# Inter-individual variability in platelet adenylate and soluble guanylate cyclase signaling: therapeutic perspectives

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# Abstract

Variability in integrity of platelet prostanoid adenylate cyclase signaling: pathogenetic and therapeutic implication

The prostanoid-adenylate cyclase (PG/AC) signaling system plays a major role in inhibiting platelet aggregation and resultant thrombosis. In the studies carried out in this thesis, it was shown that: -

(a) Integrity of PG/AC signaling exerts a major influence of individual responses to inhibitors of the $P2Y_{12}$ receptor.

(b) Patients with threatened myocardial infarction have impairment of PG/AC signaling relative to normals.

(c) Coronary artery spasms (CAS) is associated with impairment of both PG/AC and nitric oxide signaling, which may be pivotal to occurrence of CAS.

(d) PG/AC signaling is potentiated by the anti-anginal agent perhexiline.

## Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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 references has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

Because some of the experiments in this thesis, especially those in chapters 5 and 6 are the subjects of a provisional patent, the thesis should be the subject of a short-term embargo.

Therefore, I give permission for the digital version of my thesis to be made available on the web, via University digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Scholarship.

Md Hasan Imam

(April 2018)

## Acknowledgements

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# Publications, presentations and awards related to the work conducted towards this thesis

***Publication: -***

Nathan EK Procter, Nicola L Hurst, Vivek B Nooney, **Hasan Imam**, Raffaele De Caterina, Yuliy Y Chirkov, John D Horowitz. New Developments in Platelet Cyclic Nucleotide Signaling: Therapeutic Implications. Cardiovasc Drugs Ther (June 2016).

***Presentations of work related to this thesis***

(1) Platelet resistance to prostacyclin/adenylate cyclase signaling in ischaemic heart disease and diabetes mellitus: therapeutic implications. P-127, ASMR meeting, 8th June 2016, Adelaide Australia.

(2) The 10th Annual Florey Research conference, 29th September 2016, Adelaide Australia.

(3) Cardio-protective agent Perhexiline ameliorates impaired adenylate cyclase signalling in patients with cardio-vascular diseases. TQEH Research Day, Adelaide Australia 21.10.2016

(4) Adenylate cyclase signalling is an important post-receptor determinant of response to all P2Y12 receptor antagonists. ESC 2017 meeting of the European Heart Congress, Poster number P203, Barcelona, Spain.

(5) Impaired activity of platelet soluble guanylate cyclase and adenylate cyclase signaling in patients with coronary vasospasm: changes during acute crises. ESC 2017 meeting of the European Heart Congress, Poster number: P3669, Barcelona, Spain.

***Scholarship related to this thesis***

IPRS, University of Adelaide, awarded in years 2015-2018.

# Chapter 1: Introduction

***Abbreviations***

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| AC | Adenylate cyclase |
| ACS | Acute coronary syndrome |
| ADP | Adenosine di phosphate |
| CABG | Coronary artery bypass grafting |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| CHD | Coronary heart disease |
| COX | Cyclooxygenase |
| I3P | Phosphoinositide 3 kinase |
| MI | Myocardial infarction |
| NO | Nitric oxide |
| PAR | Protease-activated receptor |
| PF4 | Platelet Factor 4 |
| $PGE_1$ | Prostaglandin $E_1$ |
| $PGI_2$ | Prostacyclin |
| PKA | Protein kinase A |
| SAP | Stable angina pectoris |
| sGC | Soluble guanylate cyclase |
| SNP | sodium nitroprusside |
| $TxA_2$ | Thromboxane $A_2$ |
| UA | Unstable angina |
| VASP(P) | Vasodilator stimulated protein (phosphorylated) |
| v WF | von Willebrand factor |

## 1.1 Physiology of platelet aggregation and its limitation: signalling pathways

### 1.1.1 Aggregatory mechanisms

Platelets aggregation is the end-result a series of processes which can be initiated in the presence of agonists including ADP, thrombin, thromboxane $A_2$ ($TxA_2$), and collagen via their corresponding receptors (*Table 1.1; Figure 1.1*). ADP is released especially by damaged cells, and can bind to and act upon the nearby widely distributed purinergic G-protein-coupled $P2Y_1$ and $P2Y_{12}$ receptors (Gurbel, 2005). Stimulation of the $P2Y_1$ receptor activates phospholipase C which facilitates the breakdown of phosphatidyl inositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol. $IP_3$ stimulates the release of calcium ($Ca^{2+}$) from intracellular stores (Jin and Kunapuli, 1998; Daniel et al 1998), with a subsequent platelet activation (shape change and release of bioactive substances from alfa and dense granules) and initiation of aggregation. The continuation of these processes requires the engagement of the $P2Y_{12}$ receptor, responsible for two main signaling cascades:

(i) $Gi_{2\alpha}$ mediated inhibition of AC signaling leading to reduced generation of cAMP (which is a negative regulator of platelet aggregation) and resultant propensity towards swift acceleration of the incipient platelet aggregation enabled by the $P2Y_1$ receptor (Cattaneo, 2007).

(ii) $G_{\beta\gamma}$ mediated stimulation of phosphoinositide 3-kinase (PI-3K) signaling. Further downstream mediators of PI-3K signaling include AKT/PKB (a serine threonine kinase, also termed as protein kinase B) (Woulfe, 2010). It is now understood that activation of PI-3K via $P2Y_{12}$ receptor contributes to the ADP-mediated calcium response (via $P2Y_1$), in addition to simultaneous inhibition of AC (Hardy et al 2004).

Simultaneous stimulation of both these purinergic receptors is essential for the platelet activation and subsequent aggregation via expression of integrin $\alpha_{IIb}\beta_3$ receptors of platelets to bind with other platelets through fibrinogen bridges.

Platelets have two receptors for thrombin (PAR-1and PAR-4). The protease activity of thrombin cleaves off a component of these receptors, subsequently activating platelets (Leger et al 2006).

Another agonist that activates platelets is collagen, through its specific receptor, glycoprotein VI. The complex activation process is stimulated by a collagen-GPVI/1a interaction and subsequent release from dense granules of ADP, ATP, calcium, serotonin (Davi &Patrono, 2007). The interaction of platelets with sub-endothelial collagen and endothelium expressing von Willebrand factor (vWF) may be subjected to shear forces in the arterial lumen (Samara & Gurbel 2003). Specifically, in the narrow space of partially occluded arteries, exerting high forces (>600 $s^{-1}$), circulating vWF may transform into linear shape and bind circulating platelets through its GP1b/V/IX receptor complex (Ruggeri, 2000). Finally, platelet adhesion is regulated mainly by the collagen receptor α2β1 integrin, under low shear stress conditions (Saelman et al 1994).

$TxA_2$, an arachidonic acid derivative, is another agonist of platelet activation that activates platelets via TP receptor (Giannarelli et al 2010).

ADP has been reported to activate platelets by releasing dense granules; its activity is increased by interaction with other agonist such as Thromboxane $A_2$ (Cattaneo et al 1997; Cattaneo et al 2000; Cattaneo, 2005). Conversely, clopidogrel and other antagonists of ADP binding to the $P2Y_{12}$ receptor, and aspirin, which among other things inhibits $TxA_2$ synthesis, exert antithrombotic effects, and are widely utilized in the management of ACS.

**Table 1.1:** Selected agonists inducing platelet activation

| Agonists | Corresponding receptors |
|---|---|
| ADP | $P2Y_1$ and $P2Y_{12}$ |
| Adrenaline | α2A |
| Collagen | GPVI |
| Thromboxane $A_2$ | TP receptor |
| Thrombin | PAR-1 and PAR-4 |

### 1.1.2 Anti-aggregatory mechanisms

Platelet inactivation occurs via agents that either exert inhibitory signaling effects or degrade the stimulatory agents. Inhibitory signaling pathways may be regulated by prostacyclin ($PGI_2$), $PGE_1$, $PGD_2$ and NO, while ectonucleotidases remove the phosphate group from ADP and prevent platelet activation (Giles et al 1989; Marcus et al 1997; Iyu et al 2011). $PGI_2$ and nitric oxide (NO) are regulators of intracellular cyclic nucleotides: cGMP and cAMP respectively. Both are primarily generated by healthy intact vascular endothelial cells.

Platelet cGMP synthesis is mainly regulated by soluble guanylate cyclase (sGC), the main NO receptor. Elevation of cGMP mediates down-regulation of agonist induced intracellular calcium signaling, fibrinogen binding, adhesion, and aggregation of human platelets with the involvement of the vasodilator-stimulated phosphoprotein (VASP) (Keularts et al 2000; Schwarz et al 2001; Munzel et al 2003). On the other hand, AC is activated by Gs-protein coupled IP (prostacyclin receptor) receptors. Activation of this pathway accelerates production of cAMP which further phosphorylates cAMP-dependent protein kinase A (PKA) and vasodilator-stimulated phosphoprotein (VASP) and thus limits aggregation (Yada et al 1989; Keularts et al 2000). Thus, both cGMP and cAMP signaling pathways converge at VASP. Indeed, there is a high degree of interconnection from the receptor level to distal signaling between AC and GC signaling pathways, as reviewed by Smolenski, 2011.

$PGE_1$ and $PGD_2$ are also released from endothelium and inhibit platelet activation via increased cAMP signaling. While $PGD_2$ acts through the DP receptor (Giles et al 1989), $PGE_1$ predominantly activates the IP (receptor for prostaglandin $I_2$) receptor (Iyu et al 2011), present on the platelet membrane. However, $PGE_1$ also interacts with $EP_3$ receptor, which has opposite effects on cAMP signaling (Iyu et al 2011).

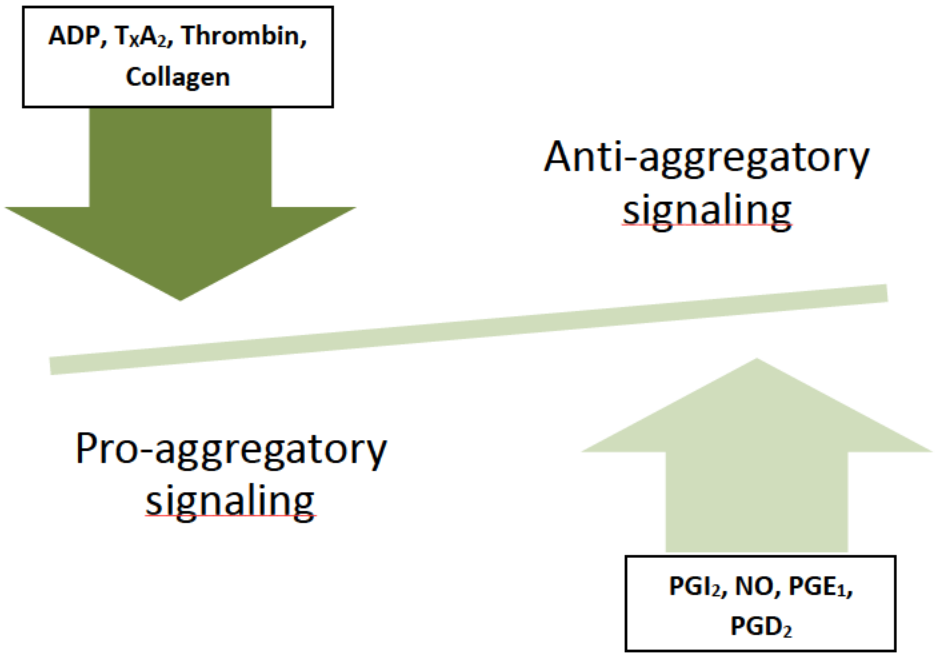



**Figure 1.1:** Schematic diagram of the balance between pro and anti-aggregatory signaling. The role of platelets in hemostasis and thrombosis is dependent on a complex balance of activatory and inhibitory signaling pathways. Pro-aggregatory signaling stimulates platelet aggregation and prevent blood loss, on the other hand, anti-aggregatory signaling prevents thrombosis.

## 1.2 Methods for evaluation of platelet aggregatory physiology

### 1.2.1 Aggregometry

#### 1.2.1.1 Platelet rich plasma

Measurement of platelet aggregability was pioneered in 1962 by Prof Gustav Born. This methodology still in widespread use, utilizes aggregation measurement in platelet rich plasma (PRP).

In that method blood samples are centrifuged to isolate platelet rich plasma, which can be utilized directly to assay responses to different agonists and inhibitors of platelet aggregation. Platelet count can be adjusted to ensure a uniform assay protocol between different subjects. However, PRP precludes assessment of effects of other cells present in blood. Indeed functional

responses of platelets depend on interactions with erythrocytes and leukocytes (De La Cruz et al 1999). Also the substantial manipulation that takes place during PRP preparation may potentially introduce artefacts.

#### 1.2.1.2 **Aggregometry in whole blood**.

Whole blood aggregometry may be considered more physiologically relevant than PRP. There are two further techniques for investigating whole blood aggregometry. These are impedance aggregometry and particle counting aggregometry. Impedance aggregometry was first described by Cardinal and Flower (1980).

The method consists of a pair of palladium-made wire electrodes placed into a tube with continuously stirred whole blood, diluted with physiological saline. The principle underlying whole blood impedance aggregometry is that platelet clumping on electrodes (*Fig. 1.2A*) during aggregation impedes the associated electrical current. During initial contact with blood, electrodes become coated with a monolayer of platelets. Platelets start clumping on the monolayer when an activator is added to the tube. Increasing platelet aggregates on the surfaces of the two wires, across which impedance is measured, causes increasing electric resistance. This impedance is recorded as computerized tracings made over time and is directly proportional to the mass of the aggregate. For example aggregation may be recorded for 7min following agonist addition, and its extent calculated in Ohms (*Fig.1.2B*).

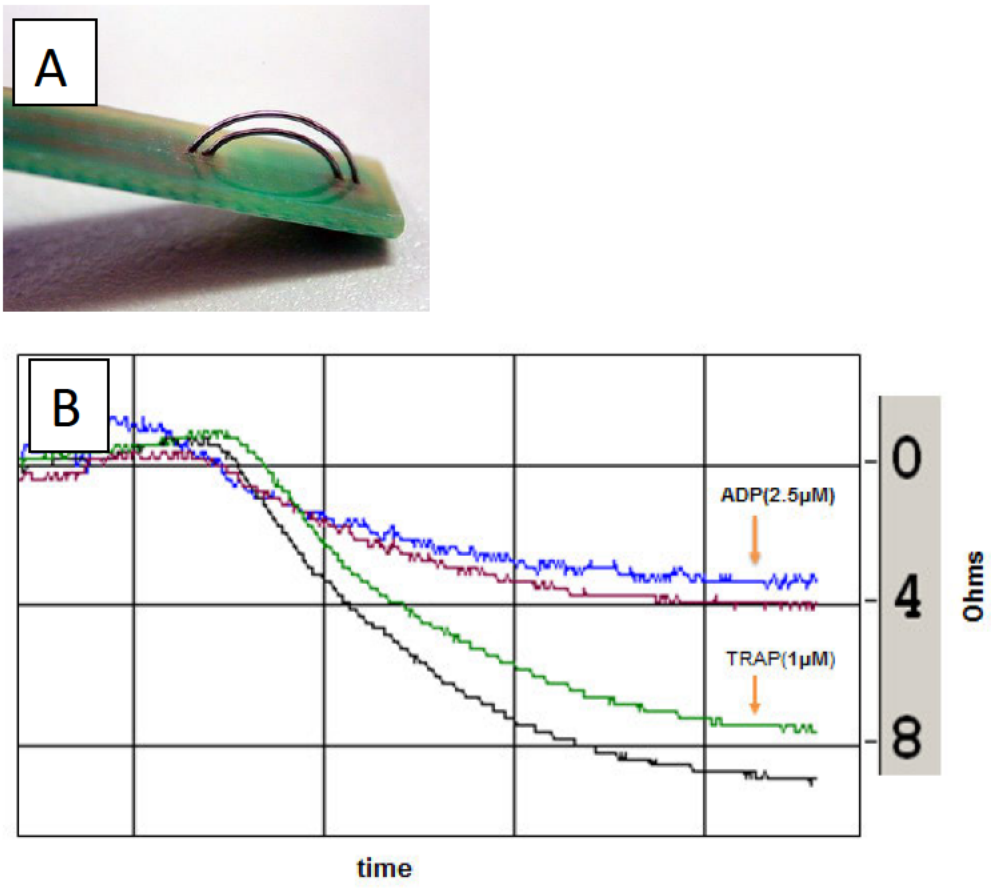



**Figure 1.2:** (**A**) Palladium-made wire electrodes inserted in sample. Increasing platelet aggregates on the surfaces of the two wires across cause increasing electric resistance. (**B**) Agonist-induced impedance aggregation tracings. The responses to 2.5μM ADP is 4.0 Ohms and 1μM TRAP is 8.0 Ohms, each tested in duplicate.

### 1.3 Markers of platelet activation/aggregation

There is increasing evidence for platelet involvement in atherosclerosis, thrombosis, inflammation and immunity in addition to the established hemostatic function (Massberg et al 2002; von Hundelshausen et al 2007). Therefore, it is worth searching a reliable platelet marker for health and disease. Possible candidates for markers platelet activation fall into three groups, as described below

i. Platelet alpha granules containing beta thromboglobulin (Moore et al 1975) and platelet Factor 4 (PF4) (Niewiarowski and Thomas, 1969). These are two chemokines which are secreted exclusively by platelets upon activation and

changes in their plasma levels have been described in various diseases (Ogasawara et al 1986). However, influence of many variables such as certain drugs, diseases and phlebotomy techniques make determination of PF4 or β-thromboglobulin potentially misleading as a good platelet activation marker (Gurney et al 2002).

ii. Platelet membrane-bound proteins including p-selectin (Hsu-Lin et al 1984), Glycoprotein IIb/IIIa, Glycoprotein V, and Glycocalicin, all are highly expressed on the platelet surface during activation. This allows convenient analysis either by flow cytometry or ELISA (Gurney et al 2002), thus offer great potential for utilization as activation markers.

iii. Metabolic products, such as members of the prostaglandin family, especially metabolite of the pro-aggregatory prostanoid $TxA_2$, show good correlation with extent of platelet activation (Schror H, 1997). Also, there are easy immuno assay-based measuring techniques for the $T_XA_2$ metabolite ($T_XB_2$). However, one problem with $T_XA_2$ is that it is not exclusively released from platelets.

## 1.4 Pathophysiological perspectives

### 1.4.1 Hyperaggregability

Platelet hyperaggregability was recently defined by Sokol et al (2017) as increased aggregation measured by light transmission aggregometry in the presence of low concentrations of ADP or epinephrine, while the effects of other agonists will remain normal. The clinical importance of hyperaggregability is undeniable and has been described in a wider range of cardiovascular disorders such as stable coronary artery disease (Diodati et al 1992), aortic stenosis (Chrikov et al 2002), chronic kidney disease (Yagmur et al 2015), obesity (Leite et al 2016). The underlying problem of hyperaggregability may stem from increased sensitivity to agonists as described in

several different disease conditions (Aaron et al 2001) There is also increasing evidence showing that impairments in anti-aggregatory signalling are associated with platelet hyperaggregability (Chirkov et al 2002; Leite et al 2016). Hyperaggregability was also associated with aspirin resistance (Kawasaki et al 2000). On treatment with clopidogrel, hyperaggregability was associated with increased risk of stent thrombosis, as reported by many authors (Gurbel et al 2005; Price et al 2008; Sibbing et al 2009), and it was suggested therefore to perform baseline platelet function testing in order to differentiate platelet resistance to clopidogrel from pre-existent platelet hyperaggregability (Nooney et al, unpublished).

### 1.4.2 Impaired function of endogenous anti-aggregatory pathways

#### 1.4.2.1 NO/sGC

Exogenous NO donors have long been used as an anti-anginal drugs with antiplatelet properties. Platelet cGMP synthesis is mainly regulated by sGC, a hetero-dimeric enzyme consisting of an alpha-and a haem-containing beta subunit, that bind with NO. Elevation of cGMP stimulates down-regulation of agonist-induced intracellular calcium signaling, fibrinogen binding, adhesion, and aggregation of human platelets (Keularts et al 2000; Schwarz et al 2001; Munzel et al 2003). Impaired responses within the platelet NO/sGC pathway in cardiovascular diseases have been described by many authors (Chirkov et al 2001 & 2002; Leite et al 2016). The putative mechanisms underlying NO/sGC resistance are reported as- heme loss from sGC (Pan et al 2016), and increased superoxide production during oxidative stress and consequent decrease of NO availability (Munzel et al 1995; Gladwin MT, 2006) etc. A number of strategies to ameliorate or circumvent NO/sGC resistance have suggested. Reversal of hyperglycaemia in diabetics (Worthley et al 2007), the ACE inhibitors perindopril (Chirkov et al 2004) and ramipril (Willoughby et al 2012) all ameliorate NO/sGC resistance.

Among strategies proposed to activate sGC while circumventing NO resistance are nitroxyl donors (Dautov et al 2013), nitrites (Borgognone et al 2018) and direct sGC activators (mechanisms are schematized in *Fig. 1.3*). Clinically, direct sGC activators are now utilized for the management of pulmonary hypertension (Simon et al 2016).

#### 1.4.2.2 PG/AC

Anti-aggregatory mechanisms may be regulated differentially under various pathologic conditions. Adenylyl cyclase is the target of one of the major signaling pathways of G-protein-coupled receptors. Upon activation by prostacyclin/prostaglandin $E_{1,}$ through the Gs-coupled IP receptor, or by direct-acting forskolin, GPCR accelerate production of cAMP, and limit aggregation (Yada et al 1989; Keularts et al 2000). Reduced responses to both $PGE_1$ and forskolin and associated diminution in cAMP formation were documented in platelets from patients with DM (Livingstone et al 1991). Chirkov et al (1995) have shown that $PGE_1$ concentration-response curves in patients with angina was shifted to the right compared with normal subjects. Impaired platelet $PGE_1$ response has also been documented in acute ichaemic heart disease patients, this anomaly was shown reversed by acute insulin therapy (Khan et al 1991).

There are also a number of other possible activating stimuli of both sGC and AC, as well as a number of potential mediators and modulators of their effects, other than the release of cyclic GMP and cyclic AMP respectively as a result of enzyme activation. Importantly, these two pathways interact at the level of VASP phosphorylation, and thus have mutually potentiating effects.

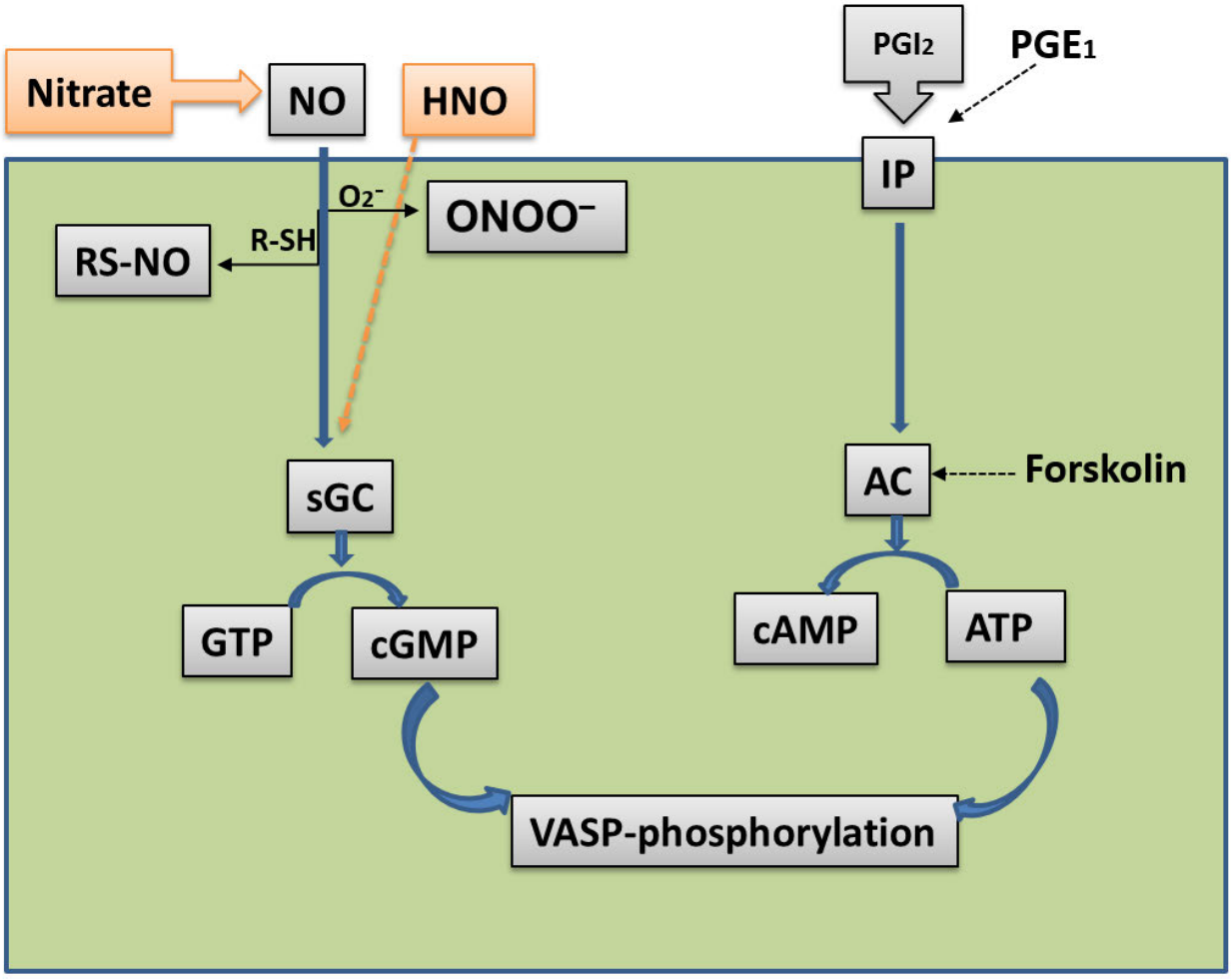



**Figure 1.3**: Schematic of the NO/sGC and $PGI_2$/AC signaling. Nitric oxide (NO) (endogenous or released from organic nitrates) has high affinity for soluble guanylate cyclase (sGC) that catalyzes cGMP formation from guanosine 5'-triphosphate (GTP). In addition, NO interacts with superoxide anion ($O_2^-$) to form peroxynitrite anion ($ONOO^-$), and in presence of sulfhydryl groups (SH) can form nitrosothiols (RS-NO) which are potentially anti-aggregant. Similarly, nitroxyl (HNO) with lack of reactivity to reactive oxygen species can also stimulate sGC. On the other hand, adenylate cyclase (AC) activation occurs via prostacyclin receptor (IP) stimulation by $PGI_2$ and $PGE_1$ and also directly by forskolin, that catalyzes cyclic AMP formation (cAMP) formation from adenosine tri-phosphate (ATP). Both cAMP and cGMP stimulate vasodilator stimulated phosphoprotein (VASP) phosphorylation and consequently inhibit platelet activation.

### 1.5 Relationship between inflammatory activation and disordered platelet aggregability

In addition to haemostatic function, circulating platelets release many proinflammatory cytokines that favor inflammation and leukocytes recruitment in the vicinity of circulatory lumen (Huo et al 2003). Almost all the receptors that activate platelets, can stimulate inflammatory cytokine release (reviewed by Vieira-de-Abreu et al 2012). These proinflammatory cytokines mediate recruitment of leukocytes in sites of vascular erosion, which is thought to be an essential part of immune surveillance. P-selectin (Huo et al 2003) and GPIb ((Massberg et al 2002) are the two important receptors for the platelet neutrophil interaction during inflammation. Clinical bases for platelet activation during inflammatory disorders are summarized in *Table 1.2*.

**Table 1.2:** Platelet activation during inflammation and malignancy

| | |
|---|---|
| Inflammatory bowel disease (IBD) | Increased CD40L (Danese et al 2003) |
| Glomerulonephritis | Increased P-selectin expression and interaction with neutrophils (Danese et al 2004) |
| Systemic lupus erythematosus | Increased: P-selectin expression; CD40L release; platelet monocyte interaction (Duffau et al 2010) |
| Rheumatoid arthritis | Increased numbers of platelet-monocyte aggregates in synovial fluid (Joseph et al 2001) |
| Inflammatory pulmonary disease | Increased platelet P-selectin expression (Moritani et al 1998) and platelet-leukocyte precipitant in lung (Pitchford et al 2005) of asthmatic patients |
| Malignancies | Increased thrombosis in malignancy (reviewed by Elamany et al 2014). Thrombocytopenia was shown to be associated with reduced metastasis in metastatic mouse model (Gasic et al 1968) This was reversed by platelet infusion (Karpatkin et al 1984) |

### 1.6 Platelet aggregability and thrombotic disorders

The role of platelets in initiation of atherosclerosis, coagulation and the process of inflammation has been reviewed by Gurbel et al (2004). Circulating platelets have little or no contact with the vascular bed under normal physiological conditions (Samara & Gurbel 2003). However, during inflammation, activated platelets can bind to intact endothelium (Gawaz et al 1996 & 1997; Bombeli et al 1998), secreting many proinflammatory cytokines, which favor leukocyte recruitment in that vicinity in a paracrine manner (Huo et al 2003). Also, under high shear force (Frenette et al 1995; Massberg et al 1998), in the narrow space of a partially occluded artery, exerting high stress (>600 $s^{-1}$) causes immobilized vWF to transform into linear shape and bind circulating platelets through its GP1b/V/IX receptor complex (Ruggeri, 2000).

On the other hand, platelet adhesion to the collagen receptor (GPVI) takes place under both high and low shear (Saelman et al 1994; Samara & Gurbel 2003). Collagen from ruptured plaque, von Willebrand factor (vWF), fibrinogen, fibronectin, thrombospondin and other sub-endothelial components cause the recruitment of more circulating platelets to the obstructed path (Gurbel & Tantry 2006; Ruggeri & Mendolicchio, 2007). Relevant receptors on the platelet surface that interact with other cells during atherosclerosis initiation are listed in *Table 1.3*.

**Table 1.3:** Platelet receptors that interact with endothelium and other cells

| Platelet receptors | Target cells/tissues |
|---|---|
| GPIIb-IIIa (αIIbβ3) | Major receptor for binding platelets to other platelets and also with vitronectin receptor (αvβ3) expressed on endothelium (Gawaz et al 1991; Gawaz et al 1997) |
| P-selectin | Inflamed endothelium, and also endothelial ligand (Huo et al 2003) |
| GPIb | Both leukocytes (during inflammation) and vascular wall (Massberg et al 2002) |
| GPVI | One of the major collagen receptors binding platelets to damaged vascular wall |

## 1.7 Therapeutic perspectives-The overall spectrum

### 1.7.1 Anti-thrombotic strategies: prophylactic

#### 1.7.1.1 Aspirin

The antiplatelet drug aspirin inhibits platelet cyclooxygenase (COX) enzyme, thereby reducing thromboxane A2 ($TxA_2$) formation and platelet activation (FitzGerald et al 1983). Prophylactic aspirin treatment, and resultant relative risk reduction of first MI risk in patients with stable angina pectoris is well described (Hartney et al 1988; Moller-Jull et al 1992). Baigent et al (2002) have shown in a collaborative meta-analysis that long term low dose aspirin therapy reduces risk of acute events (MI, stroke). Low dose aspirin therapy was also recommended by the American College of Cardiology/ the American Heart Association (ACC/AHA) as class I in ACS patients (Antman et al 2004) and class IIa in patients with SAP (Gibbons et al 2003) to reduce the risk of MI. Aspirin therapy was also shown to decrease ischaemic complications in all forms of coronary intervention (Goldman et al 1988; Kubota et al 2008; Gluckman et al 2011). Based on these mass of evidence, ACC/AHA recommended (as class I) 100-325mg/daily aspirin therapy followed by coronary artery bypass grafting (CABG) (Eagle et al 2004), and 75-325mg dose before PCI followed by 75-325mg (Smith Jr et al 2006) therapy for indefinite time. On the other hand overall therapeutic benefit of aspirin in primary prevention is less clear particularly in DM (Saito et al 2017), because the benefit in reducing infarct risk is counterbalanced by increased risk of bleeding. There is also increasing evidence that doses of aspirin no higher than 150mg/day may be optimal (Mahaffey et al 2014).

#### 1.7.1.2 ACE inhibitors

While angiotensin converting enzyme (ACE) inhibitors therapy are typically used primarily to manage hypertension or heart failure. However, in addition at least ramipril and perindopril decrease risk of cardiac events (Gomma AH and Fox KM, 2001; HOPE study investigators). The pleotropic anti-aggregatory effects of ACE inhibition and their underlying mechanisms have been known from early 1980s. James et al (1988) documented captopril-induced diminished platelet aggregation, which was associated with an implied decrease in $TxA_2$ release. After that, Zurbano et al (1999) have shown that captopril mediates a decrease in platelet surface integrin

IIb/IIIa. In addition, ACE inhibitors are also reported to increase NO production from endothelial cells and elsewhere and thereby platelet inhibition (Weir et al 1999). Willoughby et al (2012) showed that ramipril exerts a NO-sensitizing effect within platelets in high-risk patients, who would have been eligible for the HOPE trial (HOPE study investigators).

#### 1.7.1.3 Hydrogen sulphide ($H_2S$) donors

$H_2S$ is endogenous gaseous transmitter with vasorelaxant properties, produced from the β-elimination reaction of L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Kimura H, 2002). Anti-aggregatory effects with potential mechanisms involving $H_2S$ have been documented by many authors. Zhong et al (2014) have shown that NaHS induced decreases in $TXA_2$ and ATP release, and subsequent decrease in collagen induced aggregation. Using a slow $H_2S$-releasing agent, in a similar type of study, Grambow et al (2016) have also demonstrated reduced TRAP-mediated human platelet activation. The same study also documented that inflammatory binding of platelets with leukocytes decreases, as shown by expression of platelet-leukocyte binding markers, in the presence of $H_2S$. Gao et al (2015) have implicated the platelet inhibitory effects of $H_2S$ engendering from interference with gap junction channels in human platelets. Some animal studies show that pre-treatment with $H_2S$ decreases chemical-induced arterial thrombus formation (Nishikawa et al 2013; Grambow et al 2016; Qin et al 2016)

Critical relationships and potential dependencies between NO and $H_2S$ signaling have been documented. $H_2S$ mediated upregulation of NO synthase was observed in mice treated with the $H_2S$ donor $Na_2S$ (Kram et al 2013). Conversely, use of NOS inhibitor treatment decreases $H_2S$-mediated vasodilatory effects (Li Q and Lancaster JR, 2013). This research indicates that the NO/sGC signaling may be not completely self-sufficient, both in the context of platelet inhibition of aggregation and vasorelaxation.

#### 1.7.1.4 N-acetylcysteine (NAC)

N-acetylcysteine (NAC) has long been known for its antioxidant effects. Early studies from Halliwell's group demonstrated that NAC "scavenged" a number of reactive oxygen species, notably hypochlorous acid (HOCl) and hydrogen peroxide ($H_2O_2$) (Auroma et al 1989). There is also evidence that NAC, while not "scavenging" $0_2^-$, may inhibit generation by NAD(P)H oxidases (Nakagami et al 2003). NAC has been shown in many studies to potentiate NO donors' vasodilatory and anti-aggregatory effects. This has been demonstrated clinically in a wide range of cardiovascular circumstances, potentiating hypotensive responses to NTG, and reducing filling pressures (Horowitz et al 1983; Mehra et al 1994). The bases for NAC potentiation of NO response are not clear. In vitro, NAC with nitroglycerine, but not NAC alone was shown to increase sGC (purified) activation (Munzel et al 1989). NAC was also shown to increase activation of eNOS in rat myocytes (Wating et al 2016), although that effect is not obviously relevant to potentiation of NO donor effects. The NO-potentiating effects of NAC have also been shown to extend to the anti-aggregatory effects of NO donors (Loscalzo J, 1985; Chirkov et al 1996), and together with vasodilator effects formed the basis for observations of termination of cyclic flow changes after coronary artery injury in a study by Folts and Loscalzo (1991). The NTG/NAC interaction has been reported to improve efficacy of treatment in patients with unstable angina pectoris (Horowitz et al 1988), acute myocardial infarction (Arstall et al 1995; Pasupathy et al 2017) and acute pulmonary oedema (Beltrame et al 1998).

#### 1.7.1.5 New developments

Some antidiabetic, anti-hypertensive and lipid lowering drugs also exert antithrombotic effects. The biguanide derivative, metformin, was shown in rat model to increase NO production through eNOS activation (Davis, 2006) and suppression of inflammatory cytokine genes (Zhu et al 2015). Recently Krijnen et al (2012) reported that reduced expression of endothelial serine dipeptidyl peptidase 4 (DPP-4) receptor leads to increased tissue factor secretion and platelet adhesion. Given that DPP-1 is another known antidiabetic agent that inhibits glucagon like peptide-1, this warrant further research about its potential effects in cardiovascular management. Direct ex vivo antiplatelet effects of statins measured by ADP and collagen were reported by Matetzky et al (2011). Mechanisms of the "pleotropic" effects of statins have been attributed to

PAR-1 inhibition (Sikora et al 2013), but also to increased NO availability via eNOS activation (Laufs and Liao, 1998). The 3rd generation β-receptor blocker nebivolol can also increase NO synthesis, and hence β3-adrenoreceptor activation can potentially modulate platelet aggregation (Dessy et al 2005). The prophylactic anti-anginal drug, perhexiline, was also shown to increase NO-mediated inhibition of aggregation in patients with both stable angina pectories and acute coronary syndrome (Willoughby et al 2002).

## 1.8 Anti-thrombotic strategies: acute crises

### 1.8.1 $P2Y_{12}$ receptor antagonists

ADP interaction with the $P2Y_{12}$ receptor is a major platelet activating factor, acting by releasing dense granules. This process is augmented by the interaction with thromboxane A2 ($TxA_2$) (Cattaneo et al 1997 & 2000; Cattaneo 2005). Therefore, both clopidogrel for its established antagonistic activity against $P2Y_{12}$ receptor, and aspirin, an inhibitor of $TxA_2$ synthesis, have been recognized as antithrombotic dual standard therapy for patients with ACS. However, many factors, including variable bioactivation of clopidogrel (Nawarskas and Clark, 2011), co-administered drugs (Munoz-Esparza et al 2011) as well as associated diabetes (Erlinge et al 2008) may contribute to variable treatment response to clopidogrel. Specifically, patients with diabetes (Erlinge et al 2008), coronary artery disease and obesity (Gremmel et al 2013) are much described groups with a substantial risk of reduced clopidogrel response. In such cases increased dose of clopidogrel (Mehta et al 2010) or switching to more potent drugs such as ticagrelor (Cannon et al 2010) or prasugrel (Antman et al 2008) may reduce the risk of stent thrombosis.

The phenomenon of "resistance" to clopidogrel has been responsible for decline in the use of this drug in patients with acute coronary syndromes, especially since there is evidence that risk of stent thrombosis increases as clopidogrel response decreases (Iwasaki et al 2011). As stated above, the only underlying hypothesis to explain clopidogrel resistance until above 5 years ago was reduced generation of its active metabolites, either via interaction with diabetes or prescribed medications, as more usually as a result of "loss-of-function" mutations, especially in CYP2C19 (Angiolillo and Suyadevara, 2009; Iwasaki et al 2011). It is quite certain that in the presence of homozygous loss-of function genotype, clopidogrel responses are substantially impaired. In response to such findings, the FDA has issued a warning about clopidogrel,

suggesting patient genotyping be performed before it is used: - that in itself would argue against the routine use of clopidogrel in cardiac emergencies.

In the last 10 years, two double-blind controlled trials have shown that there are viable alternatives to clopidogrel therapy in patients with acute coronary syndromes. In the TRITON trial, clopidogrel was compared with prasugrel, essentially with the latter used in more active, rather that bio-equivalent, doses compared to clopidogrel (Wiviott et al 2007). The results were that prasugrel was associated with more thrombotic events that clopidogrel, but that those was a high bleeding risk, especially in the elderly (Neumann FJ, 2009). The PLATO trial compared clopidogrel with the directly acting $P2Y_{12}$ receptor antagonist ticagrelor, again in non-equivalent doses. Ticagrelor was both more effective than clopidogrel, and also remarkably safe (Wallentin et al 2009). In practice, ticagrelor has since become the predominantly used $P2Y_{12}$ antagonist in acute coronary syndromes. It should also be noted that

(1) Ticagrelor has other actions which result in potentiation of the effects of adenosine, which, like clopidogrel, indirectly results in activation of adenylate cyclase (Alsharif et al 2015)
(2) Subsequently, the results of the PEGASUS study, involving long-term treatment with ticagrelor, showed a larger bleeding risk (Bonaca et al 2015) than that seen in PLATO.

The interest of researchers at the QEH Cardiology Unit in the determinants of variability in response to clopidogrel and other $P2Y_{12}$ receptor antagonists is based primarily on the theoretical concept of a post-receptor mechanism for such variability. This concept was first outlined by Hurst et al in 2013, when it was pointed out that blockade of $P2Y_{12}$ receptors would theoretically result in increased activation on the prostanoid/adenylate cyclase (PG/AC) signaling cascade in platelets, as shown in *Fig. 3.1* (*in chapter 3*), modified from the original publication. On this basis it was hypothesized that pre-clopidogrel platelet responses to the anti-aggregatory prostanoid $PGE_1$ would be predictive of clopidogrel responses. This was tested both 4 hours (Nooney et al 2015) and 7 days (Hurst et al 2015) post clopidogrel initiation.

At 4 hours, both pre-treatment $PGE_1$ response and genotype were significant multivariate contributors to clopidogrel response. However, at 7 days, the major source of variability in clopidogrel response was PG/AC signaling (Hurst et al 2015).

These findings therefore suggest that the primary focus of clopidogrel "resistance" should be redirected from the $P2Y_{12}$ receptor to its post-receptor nexus with AC.

Indeed, this change in focus is consistent with the reported results of Frelinger et al (2013), who found that "pre-receptor" factors in combination accounted for only 18% of the variation in clopidogrel active metabolites pharmacokinetics and only 35% to 65% of inter-individual variability in clopidogrel response measured by various techniques.

A major theoretical consequence of this finding was that it theoretically should apply to all other $P2Y_{12}$ receptor antagonists, such as ticagrelor. On the other hand, if the usual doses of such agents induce submaximal responses rather than those inducing around 50% of maximal effects, heterogeneity of response will be less than obvious.

### 1.8.2 Other (PAR antagonists)

The G-protein-coupled thrombin receptor is one of the potent receptors for platelet activation (Brummel et al 2002). Two PAR receptors are so far described in platelets: PAR1 and PAR4 (Kahn et al 1998). The first one was described as more potent than the later (Chackalamannil S, 2006). Vorapaxar, a PAR 1 receptor blocker was shown to inhibit >90% TRAP-stimulated platelet activation at 40mg daily dosing (Kosoglou et al 2009). However, in The TRACER trial with 12944 patients in 818 centres, addition to vorapaxar at 40mg loading followed by 2.5mg maintenance dose along with standard therapy did not significantly reduce risk of death from cardiovascular causes, MI, stroke, ischaemia and urgent revascularization (Tricoci et al 2012).

## 1.9 Scope of the present study

Our observations [mainly by Hurst et al (2015) and Nooney et al (2015)] that individual patient responsiveness to prostacyclin receptor (IP)/AC activation predicted the majority of heterogeneity of responsiveness to clopidogrel, rather than genotype, raised the question of what controls variability in $PGI_2$ signaling. IP-receptor activation is linked via G-proteins to platelet

adenylate cyclase receptors. For example, aspirin dose cannot be relevant to this finding, because that would affect $PGI_2$ formation, but not the effect of exogenous $PGI_2$. Conversely, there could be problems at the IP-receptor, with signal transduction by the G-protein, and/or dysfunction of adenylate cyclase or even accelerated clearance of cAMP, its product.

The current research addresses the biochemical bases for variability in response to anti-aggregatory therapy. Experimentally it addresses the following objectives: -

(1) Epidemiology of response variability The extent of impairment of $PGI_2$/AC and NO/sGC systems in patients with stable or unstable IHD, and in diabetics with concurrently IHD was evaluated relative to normal subjects.

Thus we sought to determine the selectivity of both IHD and diabetes for the function of each system. These results are described in *Chapter 4.*

(2) We evaluated the impact of treatment with perhexiline on signalling of cyclic nucleotides in patients with DM and concurrent myocardial ischaemia (*Chapter 4*).

(3) Utilization of this knowledge for management of patients receiving $P2Y_{12}$ antagonists

(i) To test the hypothesis that responsiveness to $P2Y_{12}$ receptor antagonists other than clopidogrel, whether measured ex vivo or in vitro, also primarily reflects integrity of $PGI_2$/AC signalling.

(ii) Excitingly, we have used the short-acting $P_2Y12$ antagonist 2Methylthio-AMP to mimic this scenario in vitro, a very novel concept.

Patients were evaluated at $7^{th}$ day from the initiation of ticagrelor therapy and the effects of 2Methio-AMP was evaluated in an *in vitro* assay, as described in *Chapter 3*.

(4) Fortuitously, we have discovered via our epidemiology study that patients with coronary vasospasm have severely impaired platelet responsiveness to SNP and moderate impairment to Iloprost, even between symptomatic crises, which we have carried further focusing the following points-

(i) Do these anomalies worsen during spastic crises?

(ii) Does NTG/NAC infusion ameliorate the abovementioned putative anomalies?

Blood samples from CAS patients were collected in the following circumstances: during initial presentation with crisis, after 12-24 hours of appropriate treatment (NAC/GTN), and then electively about a week later. These assessments are described in *Chapter 5*.

We also investigated the molecular bases for putative changes in platelet reactivity during symptomatic crises. Specifically, we tried to determine whether there was any association of inflammatory activation with acute crises?

We also measured plasma syndecan-1 (an index of glycocalyx "shedding") and tryptase concentrations, as inflammatory markers, in CAS patients' plasma samples, collected during crises and stable phases; we compared them with values in control subjects. These results are outlined in *Chapter 5*.

(5) We also attempted to evaluate some additional putative interactions/mechanisms of NAC effects as a possible $H_2S$ donor:

(i) The precise impact of NAC on responses to SNP and Iloprost in patients with CAS in vivo and in vitro.

(ii) The potential contribution of formation of $H_2S$ from NAC to these putative interactions

(iii) The impact of a "pure" $H_2S$ donor, NaHS, in vitro on platelet responsiveness to both SNP and $H_2S$.

These in vitro experimental works are described in *Chapter 6*.

# Chapter 2: Materials and methods

### *2.1 Subject selection and blood sampling*

The study was performed within the Cardiology Unit, Queen Elizabeth Hospital, Adelaide with subjects recruited between February 2015 and March 2018.

A. Patient selection criteria were:

(1) For the $P2Y_{12}$ antagonist studies: Most of the patients in this group were being treated for STEMI (12 out of 17) and patients with stable CHD (n=5) were undergoing cardiac catheterization followed by dual anti-platelet therapy with ticagrelor and low dose aspirin. Blood samples were collected before and at $7^{th}$ days from initiation of therapy. Ticagrelor dosing was identical to that in the PLATO trial (Wallentin et al 2009).

(1) For the epidemiology of impaired cyclic nucleotides signalling assay: Patients with known coronary artery disease, diabetes mellitus, and coronary artery spasm were recruited.

(2) For coronary artery spasm (both Prinzmetal's angina and coronary slow flow): Patients were diagnosed via positive acetylcholine challenge and/or presence of the coronary slow flow phenomenon (CSFP) and were evaluated during acute (n=12) and chronic (n=54) symptomatic phases.

B. Normal subjects were recruited consecutively by local advertisement. Selection criteria were: age >30 years (to correspond to the anticipated age of patients); and the absence of anginal symptoms.

C. Exclusion criteria were:

(1) currently taking, or prior adverse reaction to clopidogrel or other $P2Y_{12}$ antagonists

(2) known bleeding diathesis

(3) Very low platelet counts (approximately <50,000/μL)

D. Almost half of our patient samples were collected from the cardiac catheterization laboratory of The Queen Elizabeth Hospital, in such cases blood samples were drawn via a femoral venous line during cardiac catheterization and the rest are collected via antecubital venesection. Participants were well informed about the study and consented before each time blood collection. Ethical approval was obtained from the relevant committee of Queen Elizabeth Hospital on 04/05/2015 (approval no. HREC/15/TQEH/75). Blood samples were collected in 10mL falcon tubes containing sodium citrate, mixed gently and kept on the bench for 20minutes before analysis.

### *2.2 Chemicals*

$PGE_1$, N-acetylcysteine, Sodium nitroprusside, Adenosine di-phosphate, Amiooxyacetic acid (AOAA, 0.5mM), DL propargylglycine (PAG, 3.3mM) were from Sigma Aldrich USA, Iloprost and NaHS were from Chayman chemical company USA. VASP kits were purchased from Biocytex (Marseille, France).

### *2.3 Platelet aggregometry in whole blood and platelet-rich plasma*

We started assaying platelet aggregability both in whole blood and PRP utilizing a four-channel impedance aggregometer (Model 700, Chrono-Log, PA, USA), following the method described by Chirkov et al (1999). Blood samples in tubes were centrifuged for 10 min at 250*g* for PRP

preparation. The method consists of a pair of palladium-made wire electrodes placed into a tube with continuously stirred whole blood, diluted 50:50 with physiological saline. The instrument is pre-heated at 37˚ C, and blood samples are taken 5minutes earlier and maintained that temperature during assessment of aggregation. The principle underlying whole blood impedance aggregometry is that platelet clumping on electrodes during aggregation impedes the associated electrical current. During initial contact with blood, electrodes become coated with a monolayer of platelets. Platelets start clumping on the monolayer when an activator is added to the tube. Increasing platelet aggregates on the surfaces of the two wires across which impedance is measured causes increasing electric resistance. This impedance is recorded as computerized tracings made over time and is directly proportional to the mass of the aggregate.

### *2.4 Aggregometry assay validation:*

The preliminary studies focusing on inhibition of platelet aggregation by $PGE_1$ were conducted both in blood and PRP and in the same subjects at two different time points in a month gap (shown in *Figure 2.1*). Concentration response curves of whole blood $PGE_1$ responses measured at the beginning did not differ significantly compared with the follow-up, which was also true for PRP ($p=0.45$ and 0.55 respectively, using paired t-test). $IC_{50}$ values of $PGE_1$ responses calculated both for whole blood and PRP were very close to each other in those two-time points (*Table 2.1*). However, $IC_{50}$ was 3.4-fold greater in PRP than the whole blood assay. Our initial study with whole blood shows high sensitivity and reproducibility and involves less labor than study with PRP, also offers more physiologically resemblance. Therefore, we chose to work with whole blood for this study.

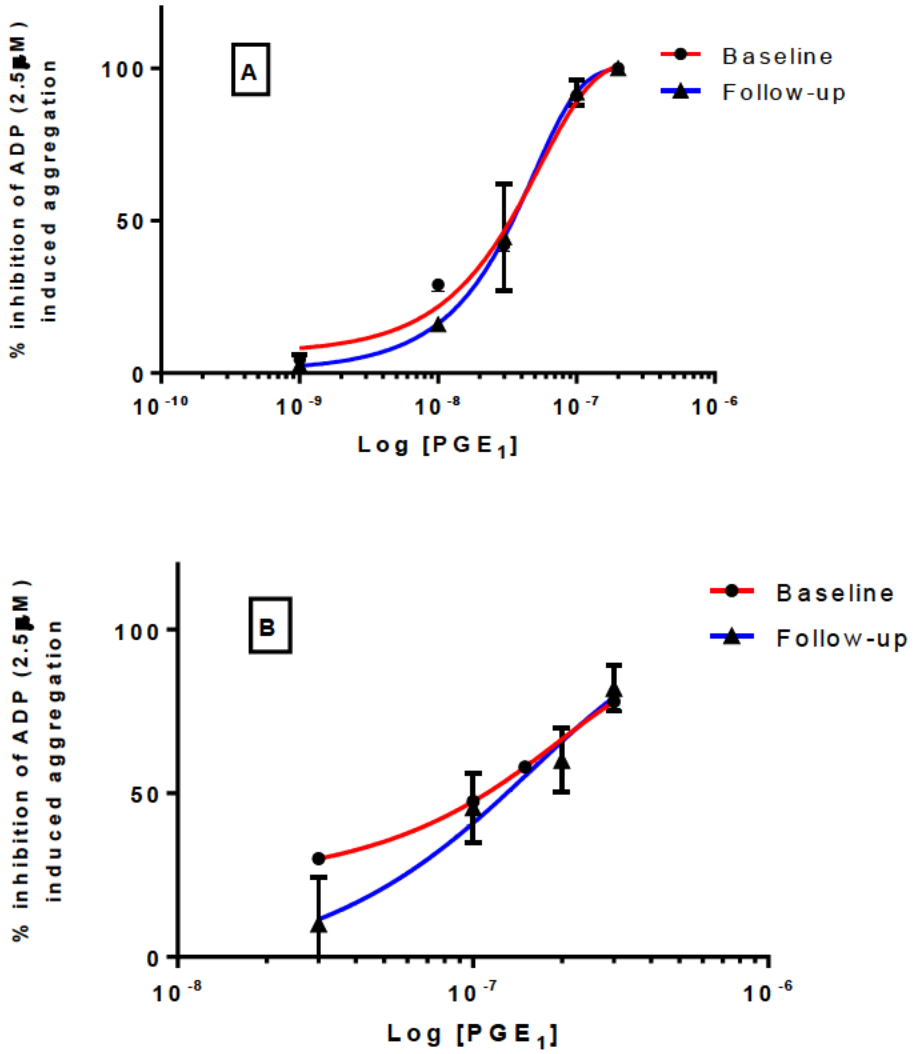



**Figure 2.1:** Dose-dependent curves for platelet responsiveness to $PGE_1$ measured twice over one-month gap in the same subject: (A) whole blood (WB) (B) platelet rich plasma. $IC_{50}$ of $PGE_1$ were measured utilizing Prism software.

**Table 2.1:** Results of duplicate assays (n=2) as outlined in *Fig. 2.1*. Responses to $PGE_1$ in whole blood and platelet-rich plasma

| | (A) WB | (B) PRP |
|---|---|---|
| | $PGE1_{IC50}$ | $PGE1_{IC50}$ value |
| **BASELINE** | 34.2μM | 113.1μM |
| **FOLLOW-UP** | 35.4μM | 128.3μM |

### *2.5 Flow cytometry-general*

Flow cytometry can characterize individual cells/particles with multiple parameters at the same time, which makes it a very powerful technique for suspended cells or cell fragments. The principle underlying flow cytometry is that laser-emitted lights are scattered through individual cells separating them as different populations on the computer screen according to their size and inner complexity. Also, any cellular markers of interest in a complex mix of cells such as blood can be counted by staining with antibody-coated fluorochromes.

### *2.6 Flow cytometry for VASP phosphorylation determination*

Vasodilator-stimulated phosphoprotein (VASP) is an intracellular profilin and actin-binding protein which is a direct substrate of both PKA and PKG and inhibits platelet aggregation upon phosphorylation (Wentworth et al 2006). Phosphorylated platelet VASP (VASP-P) was analyzed using VASP kits and following manufacturers protocol (Bio Cytex, Marseille, France), using flow cytometric analysis (Becton Dickinson FACS CANTO II flow cytometer, BD Biosciences, San Jose, California, USA). Kit reagents include the following: -

(1) $PGE_1$ to stimulate VASP-P,

(2) Red blood cell lysis buffer therefore only platelets and leukocytes are present in the cytometric analysis. However, the cytometer can separate both cell populations because of their internal complexity and size. Only platelet populations are taken in the calculation by making a "gate" around them (*Fig. 2.2A*).

(3) Antibodies for phosphorylated VASP-protein (anti VASP-P) that binds with a 2nd fluorescein isothiocyanate (FITC) tagged polyclonal antibody. Also, a fluorescent dye-phycoerythrin (PE) coated third antibody for integrin β-III/CD61 is there to distinguish platelets from leukocytes. The cytometer counts these two fluorochromes, PE and FITC, (*Fig. 2.2B*) and shows the mean in each sample (*Fig. 2.2C*).

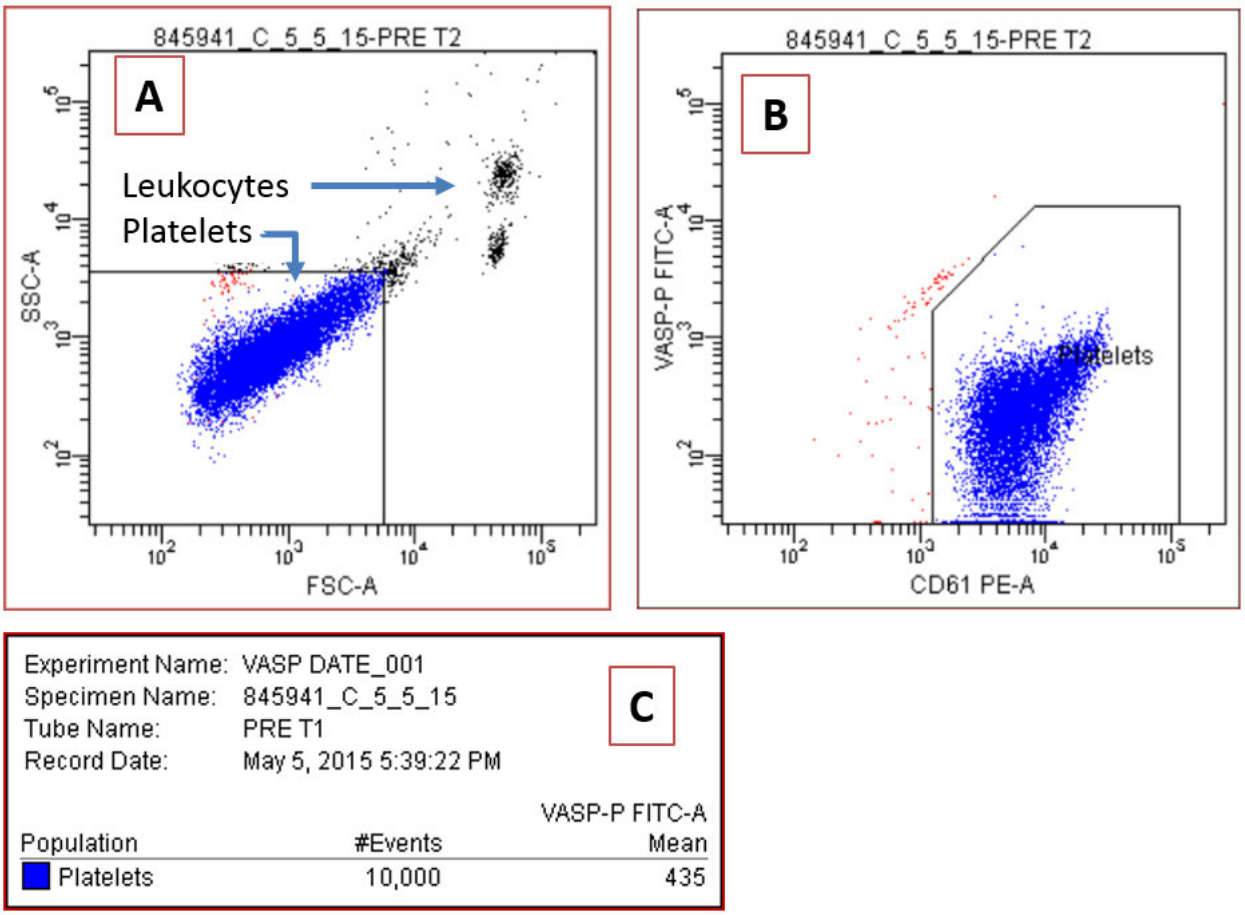



**Figure 2.2:** (**A**) Platelet population are separated from the leukocytes: leukocytes are in high field because of the internal complexity and size. Platelets are gated for further analysis. (**B**) VAS-P (only in platelets) is captured by dual color (FITC for VASP-P and PE for CD61) flow cytometry analysis, (**C**) Mean platelet VASP-P is shown among 10,000 events, which was utilized for further PRI calculation.

### *2.7 How do we measure individual response to ticagrelor?*

For ex vivo responses to ticagrelor: Blood samples were collected before initiation and 7days after therapy with ticagrelor and both aggregometry and vasodilator-stimulated phosphoprotein (VASP-P) was quantitated by flow cytometry (BD FACS II, USA). Ticagrelor response was expressed as δADP and δPRI respectively.

Won't one measure (on treatment) rather than two (to measure treatment induced change) be enough?

The problem is that residual aggregation on treatment might be due to:

**(a)** pre-treatment hyperaggregability and/or **(b)** poor response to treatment

Therefore, we are measuring both pre- and on-treatment effect in order to quantitate drug induced response changes.

### *2.8 In vitro experimentations:*

Whole blood platelet were preincubated 1min for $PGE_1$(30nM)/Iloprost(0.3nM)/NaHS(100µM), 5mins for Forskolin(5µM)/2MeSAMP(10µM), 10min for NAC(100µM), and 15mins for PAG(3.3mM)/AOAA(0.5mN) before stimulation with ADP (2.5µM) for aggregometry assay.

### *2.9 Syndecan-1 measurement:*

Given that glycocalyx shedding is activated by acute inflammation (Mulivor & Lipowsky, 2004; Nieuwdorp et al 2006**)** this was routinely quantitated in patients with coronary artery spasm and also in normals. Plasma concentrations of the glycocalyx component syndecan-1(SD-1) were determined by ELISA (Abcam biotechnology, UK). Assay was performed according to the manufacturer's instructions. Briefly, standards and samples were prepared and were pipetted into the 96 well plate coated with primary antibody for SD-1. The plate was incubated at room temperature for 60 min on a mechanical shaker followed by washing step and addition of the enzyme streptavidin-HRP. Finally, a colour-forming substrate for that enzyme was added in each well and incubated again for 15min in the dark, then absorbance was taken using a spectrophotometer using 450nm wavelength.

### *2.10 Data analysis*

One-way ANOVA was utilized to assess the differences in distribution among groups with quantitative dependent variables and the Chi-square test for categorical variables.

For patients beginning perhexiline treatment, concentration-response curves for $PGE_1$ were constructed, and $EC_{50}$ values compared by paired t-test.

Sensitivity to both ticagrelor and 2MeSAMP were evaluated by taking all covariates (response to $PGE_1$ and Iloprost, age, gender, Body weight, and cardiac risk factors) in a multivariate backwards multiple linear regression analysis. Pearson's coefficient test was performed to calculate bivariate correlations between response to Ticagrelor/2MeSAMP and $PGE_1$.

Data comparisons for SD-1 level in patients (acute with/without NTG/NAC therapy and chronic) and the control group utilized ANOVA with post-hoc specific comparisons (Bonferroni test) or Student's paired t-test as appropriate

All tests were 2-tailed, and data were expressed as mean±SEM while, p values $\leq 0.05$ were considered statistically significant. Data analysis were performed using SPSS 23 version software.

# Chapter 3- Impairment of prostacyclin/adenylate cyclase signaling in platelets: prevalence in acute coronary syndromes and impact on individual responsiveness to ticagrelor

***Abbreviations***

| | |
|---|---|
| ADP | Adenosine di phosphate |
| ACS | Acute coronary syndrome |
| AMI | Acute myocardial infarction |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| CHD | Coronary heart disease |
| Fsk | Forskolin |
| 2-MeSAMP | 2-Methyl thioadenosine monophosphate |
| MRP4 | Multidrug resistance protein |
| P-AC | Prostacyclin-adenylate cyclase |
| $PGE_1$ | Prostaglandin $E_1$ |
| PRI | platelet reactive index |
| Tx $A_2$ | Thromboxane A2 |
| UAP | Unstable angina pectoris |
| VASP | Vasodilator stimulated protein |
| VASP(P) | Vasodilator stimulated protein (phosphorylated) |

**3.1 Abstract:**

Background: Inhibition of ADP-induced platelet aggregation utilizing $P2Y_{12}$ receptor antagonists such as ticagrelor reduces risk of thrombotic complications among patients with acute coronary syndromes both in the short and long term, but increases bleeding risk. $P2Y_{12}$ blockade reverses ADP-related indirect inhibition of the prostacyclin-adenylate cyclase (P-AC) pathway, and integrity of this pathway predicts individual patient response to clopidogrel. We have now evaluated (i) differences in P-AC signaling according to presence/absence of myocardial ischemia (ii) impact of heterogeneity of $PGI_2$-AC signaling on individual responses to $P2Y_{12}$ receptor antagonists in vivo and in vitro

Methods: We compared anti-aggregatory responses to prostaglandin $E_1$ ($PGE_1$) and the direct AC activator forskolin (Fsk) in normal subjects (n=23; N), patients with stable angina pectoris (n=23; SAP) and those with acute coronary syndromes (n=23; ACS). In patients with ACS (n=18) we evaluated determinants of responses to ticagrelor, while responsiveness to the short-acting $P2Y_{12}$ antagonist 2-methyl thioadenosine monophosphate (2-MeSAMP) were evaluated in vitro.

Results: Patients with ACS exhibited both significant hyperaggregability and impairment of $PGE_1$ response relative to normal subjects. Ticagrelor response (measured as δPRI) was significantly and directly related to $PGE_1$ response both on univariate (p=0.003) and multivariate (β=0.82, p=0.002) analysis. Furthermore, there was a strong correlation (r=0.45, p=0.03) between $PGE_1$ response and that to 2MeSAMP in vitro.

Conclusions: (1) ACS is associated with impairments of platelet P-AC signaling which include diminution of response to direct stimulation of AC.
(2) Responses to both ticagrelor in vivo and to 2MeSAMP in vitro are largely determined by integrity of P-AC signaling.
These findings may facilitate individualization of $P2Y_{12}$ receptor antagonist dosing schedule to maximum patient benefit.

### *3.2 Introduction*

The establishment of coronary artery angioplasty plus stent insertion has improved short and long-term outcomes for patients with evolving acute myocardial infarction (AMI) and patients with unstable angina pectoris(UAP) in particular (Brodison et al 1999; Grines et al 1999), although its benefits in patients with stable angina pectoris (SAP) remain controversial (Boden et al 2007; Holmes et al 2008; Al-Lamee et al 2018). Part of the usual therapeutic strategy associated with coronary stenting has been the simultaneous vs introduction of "dual anti-platelet therapy", comprising the combination of low-dose aspirin plus an ADP- $P2Y_{12}$ receptor antagonist (Munoz-Esparza et al 2011), in order to reduce the short and medium-term risk of stent thrombosis.

Although clopidogrel was the first $P2Y_{12}$ receptor antagonist to be used extensively in this regard, anti-aggregatory responses to the usual (75 mg/day) treatment regimen vary widely, and this variability has been implicated as a potential risk factor for occurrence of thrombotic events (Gurbel & Trantry, 2006). Clopidogrel is a prodrug, and there is evidence to suggest that genetically determined impairment of its enzymatic bioactivation may contribute to this "clopidogrel resistance" (reviewed by Sibbing et al 2011). This variable impairment rests essentially on the integrity of CYP2C19 function in activating clopidogrel (Nooney et al 2015).

However, activation of $P2Y_{12}$ receptor -associated signaling by ADP results in extensive intracellular biochemical changes, including Gi-mediated inhibition of platelet adenylate cyclase (AC) (Storey et al 2000), the pathway involved in generation of cyclic adenosine monophosphate (cAMP) in response to anti-aggregatory prostanoids such as prostacyclin and $PGE_1$(Keularts et al 2000). Since blockade of the $P2Y_{12}$ receptor, for example by clopidogrel active metabolite, will reverse this inhibition of AC, we have previously postulated (Procter et al 2016) that the efficacy of clopidogrel in individual patients could be predicted by pre-treatment responses to prostanoid induced indirect activation of AC, such as $PGE_1$. Indeed, for both acute (Nooney et al 2015) and subacute (Hurst et al 2015) responses, a close association emerged, which in the case of weight-

adjusted subacute therapy, outstripped the importance of clopidogrel activator-genotype (Hurst et al 2015).

In the past 10years, there have been two major changes in the patterns of clinical utilization of $P2Y_{12}$ receptor antagonists. First, evidence has emerged to the effect that both prasugrel (Wiviott et al 2007) and ticagrelor (Wallentin et al 2009) may be more effective than clopidogrel in the management of patients with acute coronary syndrome. Second, it has become apparent that the use of such agents, either in the short term (in case of prasugrel [Wiviott et al 2007, Nishikawa et al 2017)] or in the long-term (in the case of ticagrelor [Bonaca et al 2015]) may increase bleeding risk. Indeed, the use of clopidogrel for the management of patients with atrial fibrillation was abandoned, mainly because of associated bleeding risk. To date, possible heterogeneity in individual patient responsiveness to $P2Y_{12}$ receptor antagonists other than clopidogrel has not been extensively studied, but the emergence of bleeding risk provides a strong rationale to establish general principles in the area.

### *3.2.1 Impaired cyclic nucleotides signalling and platelet thrombus formation*

$PGI_2$ and NO respectively stimulate AC and sGC-mediated formation of intracellular cAMP and cGMP, and thereby downregulate intracellular calcium signaling, aggregation, and thrombus formation (Munzel et al 2003, Keularts et al., 2000, Schwarz et al 2001). These cyclic nucleotides are degraded by PDEs (Schwarz et al 2001), and eliminated by nucleotide transporters and multidrug resistance protein (MRP4) (Decouture et al 2015). Therefore, any changes that take place at any point in this process controlling the formation, degradation, or efflux system may potentially affect cyclic nucleotide content and consequent platelet aggregation. Reduced responses to both $PGE_1$ and to the direct AC-agonist forskolin-induced cAMP formation was initially documented in platelets from patients with DM (Livingstone et al 1991). Earlier from our group, Chirkov et al (1995) has shown that $PGE_1$ concentration-response curves in patients with angina are shifted to the right compared with those from normal subjects. Impaired platelet $PGE_1$ response was also documented in acute ichaemic heart disease patients and was shown to be reversed by acute insulin therapy (Khan et al 1991). Another level of impairment leading to reduced cytosolic cAMP/cGMP is a defect in the ATP-dependent cyclic nucleotide transporter system, which was demonstrated in MRP4 knockout mice to be associated

with increased bleeding (Decouture et al 2015). Also, thrombin-induced PDE3 activation (Zang et al 2007), Farnesoid X receptor, a transcription factor presents in platelets, mediated cGMP signaling activation. Consequent inhibition of aggregation was documented (Moraes et al 2016), suggesting that altered transcription can also potentially modify platelet aggregation.

### *3.2.2 Clinical use of clopidogrel*

The active metabolite of the thienopyridine derivative clopidogrel inhibits the purinergic $P2Y_{12}$ receptor irreversibly, and therefore binds to platelets for their remaining survival time. It has also been found to reduce blood viscosity but the mechanism of this is unknown (Kumar et al 2009). Clopidogrel was introduced as an alternative to ticlopidine, an early $P2Y_{12}$ antagonist, on the basis of fewer side effects (Savi et al 2005). Its therapeutic applications were enhanced after the CAPRIE trial, where it showed a marginally higher therapeutic efficacy compared to aspirin (CAPRIE steering committee, 1996). Clopidogrel therapy before and after PCI in the CURE trial among12,562 patients with ACS was found to reduce the agglomerate end-point of death, MI, & stroke, compared to aspirin monotherapy (Mehta et al 2001). The randomized multi-centered double blinded CREDO trial has shown that acute clopidogrel therapy with a 300mg loading dose before stent implantation plus75mg/day maintenance therapy together with aspirin 325mg/day put patients at 38.6% lower risk of the combination of death, MI or stroke. Extending dual therapy for a 12months period was associated with a decreased risk of 26.9%.

Clopidogrel treatment also appeared to reduce infarct-related complications in patients of MI with ST elevation. It was evident in the CLARITY trial that addition of clopidogrel to aspirin, fibrinolytic agents and if needed body adjusted dose of heparin in myocardial infarction patients below 75years of age reduced ischemic complications (Sabatine et al 2005). In the COMMIT trial with patients of acute infarction, adding clopidogrel to aspirin reduced rate of stroke, further MI and death [9.2%, clopidogrel vs 10.1% placebo]. The study has therefore provided strong evidence about inpatient treatment strategy with acute MI, where patients can be treated with clopidogrel, aspirin also fibrinolytics (if needed).

### *3.2.3 What causes heterogeneity of clopidogrel response?*

There is evidence that clopidogrel resistance is associated with greater risks of coronary stent thrombosis (Gurbel&Tranty, 2006; Buonamici et al 2007; Sibbing et al 2009). Considerable evidence supports the concept that the wide spectrum of clopidogrel response variability is mainly engendered by impaired bioactivation, and consequently less active metabolite formation (Shuldiner et al 2009; Scott et al 2011; Nooney et al 2015). Polymorphism of both CYP2C19 and CYP3A4 gene has been associated with reduced clopidogrel metabolism (Mega et al 2009). Response variability has also been attributed to different conditions that cause increased platelet reactivity, such as diabetes (Ferroni et al 2004), high physical stress (Christiaens et al 2002), increased age, ACS, and renal failure (Campo et al 2011). Pretreatment platelet hyperaggregability was also associated with reduced activity of $P2Y_{12}$ antagonists (Barragan et al 2003; Michelson et al 2007; Frelinger et al 2011), although this might theoretically reflect nonspecific physiological antagonism ("yin-yang effect"). Therefore, to show the true responsiveness, state of platelet residual hyper-aggregability should be considered in study design.

Clinical intervention to minimize possible heterogeneity among study patients may also be relevant to reducing response variability. For example, it is evident that adjusting maintenance dose according to patients' body weight (Hurst et al 2015), and increasing loading dose from 300mg to 600mg (Gurbel et al 2005; von Beckerath et al 2005), may lead to diminution in clopidogrel response variability.

### *3.2.4 Possible role of "Post-receptor" factors as a partial basis for resistance to ticagrelor*

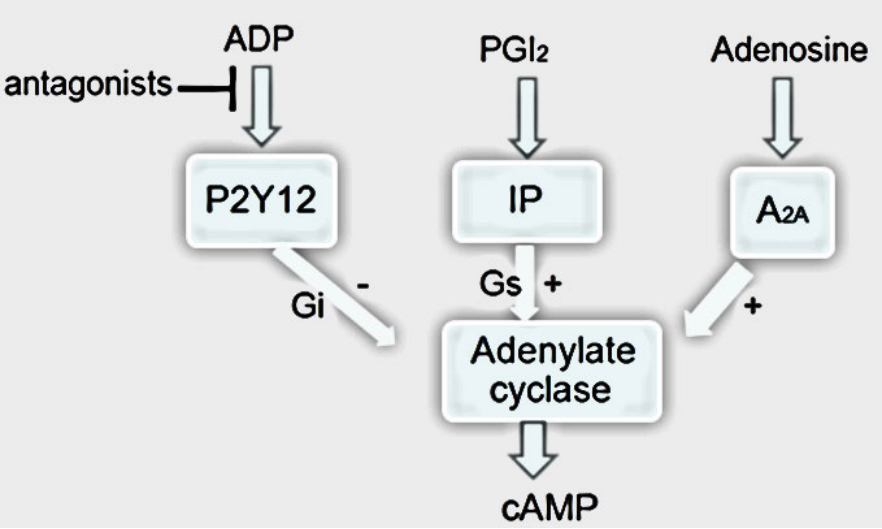



**Figure 3.1:** Figure depicts common physiological agonists such as ADP, $PGI_2$ and Adenosine signaling via their respective receptors leading adenylate cyclase enzyme activation. While the 1st one negatively stimulates AC and decrease cAMP, the latter two positively stimulate AC and increase cAMP. An ADP antagonist can set AC free to be stimulated by $PGI_2$ and adenosine. Ticagrelor differs from thienopyridines via stimulation of adenosine-receptor signaling.

Adenylyl cyclase is one the major signaling pathways of G-protein coupled receptors. Activation of this pathway accelerates production of cAMP. In platelets, the purinergic $P2Y_{12}$ receptor negatively modulates adenylyl cyclase signaling in the presence of ADP and leads to nett aggregation (Yang et al 2002,).

On the other hand, Gs coupled receptor (*Fig. 3.1*) stimulates adenylyl cyclase in the presence of prostacyclin/prostaglandin $E_1$ and thus limits aggregation (Yada et al 1989; Keularts et al 2000). The interlink between $P2Y_{12}$ and Gs coupled receptor, and further downstream adenylyl cyclase –cAMP pathway modulation in the presence of $P2Y_{12}$ antagonists, has been demonstrated by many authors (Fox et al 2004; Cattaneo & Lecchi, 2007; Iyu et al 2011; Hurst et al 2013 & 2015;

Nooney et al 2015). According to Cattaneo & Lecchi (2007), the physiological process controlling cAMP production through the adenylyl cyclase pathway can be sustained by the presence of prostacyclin. This signaling implicates the potentiation of the signal transduction of $P2Y_{12}$ receptor antagonists.

### *3.2.5 Does adenylate cyclase responsiveness to prostanoid stimulation vary between individuals?*

Chirkov et al (1995) reported results re $PGE_1$ concentration-response curves (as a surrogate for $PGI_2$). These were measured in normal subjects and in patients with angina, and were shifted to the right in the SAP patients: that implies decreased sensitivity of $PGE_1$ response, therefore a high concentration is needed to induce the same response as in normal subjects. Similarly, release of cAMP lower than that of normal individuals.

Similarly, reduced responses to both $PGE_1$ and forskolin-induced cAMP formation have been documented in platelets from patients with DM (Livingstone et al 1991). Impaired platelet $PGE_1$ response was also documented in acute ichaemic heart disease patients. This was also shown to be reversed by acute insulin therapy (Khan et al 1991).

### *3.2.6 Does this apply to clopidogrel resistance?*

As described in section 1.8, binding of $P2Y_{12}$ antagonists to their receptor leaves AC free to be stimulated by prostacyclin, resulting in subsequent increases inhibition of aggregation. Therefore, receptor occupancy by $P2Y_{12}$ antagonists paves the way for AC mediated intracellular change. A correlation between the extent of AC/cAMP impairment measured by $PGE_1$ and responses to clopidogrel was reported both by Nooney et al (2015) and Hurst et al (2015). They have addressed primarily the clopidogrel response variability, separately from CYP2C19 genotype status and $PGE_1$ signaling impairment. Nooney et al (2015), in a 4hours follow-up study, with patients receiving a 600mg clopidogrel loading dose, has shown via multivariable analysis that clopidogrel responsiveness was significantly and directly correlated with both the integrity of adenylyl cyclase-cAMP pathway and with the carriage of CYP2C19 normal gene. However, Hurst et al (2015), in a 7days follow-up study with patients receiving a weight-

adjusted maintenance therapy after a 600mg loading dose, also has shown a strong and significant correlation between $PGE_1$ sensitivity and clopidogrel response. Interestingly, there wasn't any significant variability in clopidogrel response on the basis of PYC2C19 mutation/normal genotype in a multivariable statistical analysis. It is worth mentioning that Hurst has elucidated this relationship significantly in two different ways, namely ΔADP and ΔVASP-P. Both are popular means of quantitating clopidogrel response, which increases the credibility of these findings. Therefore, platelet adenylyl cyclase-cAMP pathway integrity carries more importance during chronic therapy than the genetic variability of cytochrome 2C19 gene in clopidogrel response prediction. In contrast, impaired clopidogrel bioactivation might be important at the time of imitation of therapy with first-pass metabolism representing a basis for heterogeneity of onset of action.

These observations raised the question of what controls variability in $PGI_2$ signaling, which is via G-proteins linked to platelet adenylate cyclase receptors, and whether it correlates with all $P2Y_{12}$ antagonists or not.

Therefore, the current study had 2 major objectives: -

(1) To evaluate the hypothesis that integrity of the prostacyclin-adenylate cyclase (P-AC) pathway varied according to (a) chronic or (b) acute myocardial ischaemia
(2) To test the hypothesis that responsiveness to $P2Y_{12}$ receptor antagonists other than clopidogrel, whether measured ex vivo or in vitro, also primarily reflects integrity of P-AC signalling.

### *3.3 Methods:*

Inhibition of platelet aggregation by prostanoids ($PGE_1$/iloprost) and Fsk was evaluated in 23 normal subjects, 23 patients with SAP and 23 patients with ACS. Whole blood impedance aggregometry (Chrono-log 700 CA, USA) was utilized to assess physiological effects of $PGE_1$/Iloprost and Fsk. Blood samples were collected in 10mL falcon tube containing sodium

citrate, mixed it gently and kept on the bench for 20minutes before analysis. The agonists used to evaluate responsiveness to all inhibitors of platelet aggregation were ADP (2.5 or 5.0μM) and TRAP (1μM). Results obtained from aggregometry assays are reported as "inhibition (%) of aggregation"

Assessment of $P2Y_{12}$ receptor blockade: Blood samples were obtained from patients and utilized for the following investigations:

A. For ex vivo responses to ticagrelor: Blood samples were collected before initiation and 7days after therapy with ticagrelor and both aggregometry and vasodilator-stimulated phosphoprotein (VASP-P) by flow cytometry (BD FACS II, USA) were measured. Ticagrelor response was expressed as δADP and δPRI respectively.
B. For in vitro responses to 2MeSAMP: Whole blood platelet was preincubated for 5minutes with 10μM 2MeSAMP before stimulation with ADP (5μM) for the aggregometry assay.

***Statistical analysis:***

Oneway ANOVA was utilized to assess the differences in distribution among groups with quantitative dependent variables and the Chi-square test for categorical variables. Within subjects/patient groupings, responses to $PGE_1$ and to the direct AC activator forskolin varied substantially (*Fig.3.2*). This finding afforded us an opportunity to identify potential clinical associations of impaired responses to both activators using backward multiple logistic regression. Sensitivity to both ticagrelor and 2MeSAMP were evaluated by taking all covariates (response to $PGE_1$ and Iloprost, age, gender, Body weight, and cardiac risk factors) in a multivariate backwards multiple linear regression analysis. Pearson's coefficient test was performed to calculate bivariate correlations between response to Ticagrelor/2MeSAMP and $PGE_1$. All tests were 2-tailed, and data were expressed as mean±SEM while, p values $\leq 0.05$ were considered statistically significant. Data analysis were performed using SPSS 23 version software.

### *3.4 Results:*

#### *3.4.1 Subject/patient characteristics: implications of chronic and acute ischaemia on P-AC signaling.*

For this component of the study, a total of 23 patients with SAP and 23 with ACS were compared with 23 age and gender-matched normal (control) subjects. Demographics are summarized in *Table 3.1*.

In general, this was a middle-aged group of individuals: the patients with symptomatic ischaemic heart disease were extensively but not universally treated with anti-anginal and cardioprotective agents, but not with $P2Y_{12}$ receptor antagonists.

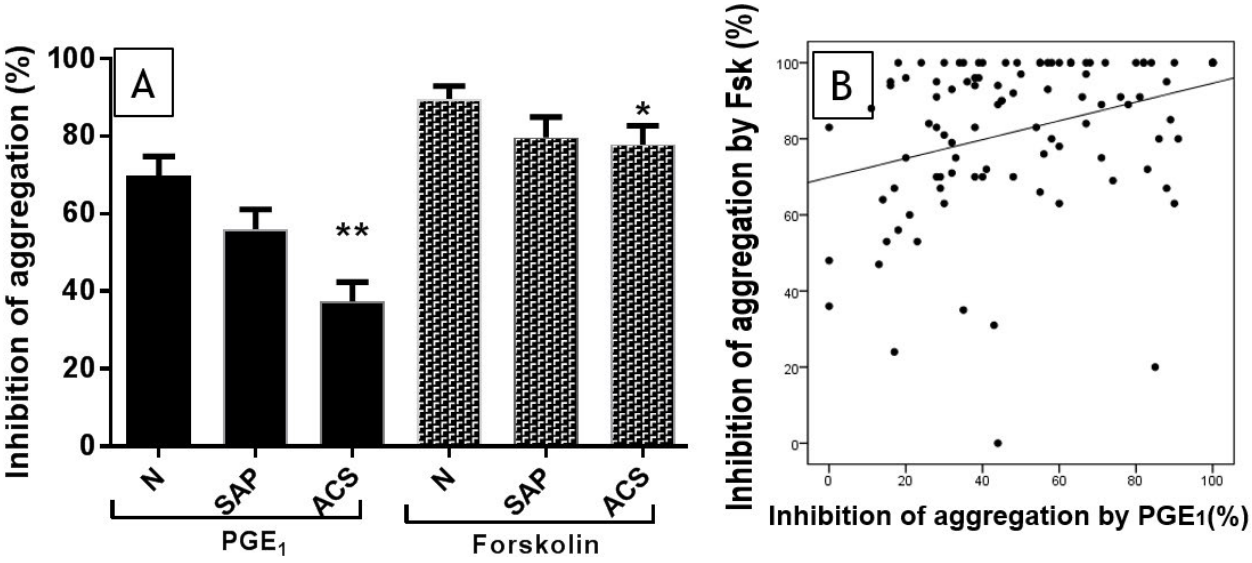



**Figure 3.2:** (**A)** Percent inhibition of aggregation by $PGE_1$(30nM) and Forskolin(5µM) in whole blood and platelet aggregation with 2.5µM ADP plotted on y-axis with mean and S.E.M shown. *p =significant, compared to the normal control using oneway ANOVA. **(B)** Correlation between response to $PGE_1$and to forskolin (r=0.27, p=0.02, n=64).

Results re-determination of responses to both $PGE_1$ and forskolin are depicted in *Fig 3.2A*. ADP responses were 6.1±0.3, 6.74±0.5 and 7.22±0.43 ohms for the normal, SAP and ACS groups respectively (p=0.06 for ACS vs normals).

There was a trend towards diminution of both $PGE_1$ and Fsk responses for SAP and ACS patients, relative to normal controls: however, this reached statistical significant only for ACS patients, as shown in *Fig.3.2A*. There was a significant (r=0.27, p=0.02) direct correlation between individual responses to $PGE_1$ and to Fsk in the entire group of subjects/patients examined (*Fig. 3.2B*).

Furthermore, multivariate analysis was performed to identify determinants of variability in individual platelet responsiveness to $PGE_1$ and to Fsk among patients with SAP and ACS. Results are summarized in *Table 3.2*, show that diabetes mellitus tended to be associated with impaired responses to both $PGE_1$ and to Fsk, while men were substantially more responsive to $PGE_1$ than women.

**Table 3.1:** Demographics of patients and normal subjects recruited

| Clinical characteristics | N (23) | ACS (n=23) | SAP (n=23) | $p^1$ | $p^2$ | $p^3$ |
|---|---|---|---|---|---|---|
| **Age (Years):** | | | | | | |
| **Mean** | 61.1±2.1 | 64±2.1 | 62.7±3.3 | 0.82 | 0.51 | 0.48 |
| **Range** | 44-71 | 35-87 | 42-77 | | | |
| **Gender(n): Male/female** | 13/10 | 16/7 | 15/8 | 0.47 | 0.77 | 0.76 |
| **Smoker (n)** | 00 | 5 | 3 | 0.02 | 0.08 | 0.48 |
| ***Laboratory data*** | | | | | | |
| **Platelet count (x109/L)** | 256±21 | 227±11 | 248±9.6 | 0.8 | 1.0 | 0.33 |
| **Total cholesterol (mmol/L)** | 5.28±0.6 | 5.2±0.3 | 4.1±0.6 | 0.6 | 0.4 | 0.004 |
| ***Medical history(n)*** | | | | | | |
| **DM** | 5 | 7 | 13 | 0.56 | 0.02 | 0.11 |
| **Hypertension** | 6 | 6 | 15 | 0.93 | 0.005 | 0.005 |
| ***Medications (n):*** | | | | | | |
| **Aspirin** | 3 | 17 | 7 | 000 | 0.17 | 0.001 |
| **β-Blocker** | 0 | 3 | 6 | 0.09 | 0.01 | 0.29 |
| **$Ca^{2++}$ channel blocker** | 2 | 3 | 9 | 0.67 | 0.02 | 0.06 |
| **ARB** | 2 | 4 | 12 | 0.41 | 0.001 | 0.009 |
| **Ace inhibitor** | 7 | 5 | 8 | 0.45 | 0.83 | 0.37 |
| **Statins** | 8 | 10 | 18 | 0.63 | 0.006 | 0.03 |

$P^1$ & $P^2$ are by comparing ACS and SAP with control

$p^3$ between ACS and SAP

**Table 3.2:** Determinants of variability in platelet response to the inhibitory effects of $PGE_1$ and to Forskolin on aggregation (multivariate analysis).

| | **$PGE_1$ response (% inhibition)** | | **Forskolin response (% inhibition)** | |
|---|---|---|---|---|
| | β | p | β | p |
| **ACS** | -0.22 | 0.055 | -0.07 | 0.54 |
| **DM** | -0.17 | 0.10 | -0.32 | 0.012 |
| **HYPERTENSION** | -0.13 | 0.39 | -0.19 | 0.26 |
| **STATINS** | -0.09 | 0.38 | 0.27 | 0.03 |
| **ACE INHIBITOR** | -0.02 | 0.83 | -0.08 | 0.44 |
| **AGE** | -0.22 | 0.18 | -0.16 | 0.45 |
| **(MALE)GENDER** | 0.42 | 0.002 | -0.06 | 0.59 |

### *3.4.2 Correlations between pre-treatment responses to $PGE_1$ and subsequent response to $P2Y_{12}$ blockade.*

#### A. Ex vivo assessment: 7 days' treatment with ticagrelor

7 days treatment with ticagrelor induced virtually complete suppression of ADP response in all ACS patients investigated (*Table 3.3*). There was also minor but significant suppression of aggregation in response to TRAP, and a marked reduction in PRI (to a mean of 13.8%, indicating very adequate inhibition of aggregation). There was a significant correlation between pre-treatment $PGE_1$ response and change in PRI on ticagrelor (*Fig.3.3*): analogous comparisons involving δADP response were precluded by virtually universal complete inhibition of aggregation on treatment.

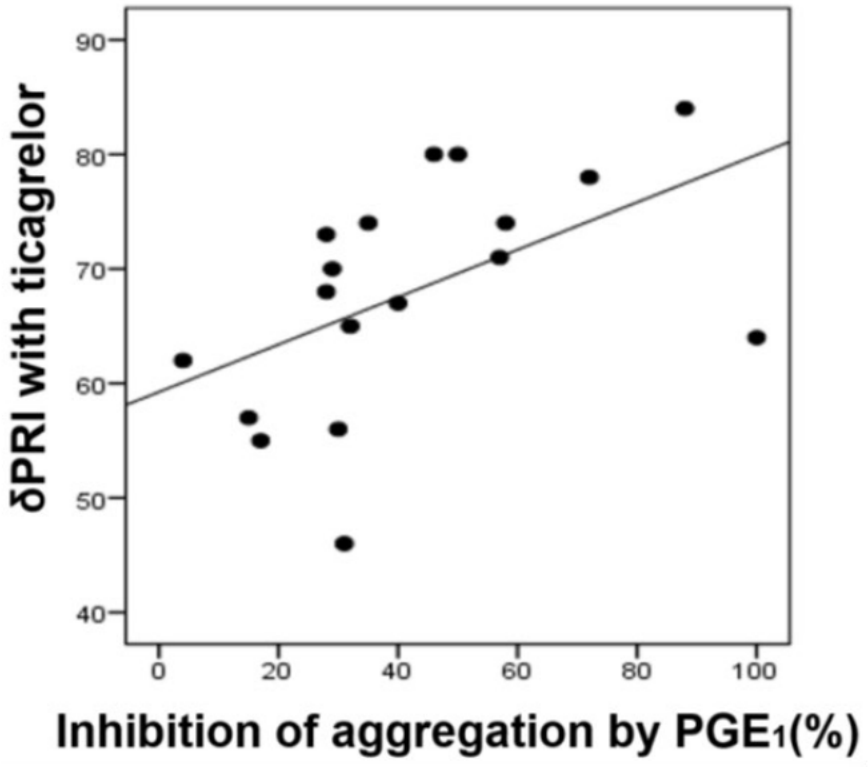



**Figure- 3.3:** Correlation of pre-treatment responsiveness to $PGE_1$ and ticagrelor therapy at 7th day from the initiation of therapy, measured by δPRI in patients with acute coronary syndrome (r=0.52, p=0.02 , n=18).

**Table 3.3:** Platelet aggregability (Ohms) (with ADP and TRAP) and platelet reactivity index (PRI) at baseline and following ticagrelor therapy for 7days in patients with acute coronary syndrome (n=18)

| | Baseline | Follow-up | P value |
|---|---|---|---|
| **ADP** | 7.8±0.5 | 0.5±0.3 | <0.001 |
| **TRAP** | 9.3±0.6 | 7.5±0.8 | 0.047 |
| **PRI** | 77.3±2.2 | 13.8±1.8 | <0.001 |

Data were also assessed by multiple logistic regression (*Table 3.4*). This showed that pre-treatment $PGE_1$ response was a major determinant of inter individual δPRI on ticagrelor. As previously reported (Hurst et al 2015), there was a trend towards an inverse relationship between platelet response to $PGE_1$ and to the nitric oxide donor SNP.

**Table 3.4:** Association of patient characteristics with ticagrelor response (δPRI%)

| | *β standardized coefficient* | *p value* |
|---|---|---|
| ***$PGE_1$ response*** | **0.82** | **0.002** |
| ***Age*** | **-0.24** | **0.22** |
| ***Gender*** | **-0.42** | **0.21** |
| ***Body weight*** | **0.11** | **0.6** |
| ***Cardiac risk factors*** | **-0.04** | **0.87** |
| ***Baseline ADP(ohms)*** | **-0.04** | **0.94** |
| ***Baseline TRAP(ohms)*** | **-0.05** | **0.95** |
| ***Platelet counts*** | **0.25** | **0.55** |

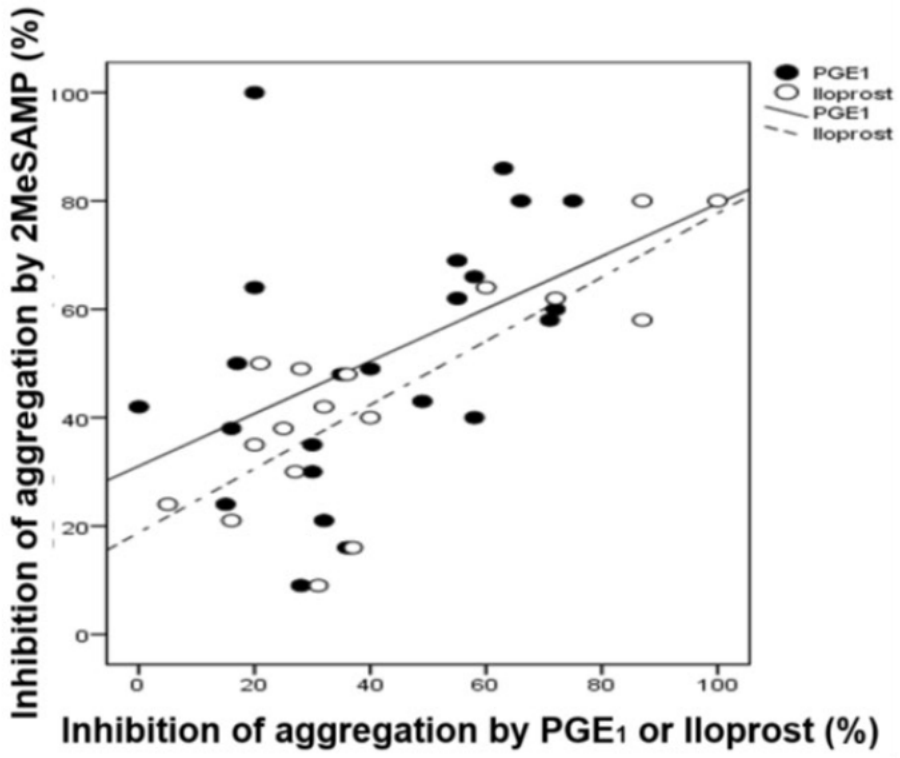



**Figure-3.4:** Correlations between anti-aggregatory effect (%) of 2MeSAMP (10μM) stimulated with ADP (5μM) and pretreatment responsiveness to 30nM $PGE_1$(r=0.45, p=0.03, n=23) and iloprost (r=0.8, p<0.001, n=17).

**B. In vitro studies**

Investigations here were carried out on blood samples both from patients with SAP (n=6) and ACS(n=17). Responsiveness to the anti-aggregatory effects of $PGE_1$ predicted (r=0.45, p=0.03) the subsequent suppression of ADP-induced aggregation in the presence of 2MeSAMP(*Fig.3.4*). Similar results were obtained when the $PGI_2$ analog Iloprost (0.3nM) was utilized instead of $PGE_1$.

### *3.5 Discussion*

The rationale underlying the conduction of the current study had two major components: -

(i) Our previous finding that platelets from patients with stable angina pectoris are hyporesponsive to the anti-aggregatory effects of $PGE_1$ (Chirkov et al 1995) and

(ii) Our recent studies focusing on the importance of post-receptor, adenylate cyclase dependent, signalling as a major source of variability in individual patients' responsiveness to the $P2Y_{12}$ receptor antagonist clopidogrel (Nooney et al 2015, Hurst et al 2015).

These data suggested that inter-individual variability in responsiveness of the P-AC signaling pathway might be of considerable importance as regards both safety and efficacy of other $P2Y_{12}$ receptor antagonists.

The first component of the study was an evaluation of the relative impact of symptomatic stable and unstable myocardial ischaemia on responsiveness to $PGE_1$. As shown in *Fig 3.2A*, the presence of ACS was associated with marked impairment of responses both to $PGE_1$ and to the direct AC activator forskolin, implying that the defect in signaling pathway was at least partially at the level of AC per se. There were also non -significant trends for a reduction in these responses to occur in SAP patients, unlike our previous data, this trend was not statistically significant, but previously entire concentration response data were examined, compared with a single point in the current study.

The second objective of the study was an investigation of the relationship between integrity of the P-AC pathway and individual responsiveness to $P2Y_{12}$ receptor antagonists other than clopidogrel. This investigation was undertaken via both a study of in vivo therapy with ticagrelor for ACS patients, and an in vitro study with the short-acting $P2Y_{12}$ receptor antagonist 2MeSAMP.

With ticagrelor, the dosing schedule for treatment of ACS patients was that utilized in the PLATO study (Bonaca et al 2015): 180 mg as loading dose and 90mg twice daily as maintenance dose for 7days prior to assessment of patient response. Given that this dosing schedule of ticagrelor induces a far greater degree of inhibition of platelet aggregation than standard clopidogrel regimens (Storey et al 2010), it is not surprising that the resultant inhibition of ADP-induced aggregation approached 100% in most patients: heterogeneity of response could be measured only via PRI determination (*Fig.3.3*). This revealed that pre-treatment $PGE_1$ response was a strong and direct correlate of δPRI on ticagrelor, both on univariate (*Fig.3.3*) and multivariate (*Table 3.4*) analyses.

In vitro analysis of the putative relationship between integrity of the PG-AC axis and response to $P2Y_{12}$ receptor blockade is difficult for prodrugs such as clopidogrel or prasugrel, but feasible for directly acting agents. In the current study, we utilized the short-acting agent 2MeSAMP for this in vitro study. As shown in *Fig.3.4*, pre-treatment responses, both to $PGE_1$ and to the "pure" IP-receptor agonist and $PGI_2$ analog iloprost were strong predictors of inhibition of ADP-induced aggregation by 2MeSAMP.

These results therefore imply that knowledge of integrity of an individual patient's responsiveness to AC stimulation by prostanoids such as $PGE_1$ or $PGI_2$ is a strong predictor of subsequent responsiveness to all $P2Y_{12}$ receptor antagonists, not just clopidogrel. Furthermore, it is demonstrated that impairment of P-AC signaling is a particularly common and severe problem in patients with ACS. On multivariate analyses, ACS and gender are two independent variables were found to predict $PGE_1$ response, while use of statins positively and DM negatively impacted forskolin response (*Table 3.2*).

In practice the main implication of these findings is that there is some diminution of ticagrelor effect in patients with ACS. While this is likely to slightly reduce the level of protection against thrombosis, the occurrence of bleeding complications in practice is a minor concern, as was observed in PLATO (Bonaca et al 2015). On the other hand, there was somewhat greater response to $PGE_1$ in patients with SAP, suggesting that the relative risk of bleeding complications be a greater concern here, as proved to be the case in PEGASUS (Bonaca et al 2015).

There are several limitations to the current study. We did not fully explore the relative importance of the various components of the PG/AC signaling cascade, such as receptor stimulation, Gi protein function and integrity of AC function, other than to ascertain from experiments with Fsk that AC stimulation was selectively impaired in ACS patients. We presume, but have not ascertained, that this might reflect dysfunction of AC due to oxidative stress.

### *3.6 Conclusions*

***(i) Ticagrelor:***

These data together have confirmed that the integrity of AC signaling predicts both in vitro responsiveness to 2MeSAMP and in vivo responsiveness to ticagrelor, findings analogous to those made previously for clopidogrel. There was not enough heterogeneity in ADP-based results with ticagrelor in usual dosing (response close to 100% for everyone). However, bleeding risk on ticagrelor, and perhaps more familiarly on prasugrel, may well be predicted by the pre-treatment $PGE_1/PGI_2$.

(iii) $P2Y_{12}$ receptor antagonist therapy in Specific Populations:

It is now well-established that the condition of "endothelial dysfunction" reflects a variable combination of disordered nitric oxide generation and soluble guanylate cyclase signaling, and that the latter component of this problem is reflected in platelets as "NO resistance" (Chirkov et al 2007): this is subject to therapeutic amelioration (Stasch et al 2011). Analogously, disordered PG/AC signaling is likely to represent a thrombotic risk factor, and its potential amelioration represents an attractive future therapeutic target.

# Chapter 4: Population studies: variability in anti-aggregatory signaling pathways

***Abbreviations***

| | |
|---|---|
| ADP | Adenosine di phosphate |
| ACS | Acute coronary syndrome |
| BSL | Blood sugar level |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| CHD | Coronary heart disease |
| HNO | Nitroxyl |
| MI | Myocardial infarction |
| MPO | Myeloperoxidase |
| Pex | Perhexiline |
| PKA | Protein kinase A |
| ROS | Reactive oxygen species |
| RS-NO | Nitrosothiols |
| Tx A2 | Thromboxane $A_2$ |
| UA | Unstable angina |
| VASP(P) | Vasodilator stimulated protein (phosphorylated) |

**4.1 Abstract:**

Background: Cardiovascular homeostasis in part depends on normal physiology of circulating blood platelets. However, impairment of platelet response to anti-aggregatory autacoids such as nitric oxide (NO, a cGMP stimulant) and prostacyclin ($PGI_2$, a cAMP stimulant) occurs in many cardiovascular disease states, including ischaemic heart disease (IHD) and diabetes mellitus (DM). We have shown previously that NO resistance at the level of platelet aggregation can be ameliorated by ACE inhibitors and perhexiline (Pex), a prophylactic anti-ischaemic agent which alters mitochondrial energy metabolism towards increased utilization of glucose. The current study sought to investigate the following issues: -

(1) extent of the impairment of $PGI_2$/AC and NO/sGC signaling in

a) patients with IHD (both acute and chronic)
b) IHD±DM, in both acute and chronic circumstances

and thus (2) Selectivity of impact on $PGI_2$/AC versus NO/sGC signaling in the presence of IHD and DM-IHD for the function of both systems.

(3) impact of Pex treatment on NO/sGC and PG/AC signaling.

Methods

We used prostaglandin $E_1$ as a receptor-based AC stimulator, and also used a direct activator of AC, forskolin (Fsk), with SNP as a sGC stimulator. Results are expressed as inhibition of ADP (2.5μM)-induced platelet aggregation in whole blood after normalized to 100%.

Six patients with DM-IHD were treated with Pex for 2 weeks, Pex dosing was adjusted to maintain plasma concentrations within the therapeutic range. $PGE_1$ concentration-response curves being performed before initiation and after 2 weeks.

Results

1) Responses to ADP tended to be greater than in normal subjects both in DM-IHD (p=0.002) and in ACS (NS) but anti-aggregatory responses to both $PGE_1$ and SNP were significantly diminished only in ACS (p=0.04 and 0.02 respectively). The effect of Fsk was diminished only in patients with DM-IHD (p=0.02, *Fig.4.1*).

2) In patients with DM-ACS (n=7), most of whom were hyperglycaemic, there appeared to be hyporesponsiveness to SNP, $PGE_1$ and Fsk (p= 0.001, 0.006, 0.17 respectively compared to normal subjects and p=0.12, 0.09, 0.41 respectively relative to non-diabetic ACS patients.
3) In patients with ACS or DM-ACS, there was marked impairment of SNP and $PGE_1$ responses. Overall impairment was selective for SNP relative to $PGE_1$, consistent with a greater degree of dysfunction of the NO-sGC pathway in ACS than that of the PG/AC pathway.
4) Pex therapy increased $PGE_1$ responses, with a 2.2-fold ($p<0.03$) decrease in $IC_{50.}$

Conclusions

ACS represents a basis for major impairment of both NO/sGC and PG/AC signaling among the conditions studied. However, in ACS, the predominant impairment is of sGC signaling. while in DM, there is additional impairment of AC enzymatic activity. Pex therapy has the potential to improve platelet AC/cAMP signaling.

**4.2 Introduction:**

Maintenance of platelets in non-activated non-aggregatory mode in the circulation is very important in the absence of a pro-aggregatory stimulus, such as a disruption of the endothelial layer of blood vessels. Just as there are multiple pro-aggregatory stimuli, there are several biochemical pathways involving activation of anti-aggregatory mechanisms. In general, these pathways are initiated by various autacoids released into the circulation primarily from the vascular endothelium. Such autacoids have long been known to include nitric oxide (NO) and prostacyclin (*see chapter 1 for review*), which are the initiating compounds for pathways centering on, but not limited to, the activation of soluble guanylate cyclase (sGC) and adenylate cyclase(AC) respectively. In the case of prostacyclin ($PGI_2$) this activation is indirect with binding of $PGI_2$ to the IP receptor as an intermediate step.

There are also a number of other possible activating stimuli of both sGC and AC, as well as a number of potential mediators and modulators of their effects, other than the release of cyclic GMP and cyclic AMP respectively as a result of enzyme activation. These mechanisms are schematized in Chapter 1 *Fig. 1.3*. Importantly, these two pathways interact at the level of VASP phosphorylation, and thus have mutually potentiating effects.

It is important to recognize the more recent identification of carbon monoxide (CO) and hydrogen sulphide ($H_2S$) as additional anti-aggregatory agents of endothelial origin. Some implications of $H_2S$ generation will be discussed in *chapter 6*.

As has already been described, there is abundant evidence that platelet responsiveness to NO is diminished (relative to that in normal subjects) in patients with hypertension, symptomatic ischaemic heart disease, hyperglycemia, heart failure and acute atrial fibrillation, as well as several other cardiovascular disease states (reviewed by Chirkov and Horowitz, 2007). Less is known about the $PGI_2$/AC system, but it has been established that stable symptomatic IHD is associated with impairment of this pathway (Chirkov et al 1995).

The objectives of the current study were (i) to evaluate the extent of impairment of each of these systems in patients with stable or unstable IHD, and in diabetics with concurrent IHD, relative to normal subjects, and thus to (ii) determine the selectivity of both IHD and diabetes for the function of each system.

Finally, preliminary studies were performed in order to attempt to identify means of ameliorating impairment of $PGI_2$/AC signaling. In view of the previously recorded effects of perhexiline as a potentiator of NO signaling (Willoughby et al 2002), experiments utilized this anti-anginal agent.

**4.3 Methods:**

For the purposes of the experiments in this chapter, the following applied: -

(1) Patient selection Patients were undergoing investigation for either acute coronary syndromes, (and were the same individuals as those assessed in *Chapter 3*) or stable angina pectoris, and are categorized according to the presence or absence of concomitant diabetes mellitus. In some cases (all ACS), there was severe hyperglycaemia at the time of study. [Please note that all similar studies with a population of platelets with coronary artery spasm are described separately (*Chapters 5/6*)]

Informed consent was obtained prior to study, and the protocol was approved by the institutional Ethics of Human Research Committee.

(2) Experimental methods Methods of venesection and of evaluation of aggregation responses to ADP were as for *Chapter 2*. $PGE_1$ was utilized predominantly instead of the $PGI_2$ analog, Iloprost. In addition to evaluation of these responses, effects of the direct AC activator forskolin (5μM) were measured.

(3) Statistical methods One-way ANOVA was utilized to assess the differences in distribution among groups with quantitative dependent variables and the Chi square test for categorical variables. For patients beginning perhexiline treatment, concentration-response curves for $PGE_1$ were constructed, and $EC_{50}$ values compared by paired t-test. Results were normalized to 100% for control and compared with others. All tests were 2-tailed, and data were expressed as mean±SEM while, p values $\leq 0.05$ were considered statistically significant. Data analysis were performed using SPSS 23 version software.

### 4.4 Results:

For this component of the study, 23 patients with SAP, 23 (the same individuals studied in Chapter 3) with ACS of whom 7 had associated DM and 25 with DM-IHD were compared with 27 normal (control) subjects. Demographics are summarized in *Table 4.1*.

In general, this was a middle-aged group of individuals: the patients with symptomatic ischaemic heart disease were extensively but not universally treated with anti-anginal and cardioprotective agents, but not with $P2Y_{12}$ receptor antagonists prior to cardiac catheterization. Importantly, the 25 DM-IHD patients all had symptomatic ischaemic heart disease. As regards diabetic treatment and control, 20% of these patients were receiving no specific treatment, 48% were on oral agents alone, and 32% were receiving insulin, while mean blood sugar level at the time of venesection was 9.1±0.67 mmol/L, with 8 patients having BSL >10mmol/L at the time of initial evaluation. Among the patients with ACS-DM, hyperglycemia was common (mean BSL 12.3±2.0 mmol/L; *Table 4.2*).

A total of 12 patients all with SAP or DH-IHD, were receiving perhexiline as a prophylactic anti-anginal treatment at the time of evaluation. A further initial 6 patients with DM-IHD started perhexiline treatment immediately after initial evaluation.

**Table 4.1**: Patients' details

| Clinical characteristics | Controls (n=27) | ACS (n=16) | DM-ACS (n=7) | SAP (n=23) | DM-IHD (n=25) |
|---|---|---|---|---|---|
| **Age (Years):** | | | | | |
| **Mean** | 57.4±2.1 | 63±2.1 | 66±6.1 | 62.7±3.3 | 70.2±3.8 |
| **Range** | 20-70 | 35-95 | 42-87 | 42-77 | 42-88 |
| **Gender(n): Male/female** | 13/14 | 13/3 | 5/2 | 15/8 | 12/13 |
| *Laboratory data* | | | | | |
| **ADP response (ohms)** | 6.4±0.3 | 6.9±0.5 | 7.8±0.7 | 6.7±0.4 | 8.7±0.6 |
| **Platelet count ($x10^9$/L)** | 251.9±13 | 222.2±10 | 248.4±23 | 249.8±19 | 237.3±23 |
| **Total cholesterol (mmol/L)** | 5.5±0.5 | 5.4±0.3 | 4.8±0.5 | 4.1±0.3 | 4.1±0.3 |
| *Medical history* | | | | | |
| **Hypertension (n)** | 6 | 3 | 3 | 15 | 15 |
| **Atrial fibrillation(n)** | 0 | 0 | 0 | 7 | 6 |
| **Smoker** | 0 | 5 | 1 | 3 | 1 |
| *Procedure* | | | | | |
| **Stent implantation/ CABG** | 0 | 16 | 7 | 7 | 3 |
| **Stable angina** | 0 | 0 | 0 | 17 | 21 |
| **Unstable angina/STEMI** | 0 | 16 | 7 | 0 | 0 |
| *Medications (n):* | | | | | |
| **Aspirin** | 2 | 16 | 7 | 7 | 11 |
| **β-Blocker** | 0 | 3 | 1 | 7 | 8 |
| **$Ca^{2+}$ channel blocker(CCB)** | 2 | 2 | 1 | 9 | 6 |
| **ARB** | 2 | 1 | 3 | 12 | 11 |
| **ACE inhibitor** | 4 | 3 | 1 | 6 | 11 |
| **Statins** | 7 | 8 | 2 | 18 | 16 |
| **Nitrates** | 0 | 3 | 1 | 4 | 6 |
| **Perhexiline** | 0 | 1 | 0 | 10 | 8 |

### *4.4.1 Findings re SNP/sGC and $PGE_1$/AC signaling*

(1) SNP responses did not vary significantly between normal subjects and those with SAP, although there was a trend towards diminution in the latter (*Fig. 4.1A*).

ACS was associated with more than 2-fold impairment of SNP responses relative to normal subjects, and this difference tended to be greater in DM.

As regards aggregability to ADP, mean response were 6.4±0.3 ohms for control subjects and 6.7±0.4, 8.7±0.6, 6.9±0.5 and 7.8±0.7 for SAP, DM-IHD, ACS and DM-ACS patients. Thus, aggregability tended to be greater in diabetic than non-diabetic subjects (p=0.01, and 0.23 for ACS and IHD populations respectively).

(2) Assessment of PG/AC signalling This was performed primarily using $PGE_1$ and forskolin, as shown in *Fig.4.1B* and *4.1.C*.

PEG1 responses were significantly impaired in both ACS and particularly in DM-ACS (*Fig 4.1B*). However, the only significant defect in Fsk responses was in DM-IHD patients (*Fig.4.1C*).

Impairment of responses shows selectively for sGC

In order to determine whether the extent of impairment of anti-aggregatory responses is similar for both SNP and $PGE_1$/forskolin, the relevant results were compared in all groups of patients, utilizing SNP: $PGE_1$(*Fig. 4.2A*), SNP: forskolin (*Fig. 4.2B*) and $PGE_1$: forskolin (*Fig. 4.2C*).

The data were remarkably similar, showing that extent of SNP resistance was disproportionately greater than that to AC activation for patients with ACS irrespectively of the comparator used (p=0.049 for $PGE_1$: p=0.006 for forskolin). Importantly, resistance to $PGE_1$ in patients with ACS was substantially greater than that to Fsk (*Fig. 4.2C*)

If the 7 patients with DM-ACS were excluded, it became apparent that much of this difference was due to impact of diabetes. For example, SNP: $PGE_1$ responsiveness changed from 0.6±0.2 to 0.75±0.3.

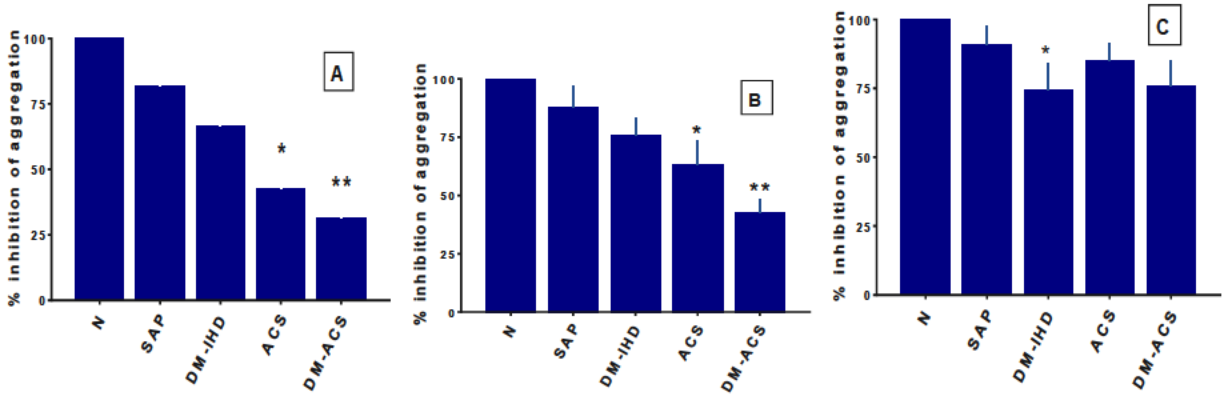



**Figure 4.1:** Inhibition of ADP-induced platelet aggregation by (**A**) SNP(10μM), (**B**) $PGE_1$ and (**C**) Forskolin, in whole blood samples from normal subjects(N), and patients with stable angina pectoris (SAP), diabetes mellitus with concurrent ischaemia (DM-IHD), acute coronary syndromes (ACS), and diabetic patients with ACS presentation (DM-ACS). Results shown are mean±SEM; *p<0.05, **$p$<0.001 *vs* normals after one-way ANOVA with Bonferroni post hoc test.

**Table 4.2:** Changes of aggregometric and flowcytometric parameters among patients with acute coronary syndrome (ACS) and ACS with concurrent DM (DM-ACS)

| | ACS (=16) | DM-ACS (n=7) | p |
|---|---|---|---|
| **ADP (ohms)** | 6.9±0.53 | 7.8±0.67 | NS |
| **SNP (%)** | 13.7±4.1 | 8.1±2.4 | NS |
| **$PGE_1$ (%)** | 43.2±6.5 | 29.1±3.7 | P=0.090 |
| **Iloprost (%)** | 53(n=9) | 23.4(n=5) | P=0.009 |
| **Forskolin (%)** | 79.4±5.6 | 70.4±7.9 | NS |
| **δPRI** | 71.3±3.5 | 54.8±3.9 | NS |
| **BSL(mmol/L)** | | 12.3±2.0 | |

### *4.4.2 Selectivity analysis*

Platelet responsiveness to SNP, $PGE_1$ and forskolin were expressed as percent inhibition of ADP-induced platelet aggregation. Ratios of anti-aggregatory effects of SNP and $PGE_1$ and/or forskolin have been calculated and control subject data represented as 100% to compare the relative impairments of SNP responses in our cohort (*Fig.4.2A and B*). Similarly, ratios of the effects of $PGE_1$ and forskolin were calculated to assess the relative impairment in $PGE_1$ signaling (*Fig.4.2C*). A low ratio therefore implies of selective impairment of the numerator parameter.

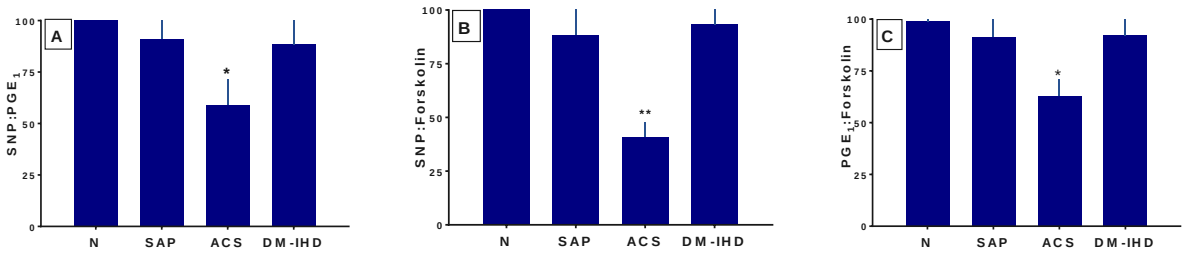



**Figure 4.2**: Selectivity calculated as a ratio of inhibition of platelet aggregation either by SNP with $PGE_1$ or forskolin. (A) SNP: $PGE_1$ (*p=0.049), (B) SNP: forskolin (**p=0.006) and (C) $PGE_1$: forskolin (*p=0.02) *vs* selectivity for normals after one-way ANOVA with Bonferroni Post Hoc test.

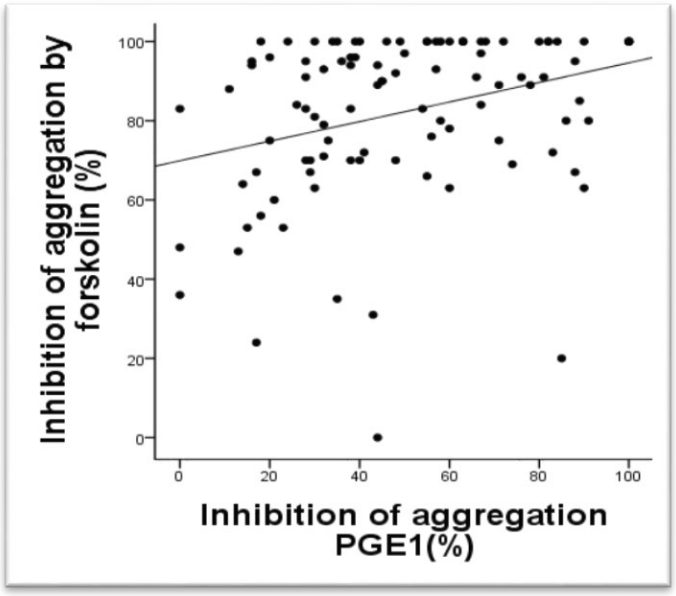



**Figure 4.3:** Correlation between inhibitory effects of $PGE_1$ (30 nM) and Forskolin (5 µM) on ADP (2.5 µM) -induced platelet aggregation in whole blood samples (r=0.27, p=0.02, n=64).

There was a trend towards diminution of both $PGE_1$ and Fsk responses in all patient groups relative to normal controls: however, selectivity of response to $PGE_1$ reached statistical significance only for ACS patients, as shown in *Fig. 4.2C*. There was a significant (r=0.27, p=0.02) direct correlation between individual responses to $PGE_1$ and to Fsk measured in all subjects (*Fig. 4.3*).

### *4.4.3 Effects of perhexiline on AC signaling*

It would theoretically be desirable to have therapeutic avenues available to reverse platelet resistance to PG/AC stimulation. The prophylactic anti-anginal drug perhexiline (Pex) has been shown to increase NO-mediated inhibition of aggregation in patients with both stable angina pectoris and acute coronary syndromes (Willoughby et al 2002). Therefore, in preliminary studies, we evaluated whether Pex therapy can affect cAMP signaling in patients with DM with ischaemia (DM/IHD). In this component of the study 6 patients of average age 72±5.3 (male: female=3:3) and well-controlled DM and associated with stable IHD were treated with Pex for 2 weeks. Daily dosing was titrated to achieve therapeutic plasma perhexiline levels (see *Table 4.3*).

$PGE_1$ concentration-response curves were plotted before and 2 weeks after imitation of therapy and $IC_{50}$ values were calculated.

There were no significant changes in blood glucose levels post perhexiline. Also, responses to ADP (2.5µM), and SNP(10µM) after two weeks Pex therapy did not change significantly compared to baseline values (*Table 4.3*). However, the extent of $PGE_1$-induced inhibition of platelet aggregation was increased: $IC_{50}$ values of $PGE_1$ were reduced 2-fold following 2weeks Pex therapy (p=0.03, *Fig. 4.4*)

**Table 4.3:** Effects of perhexiline therapy: plasma drug/metabolite concentration, blood glucose levels and parameters re aggregation

| | Before | At 2-weeks time | p |
|---|---|---|---|
| **Plasma perhexiline concentration (µmol/L)** | | 0.33±0.04 | |
| **OH-perhexiline (µmol/L)** | | 1.03±0.13 | |
| **Blood glucose (mmol/L)** | 7.1±0.46 | 7.9±0.88 | 0.31 |
| **ADP response (Ohms)** | 8.5±0.4 | 8.7±5.3 | 0.61 |
| **SNP (%inhibition of aggregation)** | 20.2±3.2 | 23±2.1 | 0.25 |

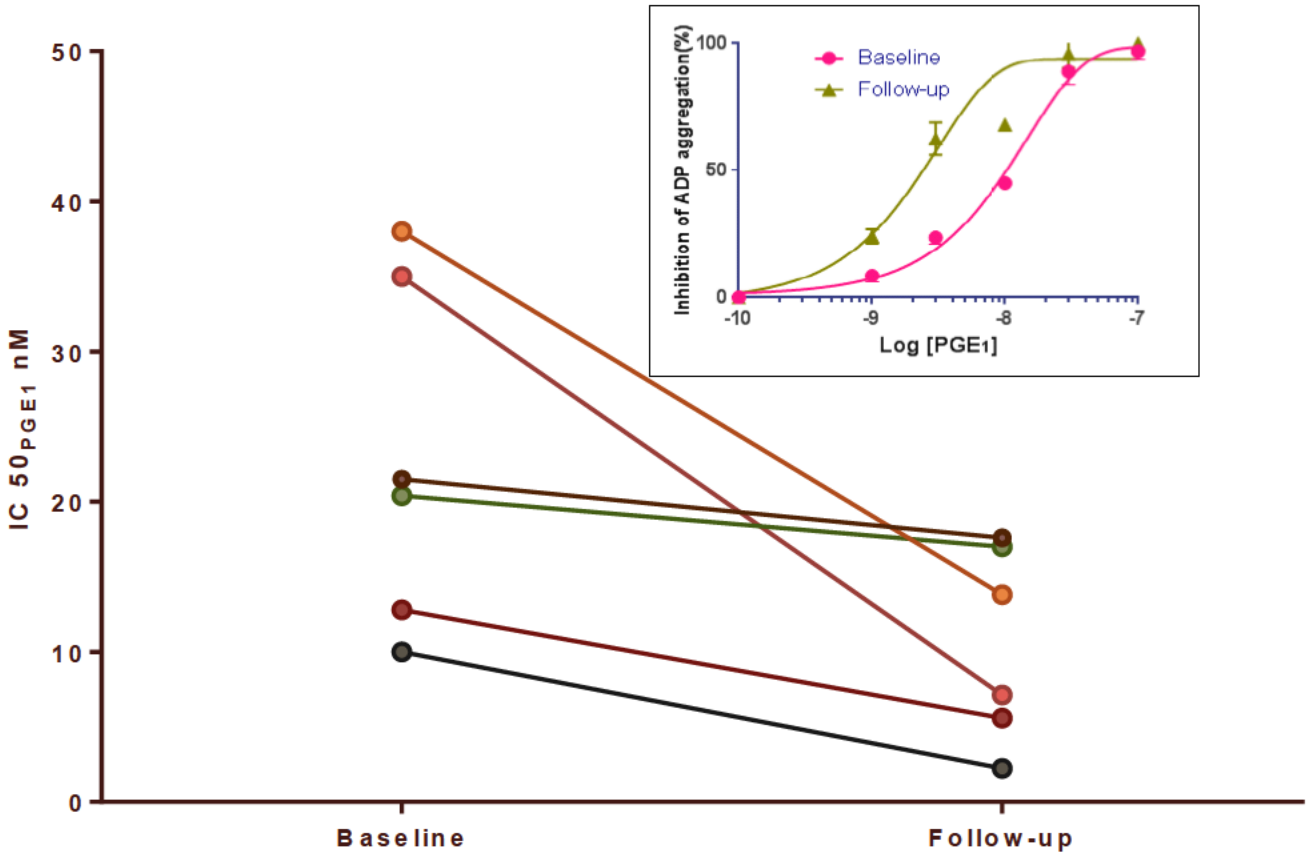



**Figure-4.4:** Platelet responsiveness to $PGE_1$ before and after initiation of perhexiline therapy: changes in $IC_{50}$ ($p<0.03$). Insert: representative concentration-response curves for $PGE_1$ effects.

**4.5 Discussion:**

(1) Clinical status and cyclic nucleotide signaling:

In the current study, we primarily examined putative differences in responses of platelets from patients with SAP and ACS ± associated diabetes mellitus and those in normal subjects to the anti-aggregating effects of SNP and $PGE_1$. There were two bases for this comparison: (1) the observations in Chapter 2 re heterogeneity of platelet response to $PGE_1$ as a basis for prediction of response to ticagrelor begs the question of what clinical factors modulate variability in $PGE_1$/AC signaling and (2) having previously reported (reviewed by Schafer et al 2008) impairment of NO/sGC signaling in IHD and DM, we wished to compare the extent of putative analogous impairment of PG/AC signaling.

We utilized $PGE_1$ which has (in theory) dual effects on AC: stimulatory via the IP-receptor and inhibitory via the EP3 receptor. However limited correlations with responses to the "pure" IP-receptor agonist Iloprost showed strong correlations ($r=0.76$; $p<0.0001$; $n=40$).

The data, in according with our objectives, can be divided into two sets of results according to the analyses performed. Therefore, the answers to the questions currently posed were:

(a) Is there variability in responsiveness to AC activators in ischaemia alone, or in the presence of diabetes?

The results are shown in *Table 4.1* and *Fig. 4.1* and can be summarized as follows:

(I) SAP and DM-IHD are associated with non-significant reductions in responses to $PGE_1$. These findings superficially contrast to previously reported results by Chirkov et al (1995) for SAP, but in that study, entire concentration-response curves were compared, a more sensitive method than a single selected concentration of $PGE_1$. It can however, be stated from the current results the impact of stable myocardial ischaemia on both receptor-mediated and direct AC stimulation is not substantial, and that in well-controlled diabetics with IHD, there is no substantial additional change.

(II) Does ACS impair PG/AC signalling? Overall, patients with ACS (including those with diabetes), exhibited significant and marked impairment of responsiveness to $PGE_1$ but not to Fsk, implying the existence of a major defect in the PG/AC pathway localized primarily or entirely at the receptor level.

(III) Is this impairment of PG/AC signalling modulated by diabetes mellitus?

Of the 23 patients in the ACS cohort (previously described in *Chapter 3*) there were 7 patients with DM. Despite these small numbers, we further partitioned the ACS data according to diabetic status. Importantly, mean blood sugar level among the diabetic patients was 12.3±2.0 mmol/L, representing hyperglycemia. These concentrations would have made patients eligible for emergency insulin infusion according to the DIGAMI protocol (Malmberg et al 1995), which we have previously shown (Worthley et al 2007) to represent a nitric oxide-sensitizing maneuver. Comparisons between ACS patients according to diabetic status suggested that the majority of the changes in responses to PG/AC signaling were driven by diabetes/hyperglycemia. These data are summarized in *Table 4.2*. Regrettably, the fact that there were only 7 diabetics

with ACS potentially limits the accuracy of data interpretations in this area, and it would be preferable for this component of study to be repeated with a large data set.

(b) Is it possible to determine the degree of selectivity of impairments of NO/sGC relative to PG/AC signalling SAP, ACS, DM-IHD?

This experience was of great theoretical importance, because (i) any marked variability in degree of impairment would suggest heterogeneity of mechanisms, and vice versa (ii) this evaluation is of great clinical importance for applying the results of Chapter 3 (re ticagrelor) as regards recommended variability in dosing.

The results strongly indicate that the defect in signaling is relatively specific for NO/sGC (*Fig. 4.2*). Indeed, one possible explanation is that there are completely different mechanisms of resistance for each pathway, with the main problem for NO/sGC residing with sGC oxidation via redox stress (Munzel et al 1995, Gladwin MT 2006), while the comparison of $PGE_1$with Fsk data suggest a defect at receptor level, of currently undefined nature.

Previously Kahn et al (1992) had shown that platelet $PGI_2$ receptors are internalized with subsequent decrease of responses to $PGI_2$ in patients with ACS. However, the cause and duration of this phenomenon of IP-receptor internalization remain to be investigated.

(2) Effects of Perhexiline therapy

Acute Pex therapy was shown to potentiate platelet AC signaling in patients with DM with ischaemia. Contrastingly, we could not find improvement in SNP responses in our patients (n=6) with Pex therapy, as was previously shown in a large cohort of SAP patients (Chirkov et al 2001). However, in this case comparisons were limited to single SNP concentrations, so potential for Type II error is large.

We did not investigate the mechanisms underlying the AC signaling increase by Pex therapy. DM is associated with increased oxidative stress, partially via increased myeloperoxidase (MPO)-mediated catalysis of reactive oxygen species (ROS) formation (Heilman et al 2009). Similarly, increased neutrophil numbers have in some studies been shown to represent sources of ROS release in DM (reviewed by Juan et al 2016). Both Kennedy (2006) and Liberts (2007) and their co-workers reported reduced neutrophil-derived superoxide generation with perhexiline

therapy. We speculate that the effects of Pex on neutrophil function, as shown by Kennedy et al 2006 and Liberts et al 2007, are relevant to the current findings, especially as neutrophils represent major source of MPO release.

### 4.6 Conclusions:

Thus, ACS and DM are associated with impairment of the platelet $PGI_2$/AC signaling pathway, but at different sites: in ACS, receptor-associated transduction is defective, while in DM, there is additional impairment of AC enzymatic activity. ACS in particular is associated with impairment of SNP/sGC signaling. Finally, Pex therapy has the potential to improve platelet AC/cAMP signaling in diabetes with ischaemia.

# Chapter 5: Impaired cGMP and cAMP signaling in patients with coronary vasospasm: changes during acute crises.

***Abbreviations***

| | |
|---|---|
| APN | Adiponectin |
| CAS | Coronary artery spasm |
| CBS | Cystathionine β-synthetase |
| CSE | Cystathionine γ-lyase |
| NAC | N-acetylcysteine |
| PA | Variant/Prinzmetal's angina |
| P-AC | Prostanoid-adenylate cyclase |
| SCFP | Slow coronary flow phenomenon |
| SD-1 | Syndecan-1 |

## 5.1 Abstract

Background: Coronary artery spasm (CAS, variant/Prinzmetal's angina) occurs relatively frequently in both the Caucasian and East Asian populations and represents a major cause of impairment of quality of life. While little is known regarding the pathogenesis of CAS or its clinical cyclical exacerbations, there is evidence supporting both an underlying pro-inflammatory state and the presence of platelet activation. In the current study we sought to evaluate both platelet reactivity and inflammatory activation during acute and chronic phases in CAS patients.

Methods: CAS patients, diagnosed via positive acetylcholine challenge and/or presence of the slow coronary flow phenomenon (SCFP) were evaluated during acute (n=12) and chronic (n=54) phases, and compared with normal subjects (N) (n=28). Platelet reactivity to ADP and its inhibition by the nitric oxide (NO) donor/soluble guanylate cyclase (sGC) activator sodium nitroprusside (SNP) and the adenylate cyclase (AC) activator Iloprost were quantitated in whole blood. Plasma concentration of syndecan-1 (SD-1), a glycocalyx shedding marker, and tryptase (a marker of mast cell activation), were measured as indices of inflammatory activation. Crises were treated with infusion of low-dose NTG plus high dose (10gm/24hours) N-acetyl cysteine (NAC).

Results: (1) CAS patients were predominantly (66%) female; mean age was 53±2.1; only 15% were smokers.

(2) Responses to ADP were greater (p=0.001) and those to both SNP and Iloprost were less (p=0.0002, p=0.0006 respectively) in chronic CAS than in N.

(3) During acute crises, responses to SNP fell from 24.6±4.5% to 6.6±2.5% (p=0.02): infusion of NTG/NAC restored responsiveness to 26.3±6.7% (p=0.02). Responses to Iloprost did not fluctuate significantly during crises.

(4) Plasma concentrations of SD-1 increased (p=0.0001) from chronic to acute phase of CAS, and tended to fall following NTG/NAC infusion (p=0.06). Tryptase concentrations were also significantly increased (p=0.02) during acute (hospitalized) phases relative to chronic status.

Conclusion: (1) Platelets from patients with CAS have markedly impaired responses to both NO/sGC and Iloprost/AC activation, and the former become more accentuated during symptomatic crises.

(2) Both SD-1 and tryptase concentrations are elevated in patients with CAS during symptomatic crises, consistent with inflammatory activation at least partially involving mast cells.

(3) NTG/NAC selectively restores NO responses towards normal and reverses glycocalyx shedding.

These data suggest that inflammatory suppression of cellular NO/sGC signaling in particular may be critical to the pathogenesis of CAS.

## 5.2 Introduction

Coronary artery spasm comprises both Prinzmetal's angina (PA: predominantly large vessel vasospasm) and the slow coronary flow phenomenon (SCFP: predominantly small coronary artery spasm). Both of these disorders are characterized by fluctuating severity of symptoms, irrespective of treatment status, and a poor clinical response to treatment with sublingual nitrates. The mechanisms underlying these clinical features remain uncertain. A number of studies show increased platelet activation and elevated release of platelet derived vasoactive substances such as circulating β-thromboglobulin [marker of platelet activation] (Ogasawara et al 1986), 5-hydroxytryptamine (Mukarami et al 1993), thromboxane $A_2$ (Hamm et al 1987, Lewy et al 1979) and increased micro-aggregates (Robertson et al 1980) in the circulating blood during acute VA. Also, systemic inflammatory markers including CRP and WBC count were shown by Cho et al (2007) to be elevated in CAS patients with ACS presentation. Correspondingly, local inflammation, shown by increased infiltration of adventitial mast cells in cadaveric coronary arteries of known variant angina subjects but not in other subjects who had died suddenly, was reported earlier by Forman et al (1985). One of the clinical characteristics of coronary artery spasm is a trend towards a cyclical symptomatic course, with frequent and prolonged episodes of pain during crises. No cause for these crises has been identified to date. N-acetylcysteine (NAC) has been shown in many studies to have beneficial effects by potentiating NO donors' effects, for example potentiating peripheral vasodilator responses to infused NTG, both in normal subjects (Horowitz et al, 1983), and in patients with chronic heart failure (Mehra et al, 1994) and also potentiating the effects of NO donors in inhibiting platelet aggregation (Loscalzo J,1985).

The bases for NAC potentiation of NO response are not clear. Given previous evidence that platelets from patients with angina pectoris exhibit resistance to the anti-aggregatory effects of NO, and that NAC potentiates vascular (Andrews et al 2001) and platelet (Chirkov et al 1996) NO signaling, largely or entirely in a endothelial NOS-independent manner (Girouard et al 2003, Andrews et al 2001), we now have sought to investigate in platelets with CAS: -

(i) Whether platelet aggregation in vitro, and its inhibition by NO donors and activators of prostanoid-adenylate cyclase (P-AC) signaling, are normal in platelet with CAS.

(ii) Whether patients with CAS, investigated during symptomatic crises, exhibit changes in the above parameters of platelet reactivity relative to their status and in markers of inflammatory activation during clinically quiescent periods.

(iii) Whether NTG/NAC infusion ameliorates the abovementioned putative anomalies.

(iv) The molecular bases for putative changes in platelet reactivity during symptomatic crises.

### 5.3 Methods

Venesection was performed in control subjects (n=28) and patients with either PA or CSFP during phases of acute symptomatic crises (n=12) and/or chronic symptomatic stability (n=53). In all 12 patients evaluated during symptomatic crises, corresponding samples were also taken during chronic status, permitting paired analysis to occur. Demographics are summarized in *Table 5.1*. The study protocol was approved by the Queen Elizabeth Hospital Ethics of Human Research committee and all patients gave informed consent before their blood collection.

#### Platelet aggregometry:

Blood samples collection and whole blood aggregometry analysis were carried out according to the method described in method section (*Chapter 2*).

#### Syndecan-1 measurement:

Given that glycocalyx shedding is activated by acute inflammation (Mulivor & Lipowsky 2004; Nieuwdorp et al 2006) this was routinely quantitated. Plasma concentrations of the glycocalyx component syndecan-1 (SD-1) were determined by ELISA (Abcam biotechnology, UK). Assay was performed according to the manufacturer's instructions. Briefly, standards and samples were prepared and were pipetted into the 96 well plate coated with primary antibody for SD-1. The plate was incubated in room temperature for 60 min on a mechanical shaker followed by a washing step and addition of the enzyme streptavidin-HRP. Finally, a colour-forming substrate

for that enzyme was added in each well and incubated again for 15min in dark, then absorbance was assessed using a spectrophotometer (Bio-Rad, USA) using 450nm wavelength.

**Plasma tryptase assay:**

Platelet tryptase assay was carried out in plasma samples collected from patients with acute and chronic phase of CAS. Samples were analyzed by SA Pathology, Adelaide, Australia following an ELISA-based protocol.

**Statistical analysis:**

Data were presented as mean values with standard error of mean. One way ANOVA was utilized to assess the differences in distribution among groups with quantitative dependent variables and the Chi-square test for categorical variables. Differences in platelet anti-aggregatory responses to SNP and Iloprost between control and patients were evaluated using ANOVA followed by Bonferroni test, and the measured differences within the patient' blood/plasma samples during acute and chronic or acute and post-NAC phases were assessed by Student's paired t-test. Data comparisons for SD-1 concentrations (acute, with/without NTG/NAC therapy and chronic) and control group utilized ANOVA with post-hoc specific comparisons (Bonferroni test) or Student's paired t-test as appropriate. All tests were 2-tailed and p values $\leq 0.05$ were considered statistically significant. Data analyses were performed using SPSS 23 version software.

## 5.4 Results:

### 5.4.1 *Patients* and control subjects: *characteristics*

In general, this was a middle-aged group of individuals: predominantly females and relatively few smokers (*Table 5.1*). Stable patients and control subjects were well-matched for age: the only major differences between these groups was a higher proportion of patients who were smokers, and more of the stable patient group tended to be female and to have a past history of atrial fibrillation (in accordance with previous reports by Kawakami et al 2014). Stable patients in particular were frequently being treated with anti-anginal and cardioprotective agents. Neither

demographic (*Table 5.2*) nor aggregometric findings (*Fig. 5.2*) varied significantly according to the type of CAS subjects.

**Table 5.1:** Demographics of coronary artery spasm patient subjects recruited

| | *CAS Clinical Phase* | | | | | |
|---|---|---|---|---|---|---|
| | N(n=28) | Chronic (n=53) | Acute (n=12) | $P^1$ | $P^2$ | $P^3$ |
| *Age* | 56.1±1.5 | 53±2.0 | 51.1±3.4 | 0.97 | 0.11 | 0.07 |
| *Male: female* | 14:14 | 18:35 | 3:9 | 0.09 | 0.19 | 0.67 |
| *Platelet count(X10⁹/L)* | 252±15 | 285±12 | 341±26 | 0.09 | 0.02 | 0.09 |
| *Total cholesterol (mmol/L)* | 5.6±0.6 | 5.4±0.5 | 5.5±0.3 | 0.76 | 0.91 | 0.58 |
| *Smoker* | 0 | 8 | 2 | 0.04 | 0.02 | 0.79 |
| *Other diseases* | | | | | | |
| *DM* | 1 | 5 | 1 | 0.38 | 0.48 | 0.97 |
| *Hypertension* | 6 | 19 | 3 | 0.25 | 0.69 | 0.59 |
| *Atrial fibrillation* | 0 | 5 | 2 | 0.11 | 0.02 | 0.39 |
| *Family history of MI* | 0 | 3 | 2 | 0.22 | 0.02 | 0.16 |
| *Osteoarthritis* | 0 | 9 | 4 | 0.03 | 0.01 | 0.20 |
| *Asthma* | 1 | 7 | 3 | 0.19 | 0.03 | 0.24 |
| *Gastro-oesophageal reflux* | 1 | 7 | 2 | 0.19 | 0.12 | 0.66 |
| *Anxiety disorder* | 0 | 7 | 4 | 0.05 | 0.01 | 0.06 |
| *Medications* | | | | | | |
| *Statins* | 7 | 14 | 4 | 0.89 | 0.48 | 0.50 |
| *$Ca^{++}$ antagonists* | 2 | 29 | 8 | <0.001 | <0.001 | 0.27 |
| *Organic nitrates* | 0 | 14 | 4 | 0.002 | 0.01 | 0.50 |
| *Aspirin* | 3 | 17 | 3 | 0.05 | 0.19 | 0.75 |
| *ACE inhibitors* | 4 | 9 | 1 | 0.56 | 0.66 | 0.51 |
| *ARB* | 2 | 6 | 2 | 0.62 | 0.31 | 0.53 |
| *Beta blockers* | 1 | 5 | 1 | 0.38 | 0.48 | 0.97 |

$P^1$ & $P^2$ are for Chronic and Acute compared with control
$p^3$ is for Chronic vs Acute CAS

**Table 5.2:** Demographic comparisons between patients with PA and SCFP during chronic phase

| | *Patients with Chronic CAS(n=53)* | | |
|---|---|---|---|
| | PA (n=34) | SCFP(n=19) | p |
| *Age* | 57.5±1.4 | 53.3±1.1 | 0.25 |
| *Male: Female* | 11:23 | 7:12 | 0.78 |
| *DM* | 2(6%) | 3(16%) | 0.24 |
| *Hypertension* | 11(32) | 8(42%) | 0.56 |
| *Total Cholesterol (mmol/L)* | 5.6±0.4 | 5.2±0.7 | 0.21 |
| *Platelet count(X10⁹/L)* | 277.4±11 | 308.2±17 | 0.54 |
| *Aspirin* | 8(24%) | 9(47%) | 0.08 |
| *ACE Inhibitor* | 6(18%) | 3(16%) | 0.86 |
| *Organic nitrates* | 11(32%) | 3(16%) | 0.19 |
| *ARB* | 4(12%) | 2(11%) | 0.89 |
| *Statins* | 8(24%) | 6(32%) | 0.52 |
| *Calcium antagonists* | 17(50%) | 12(63%) | 0.36 |
| *Beta blockers* | 2(6%) | 3(16%) | 0.24 |

### 5.4.2 ***Platelet reactivity during quiescent ("chronic") phase: CAS patients exhibit impaired responses to SNP and $PGI_2$***

Stable CAS patients exhibited significantly greater aggregability to ADP than control subjects: - mean responses were 8.7±0.4 vs 6.4±0.4 ohms (p=0.001). Conversely, there was substantial hyporesponsiveness to both SNP and Iloprost. Mean inhibition of ADP-induced platelet aggregation by Iloprost was significantly lower in patients than that in control subjects (34.1±4.1 vs. 63.7±6.7%; p=0.0002), while inhibition of aggregation by SNP was 14.1±1.4 vs. 31.1±4.1% in patients and normal control subjects respectively (p=0.0006; *Fig. 5.1*). Responses to the direct AC activator forskolin were 92.6±3.4% inhibition in normal subjects (n=23) vs 82.4±4.2% inhibition in chronic CAS patients (n=20; p=0.04).

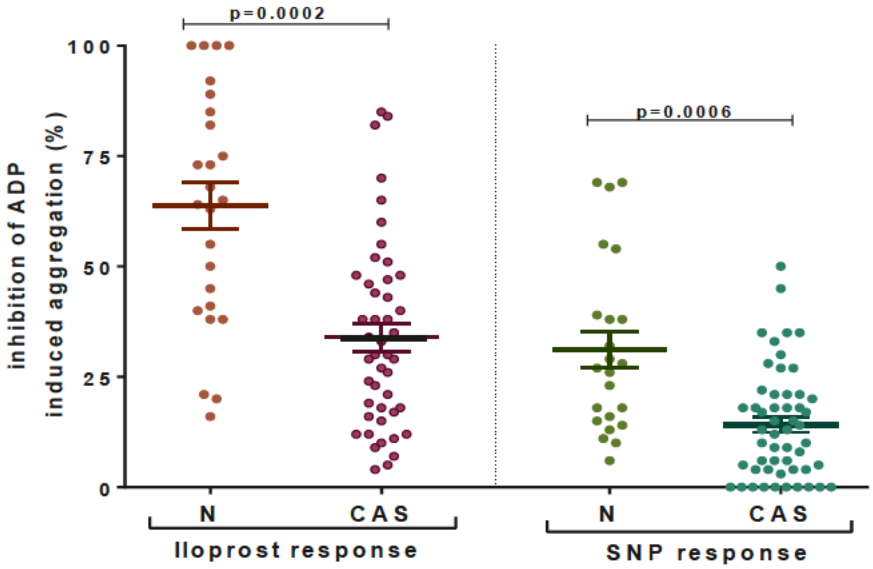



**Figure 5.1:** Inhibition of ADP-induced aggregation by Iloprost (0.3nM), and SNP (10μM) in whole blood samples from normal control subjects (N) and patients in chronic phase of coronary artery spasm (CAS).

### *5.4.3 These changes are similar for both PA and SCFP*

Results (both pro-aggregatory and anti-aggregatory effects) did not vary significantly according to the type of CAS subjects (*Fig.5.2*). Responses to ADP were 8.9±0.3 and 8.4±0.4 ohms respectively (p=NS).

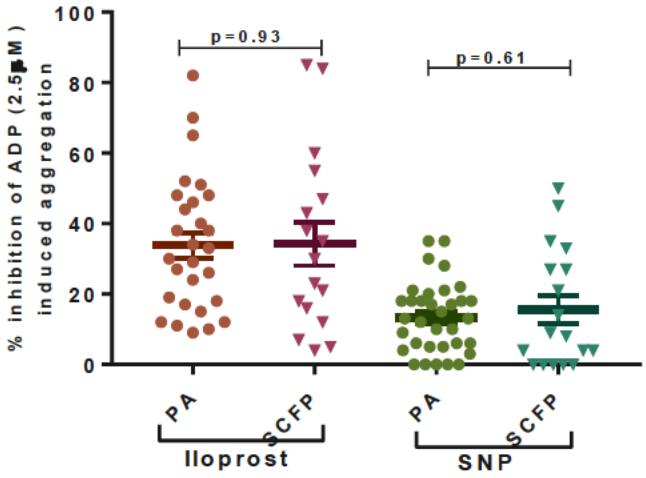



**Figure 5.2:** Inhibition of platelet aggregation by Iloprost and SNP: - comparisons between PA and SCFP patients

### 5.4.4 *Acute exacerbation of symptoms is associated with further impairment of NO/sGC signaling*

Data for patients studied during symptomatic exacerbations are compared with those for all chronic phase data and those for normal subjects in *Table 5.3*. In this table, the majority of data comparisons between acute and chronic phases are of necessity unpaired, with the blood samples for the chronic patients being collected prior to the inception of NTG/NAC therapy. These data suggest that the acute phase might be associated with incremental aggregation in response to ADP and further impairment of responses to SNP.

However, paired data (*Fig.5.3*) comparisons for chronic versus acute phases represent a more appropriate method of analysis. On this basis, there was significant (p=0.013) and selective decline in response to SNP during acute phases of CAS.

### 5.4.5 *Plasma Syndecan-1 and tryptase concentrations during acute crises*

We next evaluated potential differences between plasma SD-1 and tryptase concentrations during the acute phase of CAS and those in normal subjects, in order to determine whether endothelial glycocalyx shedding was activated, and whether mast cell activation might play a role in this process. During acute exacerbations, SD-1 plasma concentrations were four-fold higher those in normal subjects (49.5±4.3 vs. 12.7±2.4 µg/L, p=0.0001) [*Fig. 5.4A*].

**Table 5.3:** Aggregometry parameters: non-paired comparisons between all patients evaluated during chronic or acute symptomatic phases of CAS

| | *CAS* | | |
|---|---|---|---|
| *Parameter* | Chronic (n=53) | Acute (n=12) | P |
| *ADP response(Ohms)* | 8.7±0.4 | 10.5±0.5 | 0.04 |
| *SNP response (% inhibition)* | 14.1±1.4 | 8.8±3.5 | 0.07 |
| *Iloprost response (%inhibition)* | 34.1±4.1 | 35.3±9.3 | 0.88 |

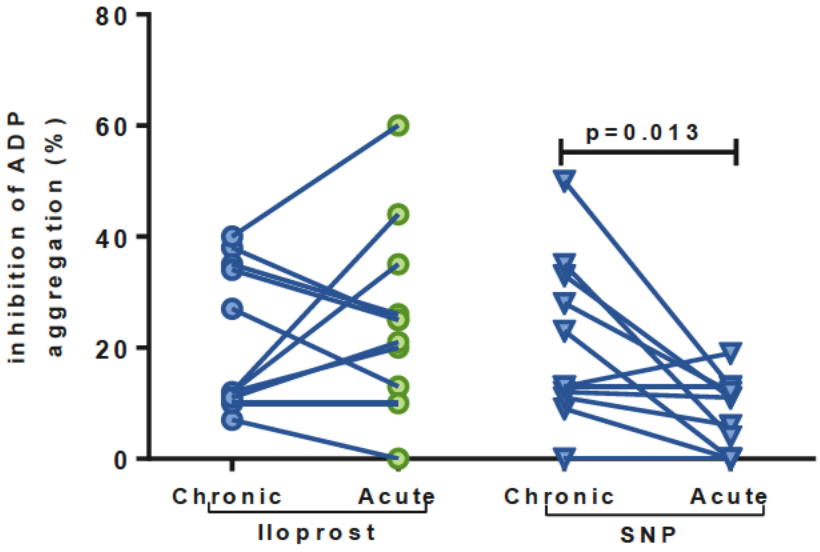



**Figure 5.3:** Changes of aggregation during acute exacerbations of CAS. During acute phase, there is significant (p=0.013) impairment of SNP responses, without significant (p=0.45) change of Iloprost responses

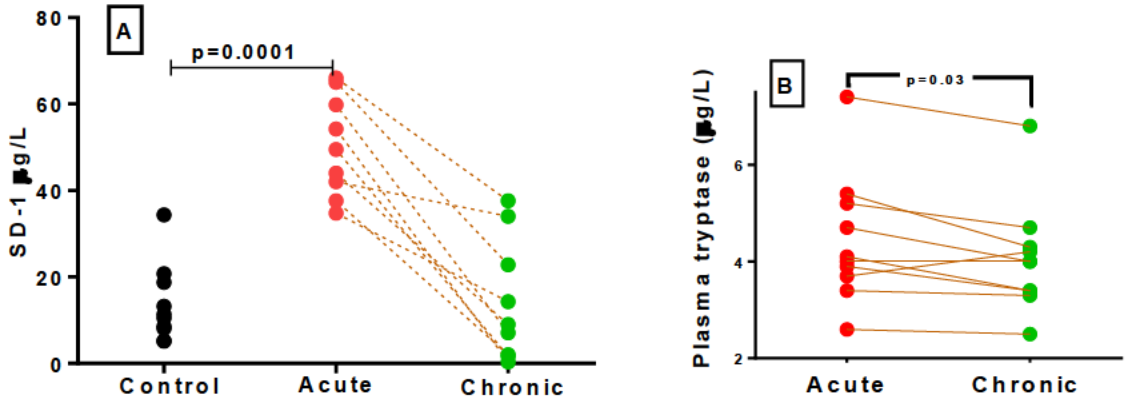



**Figure 5.4:** Changes of **(A)** Plasma concentrations of SD-1 are evaluated in acute CAS and compared with those in controls (p=0.001). Data for same patients in chronic phase in CAS are included primarily for comparison (p=0.001 versus acute phase). And **(B)** plasma tryptase during chronic and acute phases: paired analysis. The increase in tryptase concentration is small but relatively consistent.

Previous evidence of increased activation of mast cells from cadaveric coronary arteries of known variant angina subjects (Forman et al 1985). Thus, we tested tryptase level in plasma samples from chronic and acute phases for the same patients. Though systemic tryptase levels in CAS patients were very low compared to during systemic anaphylaxis(>12µg/L), there was a borderline significant difference between chronic and acute phases of CAS (4.1±0.4 vs 4.5±0.4, n=10, p=0.03, *Fig. 5.4B*).

### *5.4.6 NAC reverses platelet resistance to NO both in vivo and in vitro*

Responses to both SNP and Iloprost were studied in patients (n=12) during acute crises using whole blood obtained before and during (2-12 hrs) NTG/NAC infusion (*Fig. 5.5*). Following the imitation of infusion of NTG/NAC, platelet aggregation in response to ADP decreased marginally from 10.6±0.5 to 9.1±0.5 ohms (p=0.05). NAC therapy virtually normalized anti-aggregatory responses to SNP from [8.8±3.0% to 24.4±2.9% (p=0.0002)]. Responses to Iloprost also increased significantly (from 35.7±9.3 to 49.4±9.3%, p=0.008) on NAC.

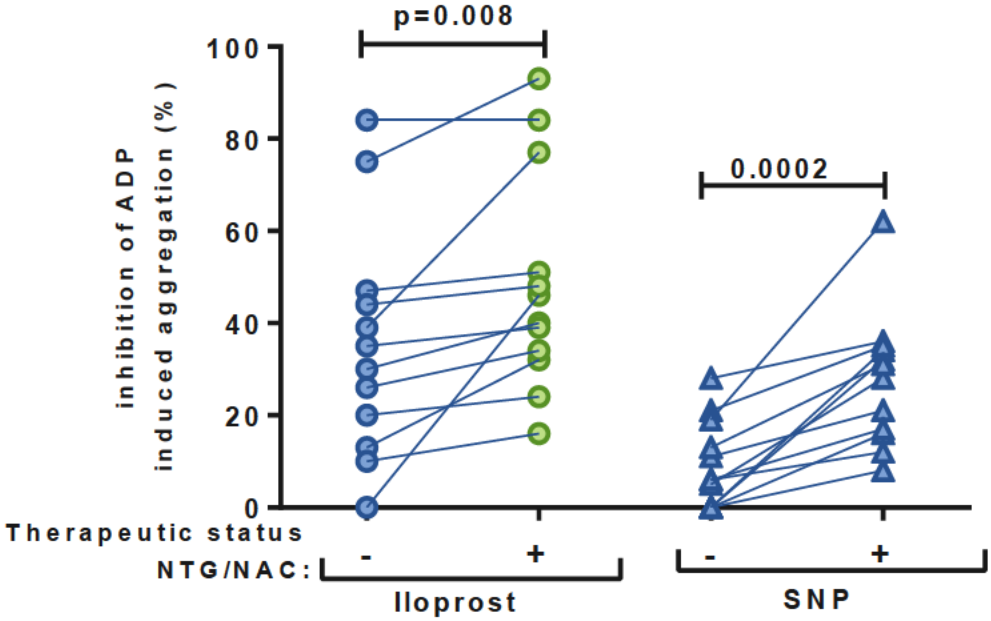



**Figure 5.5:** Comparison of platelet response to Iloprost and SNP before and after NTG/NAC therapy in acute symptomatic patients with CAS.

### *5.4.7 NAC reverses SD-1 elevation during crises*

In 9 patients, plasma SD-1 concentrations were quantitated both during symptomatic crises and 2-12 hours post initiation of NTG-NAC infusion. Results are shown in *Fig. 5.6*. There was a substantial and significant (p=0.02) fall in SD-1 concentrations post onset of NTG/NAC infusion: mean SD-1 concentrations on NTG/NAC were similar to those in the normal control group.

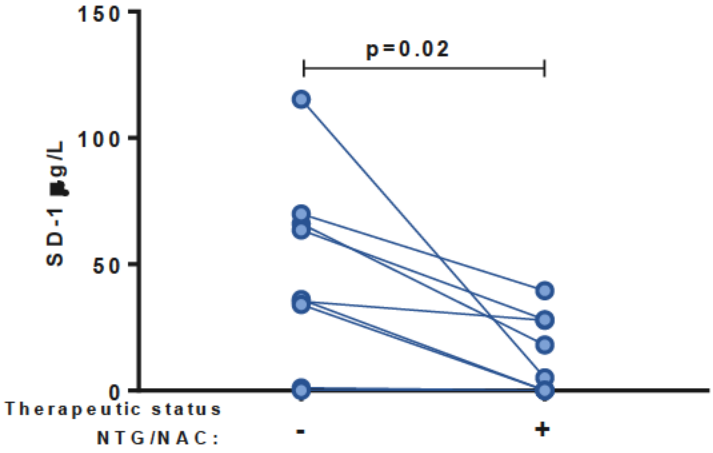



**Figure 5.6:** Comparisons of plasma syndecan-1 release before and during NTG/NAC infusion in patients with CAS. Data were compared using Student's paired t-test.

## 5.5 *Discussion:*

The main objectives of the current study were to characterize patients with CAS in attempts to answer the following questions: -

(1) Why does spasm occur?
(2) Why do patients with CAS experience crises? and
(3) Is it possible to develop a conventional method to differentiate CAS from non-cardiac chest pain (essentially normality) prior to the results of coronary angiography ± acetylcholine test?

It must also be appreciated that this is a very substantial problem for three major reasons: -

(1) CAS is notoriously under-diagnosed, and patients are often told that there is nothing wrong with them. This leads to unnecessary morbidity and anxiety.
(2) The epidemiology of CAS is poorly explored in the absence of a reliable screening test
(3) There is still no consensus as to the molecular causes of CAS, and these are difficult to evaluate without studying a large patient cohort.

In the event, the current investigations offered partial answers to many of these questions. It is appropriate to address each component experiment individually.

(1) Who were the patients and controls?

As summarized in *Table (5.1)*, we studied a large number of patients, but only 12 in the acute exacerbation phase of symptomatic status of the overall patient group. Over half were "macrovascular' spasm (Variant/Prinzmetal) angina rather than SCFP, but no significant differences were found between patients with VA and SCFP. Most of the patients were studied during treatment with calcium antagonists, and it remains possible that this might have affected results However, the main thing to observe is that the patients were mainly female, and that few were smokers (in contrast to early data on demographics). Previous series of patients with CAS have varied markedly in these respects, as shown in *Table 5.4*, and it is entirely possible that our results might have differed if there had been more males/smokers.

**Table 5.4:** Comparison of gender mix and proportion of smokers (gender-adjusted) among CAS patients in other studies

| *PATIENT ETHNICITY* | *MALE/FEMALE(N)* | *SMOKERS (N)* | |
|---|---|---|---|
| ***ASIAN POPULATIONS*** | | | |
| *JAPANESE* | 165/10 | 150/0 | ***Sugiishi & Takatsu, 1993*** |
| *JAPANESE* | 84/90 | 171/92 | ***akayama et al 1999*** |
| *KOREAN* | 83/21 | 69/4 | ***Lee et al 2009*** |
| *JAPANESE* | 1090/339 | 781/67 | ***Kawana et al 2013*** |
| *TAIWANESE* | 347/148 | 208/15 | ***Hung et al 2013*** |
| *JAPANESE* | | | |
| ***CAUCASIAN POPULATIONS*** | | | |
| *ITALIAN* | 16/7 | 7/0 | ***Perrinello et al 2014*** |
| *BOTH WHITE(CAUCASIAN) & NON-WHITE ENGLISH* | 523/947 | 53/68 | ***Murthy et al 2014*** |
| *BOTH WHITE(CAUCASIAN) & NON-WHITE ENGLISH* | 189/140 | 19/10 | ***Taqueti et al 2017*** |
| *AMERICAN* | 16/43 | 1/0 | ***Glueck et al 2015*** |

(2) What are the key observations made?

These were: -

(i) In patients with chronic CAS, compared to normal subjects, these is severe impairment of NO- and Iloprost-induced inhibition of platelet aggregation compared to normal values. Furthermore, there is platelet hyperaggregability to ADP, and hypo-responsiveness to direct AC stimulation by forskolin.

(ii) During acute exacerbations of CAS, there is aggravation of anomalies of NO/sGC and $PGI_2$/AC signaling relative to chronic disease status.
However, most importantly, the glycocalyx component SD-1 is released into plasma, with subsequent significant elevation of SD-1 concentrations relative to these seen in normal subjects. It should also be added that plasma concentrations of the mast cell enzyme tryptase rose marginally during transition from chronic to acute status as shown in *Fig. 5.5*.

(iii) Treatment of acute episodes of CAS with the combination of low dose NTG and high dose NAC infusion led to substantial increases in SNP responses and rapidly falls in SD-1 concentrations.

What conclusions can draw from these results?

**Overall, the temptation is to reach a conclusion that, irrespective of the sub-type of CAS, it is essentially a disorder of fluctuating inflammation, with inflammatory exacerbations triggering cyclical crises, as well as inducing damage to the vascular glycocalyx; there is also evidence that NAC may reverse these inflammatory crises, that some inflammation is present during chronic disease status, and possibly that inflammation involves mast cell activation.**

*Can one deduce the mechanisms underlying the observed changes?*

The central observation has been of impaired platelet signaling responses to NO/sGC and to $PGI_2$/AC signaling. This is not entirely a new observation. For example, the whole basis for the coronary vasospasm induced during provocative intracoronary injection of acetylcholine in susceptible patients is either diminished NO release or signaling. Furthermore, NOS mutations have been implicated in coronary spasm (Glueck et al 2015). The causes of these anomalies were not investigated in the current experiments, but might in theory have involved either "scavenging" of NO and/or impaired activity of both sGC and AC. Impairment of responses to forskolin strongly suggest that the latter is a component of the problem.

Overall, this finding reinforces previous observations by Chirkov et al (1995, 1996, 2001) showing that patients with angina pectoris have impairment of both NO/sGC and PG/AC signaling in platelets, despite the fact that these differences did not reach statistical significance in the studies described in *Chapter 4*.

The cause of exacerbation of abnormalities during acute symptomatic crises is of great importance. Although SNP and Iloprost responses deteriorated further during such crises, the central observation was the release of SD-1. This implicates "glycocalyx shedding", an inflammatory process (Lipowsky et al 2011) which can be triggered by release of a member of proinflammatory enzymes, such as matrix metalloproteinases (Ramnath et al 2014). In this case, the only proteolytical "sheddase" tested was tryptase, a mast cell enzyme: - the results were of borderline elevation (*Fig. 5.5*). If it were to turn out that crises of CAS are triggered by fluctuating mast cell activation, this would not be a total surprise. Specifically, Kounis syndrome, also known as allergic angina, involves inflammatory cytokines released from mast

cell activation, which may lead to coronary artery vasospasm or atheromatous plaque rupture (Kounis NG, 2006).

The extent of release of tryptase into plasma is small (for less than in anaphylaxis), and one would therefore contemplate mainly the concept of local activators of mast cells, perhaps in epicardial fat adjacent coronaries. Interestingly, there is evidence in the literature that NO may contribute to stabilization of both isolated and mixed population of mast cells, suggesting the effect of NO on mast cell is direct activation (van Overveld et al 1993; Eastmond et al 1997). This would cause the possibility of a "vicious cycle" at this level.

It remains quite probable that other mechanisms of activation may be relevant to the precipitation of crises in patients with CAS, and the simultaneous activation of glycocalyx shedding. The suggestion of an association with atrial fibrillation among CAS patients, which mirrors previous observations by other investigators (Kawakami et al 2014), suggests that activation of myeloperoxidase and consequent release of hypochlorous acid (Klebanoff SJ, 1991) may be involved: indeed NAC is known to be a hypochlorous acid scavenger (Aruoma et al 1989).

Decreased release of adiponectin (APN) has previously been reported in patients with CAS (Maruyoshi et al 2005). APN is a major adipocyte-secreted protein with anti-inflammatory, antiatherogenic and antidiabetic properties (reviewed by Chandran et al 2003), which enhances vasorelaxant effects of acetylcholine (Du et al 2016). Interestingly, L-cysteine pretreatment increases APN release from cultured adipocytes (Achari and Jain, 2016). It would therefore be desirable to additionally explore secretion of APN in the patient group currently studied.

The clinical utility of co-infusion of low dose NTG with high-dose NAC has been established in acute myocardial infarction (Pasupathy et al 2017), unstable angina pectoris (Horowitz et al 1988), and acute pulmonary oedema (reviewed by Sochman J, 2002). Experimentally, NAC has been shown to potentiate NTG effects both haemodynamically (Horowitz et al 1983) and in limiting platelet aggregation (Loscalzo et al 1985; Chirkov et al 1996). Interestingly, Folts and Loscalzo (1991) demonstrated in a canine model of intimal injury to the circumflex that NAC potentiated the effects of NTG in reversing cyclic coronary flow reductions, the letter representing periodic platelet adhesion to the injured vessel. However, there are no formal studies to date of NTG/NAC interaction in the clinical contest of CAS.

In the current study, NAC rapidly reduced intensity of chest pain, markedly potentiated SNP responses, and rapidly lowered plasma concentrations of SD-1. The potentiation of SNP responsiveness implies that this effect was not specific for organic nitrates such as NTG, while the acuity of introduction of intravenous NTG infusion imply that this potentiation had nothing to do with prevention or reversal of nitrate tolerance, which has been suggested in the past as a mechanism of NTG/NAC interaction (May et al 1987).

However, many other explanations are potentially available for this effect of NAC, as explored in *Chapter-6*.

The rapid reduction in SD-1 plasma concentrations suggests that NTG/NAC (or indeed NAC alone) rapidly reverse inflammatory shedding of glycocalyx. It has previously been shown that NO tends to limit glycocalyx shedding (Bruegger et al 2008) but the speed of this interaction is surprising. Again, this finding is worthy of further evaluation.

# Chapter 6: Mechanisms of interaction between N-acetylcysteine and nitric oxide signaling: role of hydrogen sulphide release

***Abbreviations***

| | |
|---|---|
| AOAA | Amiooxyacetic acid |
| CAS | Coronary artery spasm |
| CBS | Cystathionine β-synthetase |
| CSE | Cystathionine γ-lyase |
| $H_2O_2$ | Hydrogen peroxide |
| NAC | N-acetylcysteine |
| NaHS | Sodium hydrogen sulphide |
| NO | Nitric oxide |
| PAG | DL-propargylglycine |

### 6.1 Abstract

Background: Interplay between N-acetylcysteine (NAC) and nitric oxide (NO) is critically important in the regulation of vascular tone and inhibition of platelet aggregation. We have previously demonstrated that NAC infusion in patients with acute coronary artery spasm (CAS) improves responses to both SNP and Iloprost. However, the bases for NAC potentiation of NO/Iloprost responses are not clear. Recent studies show that NAC undergoes indirect enzymatic conversion to hydrogen sulphide ($H_2S$). We therefore tested the hypothesis that NAC potentiates anti-aggregatory response to both NO and $PGI_2$.

Methods: Pilot studies were performed in vitro to determine whether NAC exerts intrinsic anti-aggregatory effects, whether it potentiates anti-aggregatory effects of SNP and/or Iloprost, whether this putative potentiation is mediated by formation of $H_2S$ and whether the effects of NAC are mimicked by a known donor of $H_2S$, sodium hydrogen sulphide (NaHS).

Blood samples from 12 patients with CAS and two with acute coronary syndromes were evaluated in vitro, evaluating the interactions of NAC with ADP-induced aggregation and responses to SNP and Iloprost. Effects of co-incubation with inhibitors of $H_2S$ formation and NaHS were also determined.

Results: (1) NAC(100µM) which lacks intrinsic anti-aggregatory effects in this concentration, potentiates both SNP and Iloprost responses: inhibition of platelet aggregation increased from 13.8±2.9 to 37.2±5.1 % (n=13, p=0.0002) and 33.7±6.2 to 40.4± 6.6% (n=9, p=0.003), respectively.

(2) NaHS (100µM) exert similar effects but is apparently more potent than NAC at identical concentrations.

(3) Inhibition of $H_2S$ formation limits NAC-initiated restoration of SNP signaling: - Effects of SNP in the presence of NAC fell from 33.6±4.6 % to 15.2±2.8% ($p<0.03$) and 23.0±3.8% ($p=0.34$) by inhibiting $H_2S$-generating enzymes.

(4) NaHS (100µM) improves SNP (10µM) responses from 27.7±4.1 to 63.2±6 % (p=0.003), but exerts inconsistent effects on Iloprost responses

Conclusions: These results suggest that NAC potentiates SNP responses at least in part via de novo $H_2S$ formation. The apparent greater potency of NaHS is likely to reflect greater $H_2S$ release than from NAC. A number of factors remain unexplained. These include the mechanisms of potential interaction between NAC/NaHS and PG/AC signalling.

### 6.2 *Introduction:*

N-acetylcysteine (NAC) has been shown in many studies to potentiate NO donors' vasodilatory and anti-aggregatory effects. This has been demonstrated clinically in a wide range of cardiovascular circumstances potentiating hypotensive responses to NTG, and reducing filling pressures (Horowitz et al 1983; Mehra et al 1994). The bases for NAC potentiation of NO response are not clear. In vitro, NAC with nitroglycerine, but not NAC alone was shown to increase sGC (purified) activation (Munzel et al 1989). NAC was also shown to increase activation of eNOS in rat myocytes (Wating et al 2016), although that effect is not obviously relevant to potentiation of NO donor effects. The NO-potentiating effects of NAC have also been shown to extend to the anti-aggregatory effects of NO donors (Loscalzo J, 1985; Chirkov et al 1996), and together with vasodilator effects formed the basis for observations of termination of cyclic flow changes after coronary artery injury in a study by Folts and Loscalzo (1991). The NTG/NAC interaction has been reported to improve efficacy of treatment in patients with unstable angina pectoris (Horowitz et al 1988), acute myocardial infarction (Arstall et al 1995; Pasupathy et al 2017) and acute pulmonary oedema (Beltrame et al 1998).

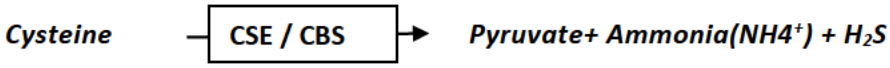
In general, the explanations offered for the observed potentiation have related to the "antioxidant" status of NAC. In this regard, early studies from Halliwell's group demonstrated that NAC "scavenged" a number of reactive oxygen species, notably hypochlorous acid (HOCl) and hydrogen peroxide ($H_2O_2$) (Auroma et al 1989). These findings imply that NAC may be particularly effective in limiting the clinical impact of activation of myeloperoxidase, which generates HOCl (reviewed by Gillissen and Nowak 1998). Indeed, in the recently published NACIAM trial, involving NAC adjunctive therapy in patients with evolving acute myocardial

infarction treated with low rates of NTG infusion (Pasupathy et al 2017), NAC was particularly effective in the context of high plasma concentrations of myeloperoxidase.

However, there is also evidence that NAC, while not "scavenging" $0_2^-$, may inhibit generation by NAD(P)H oxidases (Nakagami et al 2003). The clinical importance of this effect has never been fully investigated.

NAC is also a sulphydryl-containing compound, which raises the potential for other categories of salutary biological effects, either by reducing tissue sulphydryl oxidation, as demonstrated in patients with evolving acute myocardial infarction by Arstall et al (1995), and/or by normalising enzymatic function in the face of redox stress. For example, it has previously been shown that activity of sGC can be impaired, not only by heme depletion, but also by oxidation of critical SH groups on the enzyme molecule (Braughler JM 1980): NAC might theoretically limit this process.

Finally, NAC is metabolised to form glutathione and cysteine: the letter can represent a precursor of hydrogen sulphide ($H_2S$) via the following enzymatic reactions: -




Formation of $H_2S$ form NAC has recently been demonstrated by Ezerina et al (2018). Cystathionine γ-lyase (CSE) and cystathionine β-synthetase (CBS) are two pyridoxal 5′ phosphate (PLP) dependent enzymes. CSE is expressed predominantly in vasculature, while CBS predominantly in CNS (Leffler et al 2011) but also in arteries, including coronaries (Donovan et al 2017). CSE is regulated by intracellular calcium level: with steady low concentrations $H_2S$ formation increases, but formation of $H_2S$ is diminished with higher cytosolic calcium level (Mikami et al 2013). CBS is attached to a redox sensitive iron molecule, binding CO with CBS during hypoxia leads diminished enzymatic activity (Taoka and Banerjee 2001). During inflammatory states both CSE and CBS expression can be modulated via NF-kB (Wang et al 2014; Li et al 2012). Studies of available inhibitors such as DL-propargylglycine (PAG) and amiooxyacetic acid (AOAA) in isolated enzymes were performed by Asimakopoulou

et al (2013), showing PAG with greater selectivity for CSE with $IC_{50}$ 40±8μM, and AOAA with selectivity for both enzymes ($IC_{50}$ was 1.09±0.1 vs 8.5±0.7μM for CSE and CBS, respectively). There are further documented interactions of AOAA with many other molecular components, such as interfering with the, malate-aspartate shuttle in mitochondria (Chen et al 2015), and inhibition of binding to GABA-receptor (Carmona et al 1980), and all other PLP enzymes (reviewed by Zhao et al 2014). Therefore, the specificity of AOAA for $H_2S$ formation is questionable.

$H_2S$ is best characterized as a vasodilator and potentiator of similar effects of NO, also there are a number of studies showing that it exerts anti-aggregatory effects (*see Chapter 1 for review*). What remains unclear is the endogenous capacity of platelets to generate $H_2S$.

Initially (*described in Chapter 5*) in an ex vivo study, we have shown that patients with acute coronary artery spasm (CAS) exhibit marked impairment of both cellular NO/sGC and PG/AC signaling, while NAC therapy reverses these anomalies. Given the above-described status of NAC as a potential basis for increased $H_2S$ generation, there was a need to investigate the role of $H_2S$ in these findings. Therefore, the objectives of the current study were to determine: -

(a) The precise impact of NAC on responses to SNP and Iloprost in patients with CAS in vivo and in vitro.
(b) The potential contribution of formation of $H_2S$ from NAC to these putative interactions
(c) The impact of a "pure" $H_2S$ donor, NaHS, in vitro on platelet responsiveness to both SNP and $H_2S$.

### 6.3 Methods

***Patients: -*** Venesection was performed in patients with coronary artery spasm during acute symptomatic crises (n=8) and/or chronic symptomatic stability (n=6). Demographics are summarized in *Table 6.1*. The study protocol was approved by the Queen Elizabeth Hospital ethics committee and all patients gave informed consent before their blood collection.

**Table 6.1:** Demographics of patient subjects recruited

| | *Chronic (n=6)* | *Acute (n=8)* |
|---|---|---|
| *Age* | 50.2±12 | 60.7±2.6 |
| *Male: female* | 2:3 | 4:4 |
| *Platelet count(X10⁹/L)* | 252±52 | 284±24 |
| *Smoker* | 0 | 2 |
| *Other diseases* | | |
| *DM* | 0 | 3 |
| *Hypertension* | 2 | 3 |
| *Medications* | | |
| *Statins* | 1 | 5 |
| *Ca⁺⁺ antagonists* | 3 | 5 |
| *Organic nitrates* | 1 | 2 |
| *Aspirin* | 1 | 3 |
| *ACE inhibitor* | 1 | 2 |
| *ARB* | 1 | 1 |

***Chemicals: -*** N-acetylcysteine, Sodium nitroprusside, Adenosine di-phosphate, Amiooxyacetic acid (AOAA, 0.5mM), DL propargylglycine (PAG, 3.3mM) were from Sigma Aldrich USA, Iloprost and NaHS were from Cayman chemical company USA.

***Platelet aggregation: -*** Blood samples collection and whole blood aggregometry analysis were performed according to the method described in *Chapter 2*. Preliminary studies were conducted to determine threshold concentration for intrinsic NAC-induced anti-aggregatory effects. In vitro incubations involved adding NAC 15 minutes or NaHS 2 minutes before adding ADP.

***Statistical analysis: -***

Data were presented as mean values with standard error of mean. One-way ANOVA was utilized to assess the differences in distribution among groups with quantitative dependent variables and the Chi-square test for categorical variables. Differences in platelet anti-aggregatory responses to SNP and Iloprost within the same patient subjects were assessed by Student's paired t-test. All tests were 2-tailed and p values $\leq 0.05$ were considered statistically significant. Data analyses were performed using SPSS 23 version software.

## 6.4 Results:

### 6.4.1 *NAC potentiates both SNP and Iloprost responses*

Anti-aggregatory effects of NAC, mainly in the sense of NO potentiation have shown previously (Loscalzo J 1985; Chirkov & Horowitz J 1996). In our in vivo NAC study (*described in Chapter-5*), we also observed a diminution in ADP(2.5µM) response by 14% (n=14, p=0.05) after NAC/NTG infusion. Mean steady-state NAC concentrations during intravenous infusion have previously been documented 172±79µmol/L in patients with acute myocardial infarction at the 4th hour from initiation (15gm/24hours therapy) (Arstall et al 1995). Accordingly, in vitro 172µM NAC was initially utilized but induced 0-16% (n=6) inhibition of ADP(2.5µM)-induced aggregation. However, there were not any measurable inhibitory effects of NAC below 100µM (n=5). Therefore, we selected a 100µM concentration of NAC, to evaluate putative potentiation effects of SNP and Iloprost.

NAC improved SNP responses in all cases: - inhibition of platelet aggregation increased from 13.8±2.9 to 37.2± 5.1%(n=13; p=0.0002) after adding NAC(100µM) to SNP(10µM). This in vitro NAC treatment was also associated with significant but proportionally small improvement of Iloprost responses, inhibition of aggregation increasing from 33.7±6.2 to 40.4± 6.6%, (n=9, p=0.003, *Fig. 6.1*).

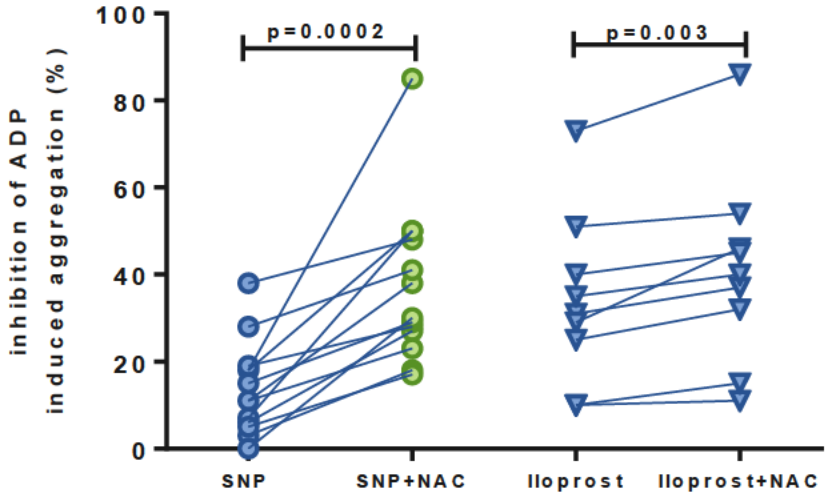



**Figure 6.1:** Effects of NAC (100µM, 10minutes incubation) on antiaggregatory effects of SNP and Iloprost. Data were compared using Student's paired t-test.

### 6.4.2 NaHS, a "pure" $H_2S$ donor, mimics some effects of NAC

Both NaHS and NAC dose-dependently increased anti-aggregatory effects of SNP (*Fig. 6.2*). Pretreatment of platelets with NAC (10-100µM) with a fixed dose SNP(10µM) led to 1.5 to 2.9-fold increases in inhibition of aggregation compared to SNP(10µM) alone, while pretreatment with same concentrations of NaHS induced a substantial increase from (3.5 to 4.5-fold).

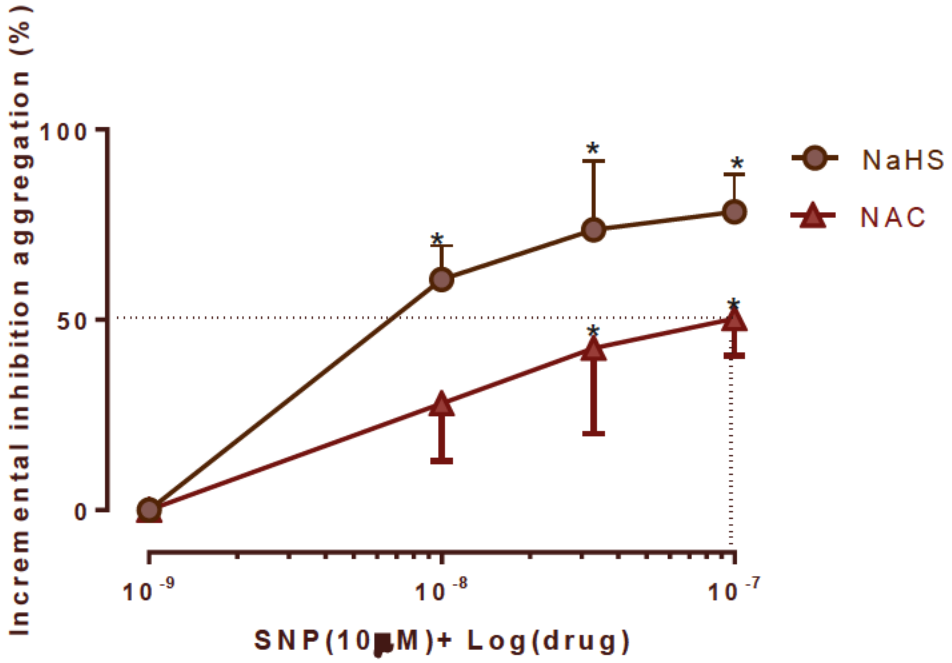



**Figure 6.2:** Concentration-response curves for potentiation of anti-aggregatory of SNP by various concentrations of NaHS (circles) and NAC (diamonds). Comparisons were limited to NAC/NaHS concentrations without intrinsic anti-aggregatory effects. Means were compared by One-way ANOVA followed by Dunnett Post Hoc t-tests taking SNP alone as a control. Data are presented as mean ±SEM; n=7; *p<0.05. NAC was pre-incubated for 15min, whereas NaHS for 1min. Because half of the $H_2S$ from NaHS can be degraded/lost in 5minutes from cell culture plates and in less than 3minutes from organ baths with turbulent oxygenation (Li Q and Lancaster JR, 2013), these short pre-incubation periods were chosen.

### *6.4.3 Preventing $H_2S$ formation limits NAC restoration of SNP signaling*

Inhibiting CSE by PAG (3.3mM) did not have any significant influence on SNP response alone (1.3±0.01% fall). However, there was a significant diminution of effects of SNP in the presence of NAC (from 33.6±4.6 % to 15.2±2.8%, p=0.03). On the other hand, CBS inhibition by AOAA (0.5mM) showed only a weak non-significant trend towards similar diminution from 33.6±4.6 % to 23.0±3.8% (p=0.34) (*Fig. 6.3*).

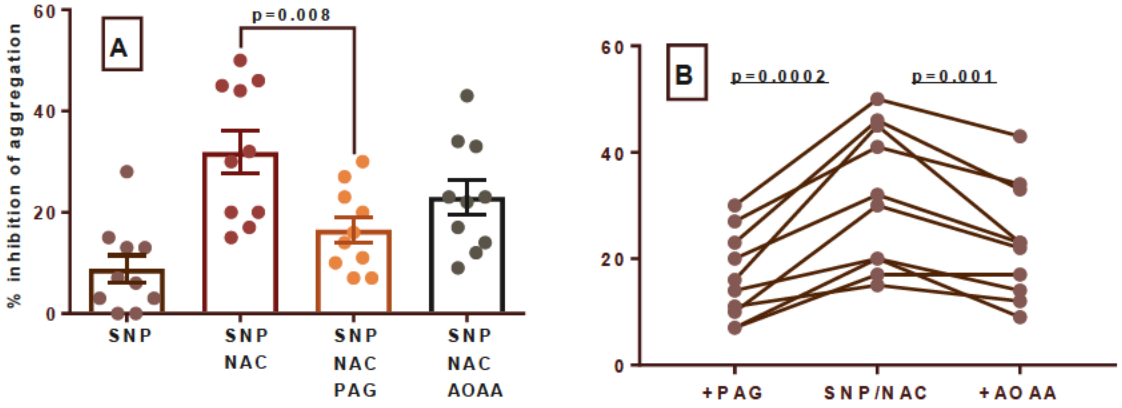



**Figure 6.3:** Impact of co-incubation with inhibitors of $H_2S$ formation [DL propargylglycine (PAG, 3.3mM) and amiooxyacetic acid (AOAA, 0.5mM)] on combined effects of SNP and NAC. Inhibitors were preincubated with blood for 15minutes. (A) Individual data points. Only PAG significantly reduced (p=0.008) the combination effects, of SNP/NAC. Results were compared using ANOVA followed by Dunnett Post Hoc test. (B) Specific paired analysis using Student t-test.

### 6.4.4 *$H_2S$ derived from NaHS potentiates SNP but exerts inconsistent interactions with Iloprost responses*

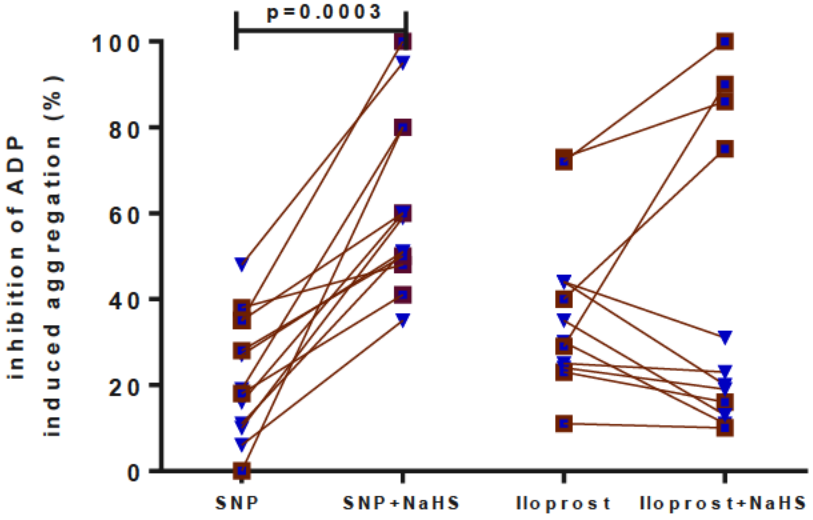



**Figure 6.4:** Effects of the $H_2S$ donor NaHS (100µM) on antiaggregatory efficacy of SNP and Iloprost. Data points shown: triangles (▼) = Patients with acute CAS/ACS and squares (□) =stable CAS. Results were compared using Student's paired t-test.

Recently, Bucci et al (2010) have shown in aortic rings that pretreatment with $H_2S$ increases SNP-stimulated accumulation of cGMP, apparently via nonspecific phosphodiesterase inhibition. In accordance with that we also found in our study that, incubating whole blood with the $H_2S$ donor NaHS (100µM) consistently increases SNP response from 27.7±4.1 to 63.2±6 %. This trend was applicable to all patient types regardless their clinical characteristics [total n=13: 7 patients with acute crises (2 ACS and 5 acute CAS) and 6 CAS patients at their chronic phase].

On the other hand, pretreatment with NaHS(100µM), while inducing no overall significant change, appeared to either decrease (n=8) or increase (n=4) of responses to Iloprost, with no significant overall change. Interestingly, all data points representing responses to Iloprost were diminished with blood samples from patients with acute crises, while those which increased were from patients with stable CAS (*Fig. 6.4*).

**6.5 *Discussion:***

We recently demonstrated (*Chapter 5*) that platelets from patients with CAS are hyporesponsive to antiaggregatory effects of both SNP and Iloprost, and during acute crises NTG/NAC infusion SNP responses return towards population norms. This is consistent with the previously documented potentiation by NAC of nitroglycerin (an organic nitrate) effects in inhibiting platelet aggregation (Loscalzo et al, 1985). The reason for this was not clear, but could involve physiological antagonism since, there was a minor fall in ADP responses following NTG/NAC infusion. However, this point was addressed and found not relevant in our in vitro study using NAC at its adjusted (intrinsically inactive) concentrations, yet it increased the anti-aggregatory response to SNP. Decreased oxidative stress by scavenging $H_2O_2$ and HOCl in the presence of NAC have been documented by many research groups (Auroma et al 1989; Gillissen A, 1997; Van Antwerpen et al 2005). Also, N-nitroso-N-acetylcysteine formation has been shown in mice receiving high dose NAC (Palmer et al 2007). More recently it has also emerged that NAC is deacetylated to form L-cysteine, which further undergoes $H_2S$ formation by enzymatic process (Chen et al 2004). Indeed, $H_2S$ formation from NAC was shown recently by Ezerina et al (2018).

In theory platelets can produce $H_2S$ from L-cysteine/cystine by enzymatic transulfuration pathways of cystathionine γ-lyase(CSE) and cystathionine β-synthase(CBS) (Loscalzo 2006; Kimura H 2011), but the extent of this conversion is not known largely because the presence of $H_2S$-activating enzymes in platelets remains poorly investigated. We evaluated in vitro potentiation of SNP effects, since the concept of mutual dependency between NO with $H_2S$ has emerged recently (Coletta et al 2012), by incubating with NAC in the presence of inhibitors of CSE and CBS. Expression of CSE enzyme has been shown in endothelial cells (Mistry et al 2016) and in vascular smooth muscle (Yang et al 2010). CSE knockout mice show diminished endothelium-dependent vascular relaxation (Yang et al 2008) which was ameliorated by NaHS treatment. Our results show that inhibiting CSE by PAG (3.3mM) did not have any significant influence on SNP response alone (1.3±0.01% fall). However, there was a significant fall in SNP/NAC interaction (from 36.6±4.1 % to 12.8±2.9%, $p<0.0001$). Though there was trend towards diminution of NAC effect by AOAA, which has been documented to inhibit both CSE and CBS (Asimakopoulou et al 2013), this did not reach a statistically significant level in

ANOVA test. We do not know the reason behind this, but we note the non-specificity for $H_2S$-forming enzymes and also cytotoxicity by interfering with the malate-aspartate shuttle in mitochondria (Chen et al 2015), and the GABA-receptor interaction (Carmona et al 1980) etc. Therefore, our result and results obtained from vascular assays by Yang et al (2008) clearly show that potentiation of SNP by NAC is mediated at least in part via de novo $H_2S$ formation. It should be noted that the effects of these two inhibitors of $H_2S$ formation, were not evaluated in the case of the NAC/Iloprost interaction.

However, the amount of $H_2S$ formation and also its interaction with antiaggregatory agents are unknown. Further that the products of CSE and CBS are not limited to the $H_2S$ but also include α-ketobutyrate, ammonia, homolanthionine are produced from L-cysteine (reviewed by Stipanuk, 2004). We therefore verified the concentration-response characteristics of NAC and the $H_2S$ donor NaHS in platelets from CAS. NaHS exhibited greater potentiation of the SNP response. Pretreatment of platelets with NaHS dose-dependently rendered them much more sensitive to inhibition of aggregation by SNP, far exceeding the potentiation seen by NAC. If the same principle were to apply in vascular response, then the combination of $H_2S$ with an NO donor would be a potential therapeutic approach in VA, diabetic peripheral vascular, and many other forms of vascular diseases.

The issue of an interaction between NAC/$H_2S$ and the activity of the $PGI_2$/AC system was not addressed by the current experiments. On the one hand NAC consistently (although to a small extent) potentiated responses to Iloprost (*Fig 6.1*). On the other hand, NaHS, a "pure" $H_2S$ donor, exerted no consistent effect on Iloprost responses, decreasing responses in in the majority of cases, but not all (*Fig 6.4*). The literature tells us that the interaction at the level of vascular smooth muscle and neuronal cells (Lim et al 2008; Nagpure et al 2014) between $H_2S$ and the AC system is dominated by $H_2S$-induced reduction in cAMP generation (Li et al 2015). This would theoretically result in inhibition of Iloprost effect. Therefore, overall, we have no adequate explanation for

(1) Iloprost potentiation by NAC
(2) Heterogeneity of response to NaHS.

Addressing this problem will require monitoring of cAMP generation, as well as of changes in redox stress.

Clinical considerations These data suggested that NAC may act largely as a pro-drug of $H_2S$ which potentiates NO signalling. These conclusions, if reinforced by additional data, raise the possibility that NaHS might represent a more predictable means of achieving rapid restoration of cardiovascular homeostasis in CAS.

# Chapter 7: Summary, significant contributions to the discipline and future directions

The overall purpose of the experiments undertaken in this thesis was to evaluate the relationships between impairment of function of platelet NO/sGC and PG/AC signaling in a wide range of patients, including those with acute and myocardial ischaemia, diabetes and coronary artery spasm. In particular, I focused on implications of PG/AC functionality on individual patient responses to $P2Y_{12}$ receptor antagonists, and on the potential role of platelet dysfunction in the pathophysiology of coronary artery spasm.

### 7.1 *The major results are as follows: -*

(1) We have confirmed that in vivo platelet anti-aggregatory responsiveness to ticagrelor, like that to clopidogrel, reflects integrity of AC signalling (*Chapter 3*). Additionally and excitingly, we have used the short-acting $P_2Y12$ antagonist 2MeS-AMP to mimic this scenario in vitro, a very novel concept.

The epidemiology of impairment of NO/sGC and PG/AC signaling was explored.

(2) ACS was associated with more than 2-fold impairment of SNP responses, but diabetes did not represent an obvious increment to this anomaly, similar results were obtained for $PGE_1$. However, forskolin responses were impaired only among diabetics (*Chapter 4*).

(3) Overall specificity assay shows, both SNP and $PGE_1$ resistance were disproportionately greater for patients with ACS (*Chapter 4*).

(4) Perhexiline therapy sensitises platelet adenylate cyclase to activation by $PGE_1$ in patients with diabetes and concurrent ischaemia (*Chapter 4*).

(5) Platelets from patients with CAS have markedly impaired responses to both NO/sGC and Iloprost/AC activation, and the former become more accentuated during symptomatic crises. Inflammatory activation involving glycocalyx shedding and probably mast cells degranulation were associated with acute exacerbation phases. NTG/NAC infusion effectively reverses those molecular anomalities (*Chapter 5*).

(6) Preventing $H_2S$ formation limits NAC restoration of SNP signalling. With equal concentrations, the $H_2S$ donor NaHS induces greater potentiation effects of SNP compared to those NAC. Interestingly, NAC *in vivo* increases responses to both Iloprost and SNP, whereas NaHS increases SNP response but tends to decreases Iloprost responses (*Chapter 6*).

### 7.2 *Significance/ Contribution to the discipline:*

Results obtained from Ticagrelor and 2MeS-AMP assay (*Chapter 3*): The findings of this work will contribute significant knowledge on the processes controlling individual patient responses to anti-aggregatory agents. The results may help to reduce the risk of stent thrombosis in patients with ACS.

Results obtained from coronary artery spasm patients (*Chapter 5 & 6*): These data suggested that NAC may act largely as a pro-drug of $H_2S$ which potentiates NO signalling. These conclusions, if reinforced by additional data, raise the possibility that NaHS might represent a more predictable means of achieving rapid restoration of cardiovascular homeostasis in CAS. The findings also strengthen the implication that crises of coronary spasm may be triggered by mast cell degranulation and resultant damage to the coronary endothelial glycocalyx. These results may form the basis of development of strategies to prevent crises in CAS patients.

### 7.3 *Future directions*

There are several priorities for future investigations. These can be summarized as follows: -

(1) First, we need to extend our findings of CAS in vascular work to demonstrate whether CAS patients have permanently disordered vascular cyclic nucleotide signalling.

Second it is important to investigate whether patients with coronary spasm have oxidised sGC, even without crisis status. It would also help if we knew the status of heme-conjugation of the enzyme in this circumstance. Investigations along these lines could be extended from coronary spasm to both acute infarction and uncontrolled diabetes. Similarly it would be important to determine whether NAC can reverse sGC oxidation in each of the proposed models (and also what are the concentration-response characteristics involved).

(2) These findings may also be relevant to contrast-induced renal damage, and also to the protective effects of NAC in other acute disease states associated with oxidative stress, such as ischaemic stroke and peripheral arterial occlusion.

(3) The findings regarding the role of $H_2S$ as a factor which reverses abnormalities in NO/sGC signalling are relatively novels, and should be explored more extensively. For example it, may be possible to increase $H_2S$ generation within tissues on a permanent basis, by stimulating one or more of the $H_2S$-generating enzyme systems, or by inhibiting $H_2S$ degradation. The priority for such investigations is now critical.

(4) The relative lack of specificity of disease-related impairment of sGC and AC-related anti-aggregatory effects and the extraordinary impact of ACS and CAS on these systems should inspire searches for commonalities of pathogenesis, and therefore potentially of amelioration. For example, if acute inflammatory activation inhibits activity of both sGC and AC via induction of redox stress, it is conversely possible that anti-inflammatory treatments such as ACE inhibitors (Willoughby et al 2012) and mineralocorticoid inhibitors rather than only perhexiline, will prove beneficial.

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