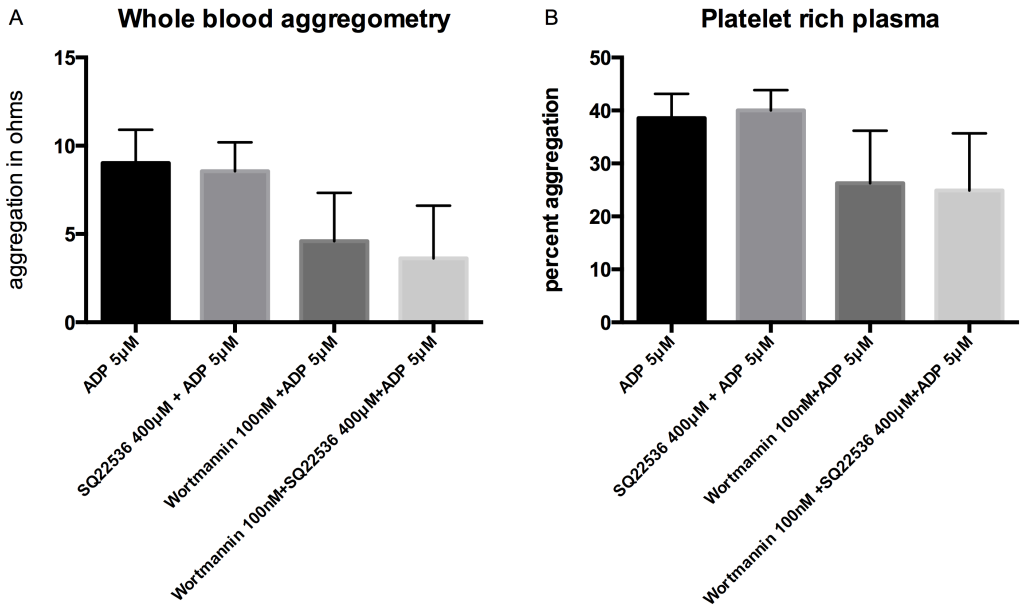


Figure 5.7: Theoretical actions of adenylate cyclase blockade via SQ22536 and myosin light chain kinase blockade using wortmannin. ADP stimulated signalling via G protein coupled $P2Y_1$ receptor activation leads to myosin light chain phosphorylation leading to platelet shape change and platelet aggregation. PGE_1 inhibits myosin light chain phosphorylation through ongoing activation of myosin light chain phosphatase activity[279]. Theoretically, if adenylate cyclase activity could be inhibited, myosin light chain phosphatase activity would also be inhibited and myosin light chain phosphorylation and platelet activation may be able to be at least partially restored in the presence of wortmannin.

In practice, both in whole blood and in platelet rich plasma, the inhibitory effect of wortmannin did not vary significantly in the presence of SQ22536 (Figure 5.8A and B)

Figure 5.8A and B: Whole blood (A) and platelet rich plasma (B) aggregometry responses to ADP 5µM, effects of co-incubation with (i) SQ22536 400µM (ii) wortmannin 100nM (iii) wortmannin 100nM plus SQ22536 400µM. Aggregation was significantly inhibited by wortmannin ((A) and (B) P=0.03) with no significant difference with co-incubation with SQ22536 ((A) and (B) P>0.4).



5.5 Discussion

The original purpose of investigating the impact of SQ22536 on ADP-related signalling was primarily two fold:-

(i) in order to detect a putative "tonic" effect of adenylate cyclase signalling on ADP responsiveness

(ii) in order to determine whether extent of inhibition of PGE_1-induced effect on aggregation by SQ22536 was also predictive of clopidogrel effect.

In practice, the observed effect of SQ22536 were very limited with

(i) no effect on ADP-induced aggregation

(ii) inhibition of PGE_1 effect only in platelet-rich plasma under carefully controlled conditions

(iii) finally, SQ22536 did not modify wortmannin effect.

Interpretation of these findings is limited by the probability that SQ22536 is a relatively weak inhibitor of adenylate cyclase in whole blood[327]. Therefore the "negative" findings do not amount to exclusion of a tonic role of cAMP generation. The relatively weak inhibition observed with SQ22536 in platelet rich plasma made it unsuitable for detailed evaluation of the PGE_1/adenylate cyclase signalling pathway. Indeed, the effects of SQ22536 could only be demonstrated under carefully controlled conditions.

Availability of a more potent, specific inhibitor would have been conceptually useful here. However, there was no clear-cut alternative: the majority of analogous studies attempting to inhibit adenylate cyclase in the literature utilise SQ22536[328-332] although dideoxyadenosine has also been utilised[333-335].

CHAPTER 6: SUMMARY AND FUTURE PERSPECTIVES

At the time the hypotheses tested in this thesis were first postulated, loss-of-function genotypes, particularly of CYP2C19, had gained considerable prominence as the major factor modulating the development of clopidogrel "resistance", or as it has come to be more commonly termed, high on treatment platelet reactivity. This was despite epidemiological evidence that clopidogrel "resistance" was more commonly found in various disease states, including ischaemic heart disease, obesity and Type 2 diabetes and that these disease states were not associated with major differences in the distribution of genotypes compared with the normal population[153].

The $P2Y_{12}$ receptor is linked, via Giα protein, to adenylate cyclase, inducing effective inhibition of cAMP mediated negative control over platelet aggregation[336]. Blockade of $P2Y_{12}$ receptors by clopidogrel therefore in principle restores this negative control (reviewed[337]). A number of prostanoids, including prostacyclin and prostaglandin E_1, function as physiological activators of adenylate cyclase. Furthermore, PGE_1 has previously been shown to potentiate the effects of $P2Y_{12}$ inhibitors [156,283] as have increasing cAMP levels (OPTIMUS-2)[338]. The integrity of adenylate cyclase signalling is impaired in patients with stable and unstable angina [138], obesity and diabetes mellitus. We therefore tested

the hypothesis that the integrity of the PGE_1/cAMP pathway is a genotype-independent correlate of clopidogrel effect. The cGMP pathway was also investigated using SNP as a NO donor, given that cGMP formation contributes to VASP phosphorylation and hence potentially to measured clopidogrel effect. High on treatment platelet reactivity is measured, as the name suggests, only once the patient is treated with the drug and does not take into account baseline platelet responsiveness, a significant potential source of inter-individual heterogeneity. The majority of studies have been performed using "on treatment" measures[193] and at times selecting only one kind of measuring system[193]. We wished to determine the effect of clopidogrel as a change from baseline platelet activity by testing platelet responses before and after clopidogrel treatment and then correlating these responses with clopidogrel effect. To determine clopidogrel effect we performed ADP induced platelet aggregation in whole blood as well as the measurement of VASP PRI before and after one week of weight adjusted clopidogrel dosing. Furthermore we wished to investigate the pre clopidogrel platelet responses to PGE_1 and SNP to determine the integrity of the cAMP and cGMP platelet pathways.

Regarding obesity and the previous literature reporting clopidogrel "resistance", the problem was two-fold. The first was the absence of studies where clopidogrel dosing is adjusted for increased body weight/BMI. This could potentially lead to results where the detected increased rates of high on treatment platelet reactivity could be the result of a "dilutional" effect of increased body weight. Secondly,

there is also evidence, in particular for central obesity, that platelet sensitivity to the inhibitory effects of anti-aggregating autocoids such as prostacyclin and NO donors is impaired and that these effects could be reversed with weight loss. Insulin-resistant Type 2 diabetics lose the modulatory inhibitory effects of insulin on platelet aggregation with insulin resistance also known to develop with increased body weight. We therefore designed a study that incorporated (1) weight adjusted clopidogrel dosing (2) measurement of the pre clopidogrel integrity of the cAMP and cGMP pathways and (3) fasting glucose and insulin levels to determine a measurement of insulin resistance for each subject. We then correlated these responses with clopidogrel effect.

6.1 Key Investigation Findings

We found evidence that post-$P2Y_{12}$ receptor signalling was associated with clopidogrel effect. The key findings of these investigations were:

(1) Pre-clopidogrel platelet sensitivity to PGE_1 predicted clopidogrel effect. Loss of integrity of the cAMP pathway as measured by pre-clopidogrel platelet response to PGE_1 was a significant predictor of clopidogrel effect as measured by platelet aggregometry (ΔADP) and VASP-PRI (ΔVASP-P). This was strongly significant using univariate and multivariate analysis. CYP 2C19 genotype was not found to be a significant multivariate predictor of clopidogrel effect.

(2) Pre-clopidogrel platelet response to SNP, was inversely related to ΔVASP-P alone on multivariate analysis.

(3) Increased BMI was not significantly associated with decreased sensitivity to PGE_1 nor, after weight adjusted dosing, was BMI a predictor of clopidogrel effect using univariate or multivariate analysis.

(4) Insulin resistance was associated with decreased clopidogrel effect
In non-diabetic subjects, there was a significant inverse relationship found on univariate analysis via linear regression between HOMA-IR and ΔVASP-P ($r = -0.29$, $P=0.03$). On multivariate analysis however, HOMA-IR was not found to be an independent determinant of VASP-P.

(5) Under carefully controlled experimental conditions, it could be demonstrated that thrombospondin-1 at concentrations between $1x10^{-10}$M to $1x10^{-11}$M was able to reverse the inhibitory effect of PGE_1 on platelet aggregation using ADP induced aggregometry.

(6) Wortmannin at a concentration of 100nM, was found to have variable inhibitory effects on ADP induced platelet aggregation and these correlated directly with PGE_1 effect on platelet aggregation. One possible explanation of these data is that, wortmannin is inhibiting a kinase which is also inhibited by

PGE_1. It is suggested that the effects of 100nM wortmannin are not specific to PI3-kinases.

6.2 Future perspectives.

1. Methodological Issues

   In the preliminary thesis proposal, our intention was to study the effect of clopidogrel active metabolite in vitro, as well as to study subjects before and after taking clopidogrel, in order to eliminate any potential variability of effect due to the metabolism of the drug. Despite numerous attempts to secure a functional form of the clopidogrel active metabolite, we were unable to do so. We further considered the development of a mass spectrometry assay to detect clopidogrel active metabolite for correlative purposes. However the assay is reputed to be extremely difficult and was not attempted.

2. Post-$P2Y_{12}$ receptor signalling, detected by loss of integrity of the platelet cAMP pathway, was found to be significantly associated with clopidogrel effect. This raises whether loss of platelet sensitivity to inhibitory prostacyclins will also have an effect on the efficacy of other $P2Y_{12}$ antagonists such as prasugrel and ticagrelor. A significant number of patients taking prasugrel undergoing PCI with acute coronary syndromes did not achieve "optimal" platelet inhibition with 25% VASP PRI >50%. This "high on treatment" group were found to have a significantly higher risk for MACE after PCI[339]. Patients with diabetes also have significantly

higher rates of prasugrel "resistance" with increased platelet reactivity as measured by platelet aggregation and VASP PRI compared with non diabetics[254]. Whether this is the result of loss of integrity of the cAMP pathway is worthy of investigation. Furthermore, there is a consistent inverse relationship between body size and prasugrel response[277]. Whether this represents "underdosing" of prasugrel or modulation of post-$P2Y_{12}$ pathways is also of considerable interest. Furthermore, the finding in the PLATO study that high-dose aspirin was associated with inferior responses to ticagrelor has raised the possibility of an effect of aspirin on PGI_2 generation as doses of aspirin greater than 100mg have been demonstrated to further reduce PGI_2 formation[340] .

3. We were able to predict clopidogrel effect based on pre-clopidogrel sensitivity to the effects of PGE_1. While this approach allowed us to detect inter-individual heterogeneity with regard to the integrity of the cAMP pathway by the measurement of platelet responses, it did not allow us to determine where the "defect" lay. In previous work with regard to diabetes, comparisons of platelet reactivity in diabetic versus healthy subjects revealed impairment of adenylate cyclase stimulation by PGE_1 and in response to forskolin[259]. A similar approach to investigate subjects and without sensitivity to prostacyclin/PGE_1 to determine whether impairment of adenylate cyclase stimulation is associated with decreased clopidogrel effect would be of considerable interest.

4. The “salvage” of potentially reduced signalling of the adenylate cyclase/cAMP pathway would be of therapeutic benefit. The OPTIMUS-2 study[338] demonstrated that the PDE3 inhibitor cilostazol, through decreased degradation of cAMP which was detected via increased VASP phosphorylation, was able to improve cardiovascular outcomes on clopidogrel. Cilostazol has a significant side effect profile and can be difficult for patients to tolerate. Development of additional medications to salvage adenylate cyclase signalling may lead to significant clinical benefits.
5. There was a significant inverse relationship between subject HOMA-IR and ΔVASP-P, which was present in subjects who were not diabetics. While the presence of diabetes has a well established relationship with high on treatment platelet reactivity, such a relationship in subjects who are insulin resistant but not diabetic has not been established. Relative to mice fed low fat diets, obese mice fed high fat diets had dramatically reduced VASP phosphorylation in aortic tissues. Conversely, over expression of VASP in endothelial cells blocked inflammation and insulin resistance induced by palmitate[341]. Given our findings in humans that a decreased change in VASP-P was detected with increasing insulin resistance, further investigation of subjects with known increased rates of insulin resistance such as obesity and diabetes would be worthwhile.

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Appendix

Published work in whole contained within this thesis.

Hurst, N.L., Nooney, V.B., Raman, B., Chirkov, Y.Y. and Horowitz, J.D. (2013)
Clopidogrel “resistance”: Pre- vs post-receptor determinants.
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