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Department
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Immunity and
Cardiovascular
Disease.

Assessing the Efficacy of Aspirin and Clopidogrel in Patients with Acute Coronary Syndromes

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Statement of Originality

I hereby declare that this submission is my own work and to the best of my knowledge, it contains no materials previously published or written by another person, except where due acknowledgement is made in the thesis. The material in this thesis has not previously been submitted for a degree to any other University. Any contribution made by others, with whom I have worked, is clearly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except that full assistance from my supervisor in the project's design, conception, execution, presentation and linguistic expression is acknowledged.

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ABBREVIATIONS

AA	Arachidonic acid
ACC	American College of Cardiology
ACE-I	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AHA	American Heart Association
ARB	Angiotensin receptor blockers
ARU	Aspirin reactive units
AS	Average size
AT	Antithrombin
ATP	Adenosine triphosphate
ATT	Antiplatelet trialists' collaboration
AUC	Area under the curve
BCSH	British Committee for Standards in Haematology
BMI	Body mass index
BMS	Bare metal stent
BT	Bleeding time
C/ADP-CT	Collagen /adenosine diphosphate -closure time
C/EPI-CT	Collagen epinephrine-closure time
Ca ²⁺	Calcium
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease

CADP	Collagen and adenosine diphosphate
cAMP	cyclic Adenosine monophosphate
CCB	Calcium channel blocker
CEP	Collagen and epinephrine
cGMP	cyclic Guanosine monophosphate
CLSI	Clinical and Laboratory Standards Institute
COX	Cyclooxygenase
CPA	Cone and Plate(let) Analyzer
CPTP	Cyclopentyltriazolopyrimidine
CRP	C-reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
CYP	Cytochrome P450
DES	Drug eluting stent
DTI	Direct thrombin inhibitor
DTS	Dense tubular system
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESC	European Society of cardiology
GP	Glycoprotein
GPI	Glycoprotein inhibitors
GT	Glanzmann thrombasthenia
HPR	High-on treatment platelet reactivity
HT	Hydroxy tryptamine

ICAM	Intracellular adhesion molecule
IL	Interleukin
IQ	Interquartile
ISTH	International Society of Thrombosis and Haemostasis
IV	Intravenous
LDL	Low density lipoprotein
LMWH	Low molecular weight heparin
LOE	Level of evidence
LPR	Low platelet reactivity
LTA	Light transmission aggregometry
MA	Maximum amplitude
MACE	Major Adverse Cardiovascular Events
MCP	Monocytes chemoattractant protein
MEA	Multiplate Electrode Aggregometry
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MNS	Methylene dioxy-beta-nitrostyrene
NSAIDs	Non-steroidal anti-inflammatory drugs
NSTEMI	Non-ST-elevation myocardial infarction
OCS	Open canalicular system
PAI	Plasminogen activator inhibitor
PAP-4	Platelet aggregator profiler-4 channels
PAR	Protease activated receptor
PAU	Platelet aggregation unit
PCI	Percutaneous Coronary Intervention

PDGF	Platelet-derived growth factor
PFA	Platelet function analyzer
PFT	Platelet function test
PGE	Prostaglandin E
PGHS	Prostaglandin G/H synthase
POCT	Point-of-care test
PON	Paraoxonase
PPACK	D-Phenyl-Alanyl-L-Prolyl-L-Arginine
PPI	Proton pump Inhibitors
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRU	P2Y ₁₂ reactivity units
PSGL	P-Selectin glycoprotein ligand
PTFE	Poly-tetra-fluoro-ethylene
PVD	Peripheral vascular disease
RANTES	Regulated on activation normal T cell expressed and secreted
RBC	Red blood cells
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
ROTEG	Rotational thromboelastography
ROTEM	Rotational thromboelastometry
SEM	Standard error of the mean
SPSS	Statistical Package for Social Sciences
STEMI	ST-elevation myocardial infarction

TEG	Thromboelastography
TFPI	Tissue factor pathway inhibitor
TGF- β	Transforming growth factor- β
TIA	Transient ischaemic attack
TIMI	Thrombolysis in Myocardial Infarction
TNF- α	Tumor necrosis factor- α
TRA	Thrombin receptor antagonist
TRAP	Thrombin receptor activating peptide
TSP	Thrombospondin
TX	Thromboxane
TXA ₂	Thromboxane A ₂
TXB ₂	Thromboxane B ₂
UA	Unstable angina
UFH	Unfractionated heparin
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
WBC	White blood cells
WBSPCA	Whole blood single platelet counting assay

Chapter 1: Introduction

1.1 Introduction

The development of thrombus is a complex and dynamic process. This involves platelet aggregation and generation of thrombus. Platelet aggregates are strengthened by a network of fibrin during the formation of thrombus. Thrombus predominantly forms at the site of plaque rupture or erosion in diseased atherosclerotic arteries. This process is further compounded by inflammatory response at the site of plaque disruption and thrombus formation. This leads to various clinical presentations depending on the affected arterial bed such as acute coronary syndrome (ACS, consisting of unstable angina (UA), ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI)), acute cerebrovascular events, such as ischaemic stroke and transient ischaemic attacks (TIA), and occlusive peripheral vascular disease (PVD). These presentations of atherothrombosis are major causes of death and disability worldwide(1, 2).

1.2 Platelets and Atherothrombosis

1.2.1 History of Platelets

William Osler was probably the first to notice platelets and described them in his 1873 and 1874 papers(3). However the first accurate description of platelets was given by Max Schultz, who also recommended further studies of them(4). Subsequently Giulio Bizzozero, in 1881 and 1882, identified the platelets anatomically. He called them “blood plates” and was the first to describe the adherence and aggregation of platelets at the site of vessel wall injury; he also identified bone marrow megakaryocytes but the discovery that megakaryocytes are precursors of platelets was made by Wright in 1906(5).

1.2.2 Structure of Platelets

Platelets are small a nuclear cellular component of the blood that are released from the megakaryocytes. They measure 2.0 to 5.0 μm in diameter, 0.5 μm in thickness and have a mean cell volume of 6 to 10 femtoliters. One giant megakaryocyte produces over 1000 platelets upon fragmentation. The lifespan of each platelet is between 7-10 days. Platelet structure can be broadly divided into peripheral zone, sol-gel zone, and organelle zone and platelet membrane system (6-9). The structure of platelets is presented in figure 1.1

Figure 1.1: Structure of Platelets

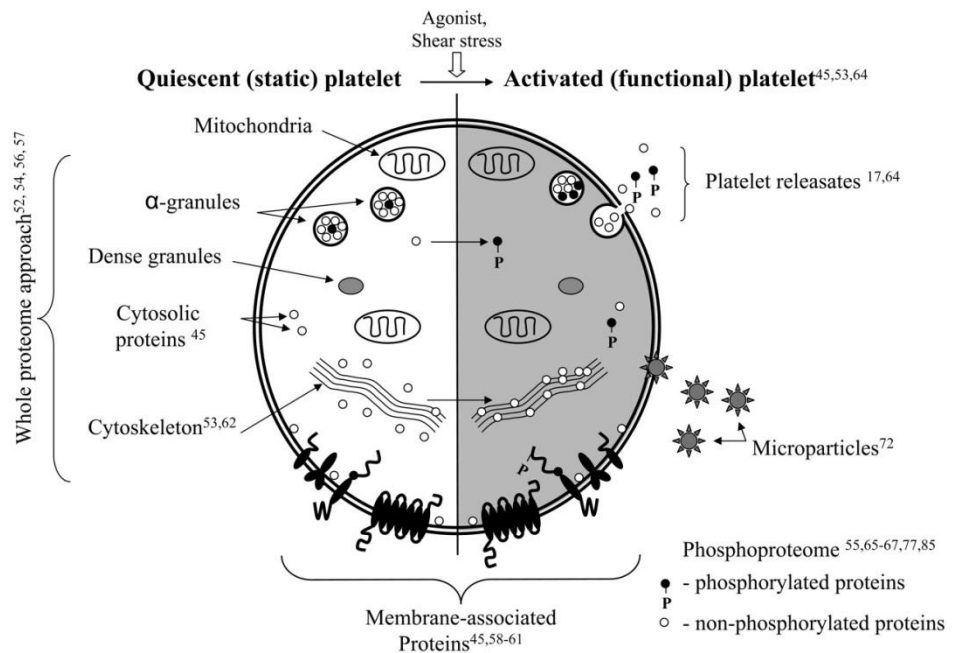


Figure 1.1

Schema of platelet ultrastructure integrated with proteomic studies.

The unshaded panel delineates the proteomic studies involving quiescent platelets. The shaded panel represents those studies focusing on activation-dependent platelet end points (eg, microparticles, exosome/releasates) (10). (Reproduced from Dmitri V. Gnatenko, Journal of Blood 2006, DOI 10.1182/blood-2006-06-026518, Copyright @ 2006 by The American Society of Haematology)

The peripheral zone contains a thick exterior coat or glycocalyx, a lipid bilayer upon which the glycocalyx rests and a submembranous area. The glycocalyx is a dynamic structure which is constantly in contact with the other blood components and hence has major and minor glycoprotein receptors necessary for platelet adhesion, aggregation and clot retraction. The lipid bilayer is a typical unit membrane that cannot stretch and has an open canalicular system (OCS) that provides increased surface area to it. The submembranous area has actin filaments that play an important role in shape change and translocation of receptors and particles over the exterior surface of the cell (11).

The sol–gel zone is the main matrix of the platelet. It is made of a dense mat of fibrous elements that supports the platelet discoid shape and internal contraction. As this resembles a liquid gel, it has been renamed as the sol–gel zone from hyaloplasm. The main components of the sol-gel zone are circumferential coil microtubule, actomyosin filament system, platelet glycosome and smooth and coated vesicles. The microtubule acts as a cytoskeletal support system whereas the actomyosin filament is involved in shape change (7).

The organelle zone contains secretory structures, namely α granule, dense bodies (δ granules) and lysosomes. The main secretions from these structures are discussed below (12, 13). They also have mitochondria in small numbers that play a significant role in the energy metabolism of the cell, and other membrane-enclosed organelles, namely glycosome, electron-dense chains and clusters, and tubular inclusions (7).

The platelet membrane system contains Golgi zones, surface-connected OCS and the dense tubular system (DTS). The Golgi zone is normally confined to the megakaryocytes and is found only in 1 out of every 100 to 500 platelets. The OCS and DTS are anatomically situated in the peripheral zone (14, 15).

1.2.3. Platelet Secretions

When platelets are activated, they release several granule components. The dense bodies are the main storage pool of platelets and release adenosine diphosphate (ADP) and serotonin upon activation. ADP, which is a prominent amplifier of initial platelet activation, acts on two main ADP receptors namely P2Y₁ and P2Y₁₂ situated on the platelet surface.

Table 1.1: Major Secretions from the α - Granules

Large adhesive proteins	von Willebrand factor (vWF) Thrombospondin-1 (TSP 1) Vitronectin Fibronectin
Mitogenic factors	Platelet-derived growth factor (PDGF) Vascular endothelial growth factor (VEGF) Transforming growth factor β (TGF- β)
Coagulation factors	Factors V, VII, XI, and XIII
Protease inhibitors	Protein C Platelet plasminogen inhibitor -1 (PAI-1) Tissue factor pathway inhibitor (TFPI)
Others	P- selectin Platelet factor 4

P – Selectin (CD62P), a glycoprotein, is localized on α - granule membrane of resting platelets. This, along with other glycoproteins including sCD40L, mediates platelet binding to neutrophils and monocytes. Platelet factor 4, an α -granule protein, is synthesized by megakaryocytes (16).

Serotonin (5-hydroxytryptamine, 5-HT), a strong vasoconstrictor, binds to the Gq-coupled 5HT_{2A}-receptors. The DTS also contains a calcium (Ca²⁺) pool which is mobilized during platelet activation. Ca²⁺ fluxes are central triggers in platelet activation, secretion and aggregation(13, 16). The major secretions from the α-granule are summarized in table 1.1. Platelet lysosomes liberate clearing factors that facilitate the digestion and resolution of thrombi (17). A schematic representation of platelet secretion is depicted in figure 1.2.

Figure 1.2: Platelet Secretions

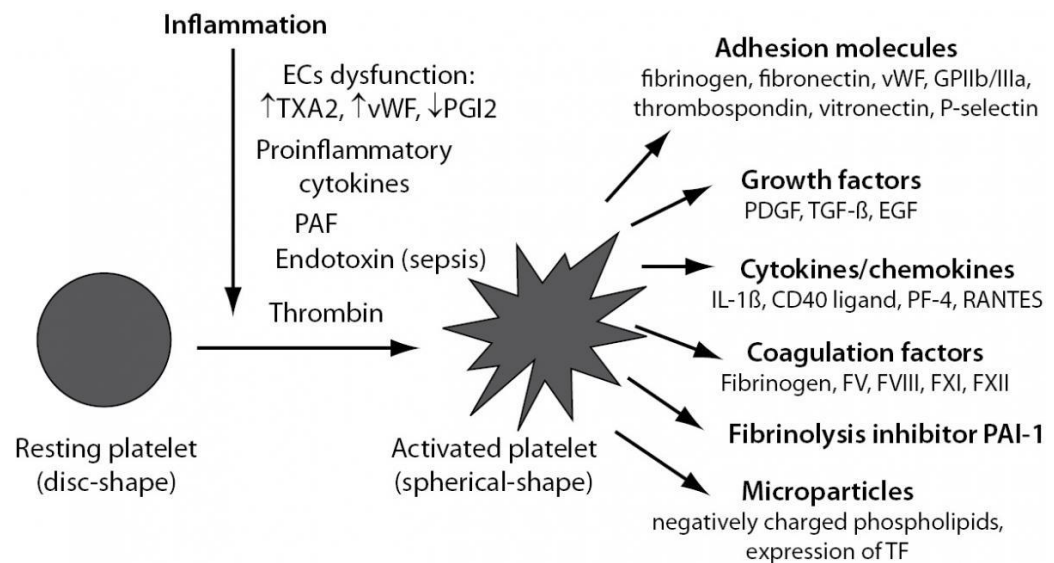


Figure 1.2:

Platelet activation induced by inflammation. EC – endothelial cell; TXA2 – thromboxane A2; vWF – von Willebrand factor; PGI2 – prostacyclin; PAF – platelet activating factor; GPIIb/IIIa – glycoprotein complex IIb/IIIa; PDGF – platelet derived growth factor; TGF-b - transforming growth factor-beta; EGF – epidermal growth factor; IL-1b - interleukin 1beta; PF-4 – platelet factor 4; RANTES – CCL5 chemokine; PAI-1 – plasminogen activator inhibitor 1; TF – tissue factor (Figure taken from Sandra Margetic. Inflammation and haemostasis. Biochemia Medica 2012; 22 (1):49-62. <http://dx.doi.org/10.11613/BM.2012.006>)

1.2.4. Platelet Receptors

A number of major platelet receptors have been described and detailed discussion of all of them is beyond the scope of this review. Important receptors relevant to my study are briefly described below.

1.2.4.1. Glycoprotein (GP) Ib-IX-V Complex

The GP Ib-IX-V complex is a pivotal platelet receptor in initiating platelet attachment to injured vessel wall and thus plays an important role in both haemostasis and thrombosis. This is the main receptor for vWF and deficiency of this receptor causes Bernard Soulier disease, first described by Dr. Jean Bernard and Jean Pierre Soulier in 1948 (18). GP Ib-IX-V is also a major receptor for TSP, the leukocyte integrin $\alpha M\beta 2$, P-selectin, and the platelet integrins $\alpha IIb\beta 3$ and $\alpha 2\beta 1$. It is essential for platelet adhesion under high shear conditions. In addition, it plays a key role in promoting procoagulant activity, controls platelet size and shape and regulates clearance of platelets from the circulation (19).

1.2.4.2. Integrins

Integrins are a major class of adhesive and signaling molecules that contains heterodimers of α and β subunits and are generally involved in linking adhesive molecules to the cellular cytoskeleton. Platelets have members of three families of integrins ($\beta 1$, $\beta 2$, $\beta 3$) and in total six different integrins $\alpha 2\beta 1$, $\alpha 1\beta 5$, $\alpha 6\beta$, $\alpha_L\beta 2$, $\alpha IIb\beta 3$, and $\alpha_V\beta 3$. $\alpha IIb\beta 3$, (GP IIb/IIIa) is the major platelet integrin that is expressed uniquely on platelets. Deficiency of the GP IIb/IIIa complex leads to Glanzmann thrombasthenia, originally described by Eduard Glanzmann, a Swiss pediatrician (20-23). The platelet receptors are shown in figure 1.3.

Figure 1.3: Platelet Receptors

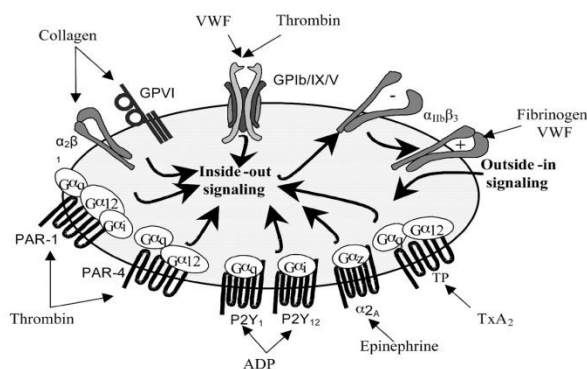


Figure 1.3 : Platelet receptors and its interactions with major ligands (24). Reproduced from *Haematologica*, Journal of The European Haematology Association May 2009 94: 700-711; DOI 3324/haematol.2008.003178 Jose Rivera, Maria Luisa Lozano, Leyre Navarro-Nunez, Vicent.

1.2.4.3. Thrombin Receptors

Protease-activated receptors (PARs) are the seven transmembrane receptors that play dominant roles in the action of proteases such as thrombin. 4 PARs have been identified, namely PAR1, PAR2, PAR3 and PAR4. PAR1 and PAR4 are present on human platelets. PAR3 is present only in mouse but not in human platelets. PARs are activated by a tethered-ligand mechanism where a receptor carries its own ligand that is silent until the receptor is cleaved. In the case of PAR1 and PAR4, thrombin acts as an agonist to the receptors. When these receptors get exposed to thrombin, a new amino terminus is revealed due to cleavage within the extracytoplasmic portion of the PAR. This ultimately causes transmembrane signaling through intramolecular ligation. PAR4 needs 10 times higher concentrations of thrombin to be stimulated than that needed for PAR1. PAR2 is cleaved and activated by trypsin but not by thrombin. The selective PAR1 antagonist vorapaxer has shown efficacy in patients with a history of atherosclerotic disease, albeit at the expense of increased major bleeding, including intracranial haemorrhage (25-30).

1.2.4.4. Platelet Purinergic Receptors

The platelet purinergic receptors that are recognized to have functional roles are of three subtypes, namely P2X₁, P2Y₁, and P2Y₁₂. P2Y₁ and P2Y₁₂ are ADP receptors, ADP being the primary platelet agonist that is present in abundance in the platelet dense granules. P2X₁ is an ATP receptor and structurally belongs to a different class of receptors. P2Y₁ and P2Y₁₂ are seven transmembrane G-protein-coupled receptors. P2Y₁ is linked to G_q protein. ADP stimulation of this receptor results in calcium mobilization, platelet shape change, and rapidly reversible platelet aggregation. On the other hand, P2Y₁₂ receptor is linked to an inhibitory G protein (G_i) and, when stimulated by ADP, results in amplification of platelet aggregation and secretion and consequent stabilization of platelet aggregates (31-33).

1.2.5. Platelet Functions

Platelets play a key role in protective haemostasis and pathogenic thrombosis. Furthermore, they are mediators of inflammation, have immunomodulatory activity, and are involved in wound healing and haematological malignancies. Platelets are recruited at the site of vessel damage to form a haemostatic plug and at the site of ruptured atherosclerotic plaque to form thrombus. Both these processes mainly involve platelet adhesion and aggregation mediated by several agonists that interact with the platelet surface receptors. Platelets thus link inflammation, thrombosis and atherogenesis by interacting with endothelial cells and leukocytes (34).

1.2.5.1. Role of Platelets in Haemostasis

Damaged vessel wall exposes subendothelial matrix. Adhesion of platelets at this site is the initial step in the formation of haemostatic plug. The adhesion of platelets to the subendothelial matrix happens in two naturally existing physiological environments, namely high shear conditions as found in arteries and low shear conditions as present in

veins. The former is mediated mainly by several platelet surface receptors that bind to collagen either directly through GPVI and the integrin $\alpha_2\beta_1$ or indirectly with the GPIb/V/IX complex and the integrin $\alpha_{IIb}\beta_3$ via vWF (16,28,34,35). This leads to the formation of a platelet monolayer attached to each other by collagen on the subendothelial matrix. Activated platelets are involved in a series of further complex dynamic processes. These include: a) Shape change contributed to by the formation of pseudopodia originating from the plasma membrane due to the change in the configuration of the cytoskeletal proteins (36), b) secretion of various granular components as discussed above (34), and c) Rolling of platelets on the endothelium and their binding to neutrophils and monocytes facilitated by endothelial P-selectin. These lead to accumulation of activated platelets on the already formed monolayer of platelets and collagen (34).

A haemostatic plug caused by accumulation of platelets is the hallmark of platelet aggregation. The key receptor in this process is GP IIb/IIIa which links activated platelets through fibrinogen bridges. The GP IIb/IIIa receptors are inactive in a resting platelet. However, when platelets are activated this leads to augmentation and activation of surface exposed GP IIb/IIIa molecules via α -granule exocytosis. Activated GP IIb/IIIa complexes interact with ligands leading to molecular conformational changes that end in firm connection and high affinity binding sites for fibrinogen. Thus, a primary haemostatic plug is formed. This process is commonly called inside-out signaling (28, 37).

The haemostatic plug is further strengthened by the mechanisms called outside-in signaling mediated by integrins. In this process direct interaction between platelets occurs, ultimately leading to stabilization of the platelet plug that prevents premature disaggregation of platelets (28, 38).

1.2.5.2. Role of Platelets in Thrombus Formation

Platelets play an extended role in converting the protective haemostatic plug into a pathological occlusive platelet rich thrombus. Various factors play a key role in this process. In addition to platelet shape change, platelet secretions, activation of GP IIb/IIIa receptors mentioned above that are important in the formation of haemostatic plug, expression of proinflammatory molecules such as P-selectin and CD40 ligand (CD40L) and expression of platelet procoagulant activity are important in the stabilization of the growing haemostatic plug and progression to pathological thrombus. Fibrin generated from fibrinogen that is cleaved by thrombin is also important in the formation and consolidation of the haemostatic plug (39, 40).

1.2.5.3. Role of Platelets in Coagulation

Activated platelets have an ability to support the coagulation process. Coagulation factors bind to the platelets either by their own glycoprotein receptors or indirectly to the platelet membrane facilitated by aminophospholipid exposure on the surface of activated platelets. The interaction between collagen and platelet GPVI plays a crucial role in the above-mentioned process and also in the formation of membrane blebs that provides procoagulant microvesicles.

Intracellular increase in calcium is important for bleb formation and exposure of aminophospholipids such as phosphatidylserine. The increase in intracellular calcium is, in turn, caused by activation of platelet receptors for ADP, thromboxane A_2 (TXA₂), thrombin and collagen. Shedding of membrane blebs into the circulation initiated by the collagen - GPVI interaction and the interaction of ADP with P2Y₁ and P2Y₁₂ supports this platelet procoagulant activity. Activated platelets also play an extended role in coagulation by secreting factor V, factor VIII and fibrinogen and by providing binding sites for factor XI and prothrombin (33, 34, 41, 42).

1.2.5.4. Role of Platelets in Inflammation

Platelets in general do not have any effect on the intact and inactive endothelium. Platelets adhere to the inflamed endothelium in a similar fashion to damaged endothelium by a multi-step process that involves tethering, rolling and firm adhesion (16, 43). Platelet tethering is mediated by P-selectin and E-selectin on the endothelial cells, whereas platelet rolling is caused by the interaction of GPIb and P-selectin glycoprotein ligand-1 (PSGL-1). Both these processes are reversible. The GP IIb/IIIa receptor along with fibrinogen plays a vital role in the firm adhesion of platelets to the inflamed endothelium which is an irreversible process (34,44,45).

CD40L present on the activated platelets causes the endothelium to produce reactive oxygen species (ROS), adhesion molecules, chemokines and tissue factor. The ROS may have a role in recruiting more platelets to a growing thrombus. The interaction between CD40 on the endothelium and the CD40L on the platelet surface results in the recruitment of neutrophils and monocytes. This mediates the release of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1). Ligation of CD40L also results in release of matrix metalloproteinase (MMP)-2 which is a platelet stimulator and MMP-9 that has an inhibitory role on platelets (39). The role of platelets in inflammation is shown in figure 1.4.

Activated platelets secrete various inflammatory factors that augment inflammation and thrombosis. These factors include growth factors, chemokines, cytokines and coagulation factors. Regulated on activation, normal T-cell expressed and secreted (RANTES), secreted by the activated platelet, causes monocytes to arrest, either on its own or in conjunction with platelet factor 4 (47). Thus platelets play an important role in attracting leukocytes including monocytes and neutrophils at the site of endothelial inflammation. Platelet P-selectin is vital for this recruitment. The end result of these events is the infiltration of inflammatory cells into the vessel wall which is critical in atherosclerosis (48).

Figure 1.4: Role of Platelets in Inflammation

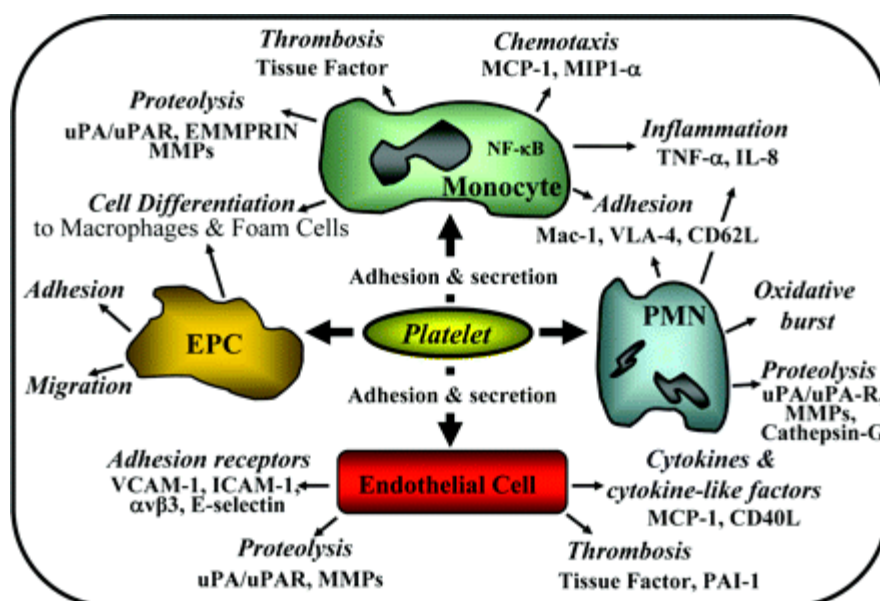


Figure 1.4:

Platelets inflame their cellular interaction partners. Platelets can induce a variety of inflammatory responses in monocytes, neutrophils (PMN), endothelial cells, or endothelial progenitor cells (EPC), resulting in key inflammatory processes, such as adhesion, chemotaxis, migration, proteolysis, thrombosis, or even cell differentiation to macrophages or foam cells. These processes provide an atherogenic milieu at the vascular wall that supports plaque formation (46) . Reproduced from the Journal of Arteriosclerosis, Thrombosis, and Vascular Biology published 3 Jan 2008; 28; s5-s10. Andreas E.May, Peter Seizer and Meinard Gawaz.

1.3. Process of Atherothrombosis

Atherogenesis, the development of atheromatous plaques, evolves through various phases, including the initial phase of atherosclerosis that ultimately leads to the formation of occlusive thrombus called atherothrombosis (1, 49).

Atherosclerosis is fundamentally a disease involving the intima of the muscular and elastic arteries that are lined by endothelial cells. These

cells, under normal physiological conditions, provide a nonthrombogenic surface that acts as an effective permeability barrier (50). Many factors can damage the endothelium including oxidized lipoproteins, smoke, glycosylation products in diabetes mellitus, infection by Chlamydia or viruses and elevated shear stress. When the endothelium is damaged, a series of changes takes place that results in intimal proliferative lesions of atherosclerosis (50, 51).

The initial step is an inflammatory process that is mediated by proinflammatory cytokines that have both autocrine and paracrine actions. Arfors et al in 1987 first demonstrated that gradual recruitment of white blood cells is the primary event in the inflammatory atheromatous sequence (52). Due to the high velocity of the red blood cells (RBCs) compared with the white blood cells (WBCs) in the vessel lumen, the RBCs tend to move to the centre of the vessel lumen whereas the WBCs are naturally pushed towards the periphery of the vessel lumen.

This enables the leucocytes to be in contact with the endothelial cells. Under pathological conditions, further contact of the leucocytes with the vessel wall endothelium is facilitated by L-selectin, E-selectin, and integrins ($\beta 2$ - $\beta 3$ and $\alpha 4$). IL-8, MCP-1 and RANTES make the leucocytes roll over the endothelium at a very slow speed. Also, activation of integrins and intracellular adhesion molecules (ICAM)-1 results in synthesis of various chemokines by the inflammatory cells that not only causes adhesion of leucocytes to the endothelium but also results in shape change of the leucocytes enabling increased surface area contact of the leucocytes with the endothelium. The process of atherothrombosis is shown in figure 1.5.

Figure 1.5: Role of Platelets in Thrombus Formation

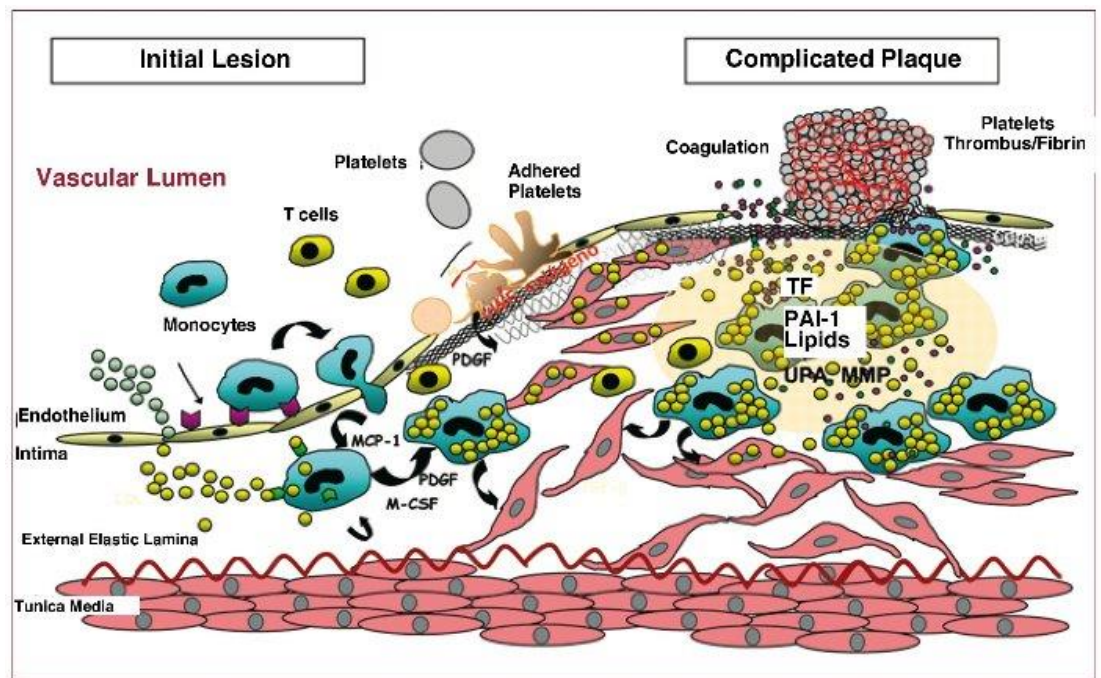


Figure 1.5:

Schematic representation of the progression of atherosclerotic plaque from initial stages of endothelial dysfunction to advanced stages with the presence of complicated plaques. M-CSF indicates macrophage colony stimulating factor; MCP-1, monocyte chemotactic protein 1; MMP, metalloproteinases; PAI-1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; TF, tissue factor; UPA, urokinase plasminogen activator (53). Reproduced from *Rev Esp Cardiol.* 2009; 62 (10); 1161-78-vol.62 Num.10 DOI; 10.1016/S1885-5857 (09)73331-6.

5-Hydroxytryptamine, histamine, bradykinin, prostaglandins and nitric oxide along with proinflammatory cytokines and growth factors increase the permeability of the endothelium, causing extravasation of the leucocytes into the subendothelial matrix. The differentiation of the monocytes into macrophages and subsequent loading with oxidized low-density lipoprotein (LDL) results in the formation of foam cells, a hallmark of atherosclerosis. Fatty streaks, the first macroscopic appearance of atherosclerosis, are due to foam cells (51, 54-56).

The next step involves activation and proliferation of smooth muscle cells. This is facilitated by the growth factors. Accumulation and concentration of more inflammatory cells leads to the formation of atheromatous plaque, the typical feature of advanced atherosclerosis. The fibrous plaque consists of a thin fibrous cap of proliferated smooth muscle cells surrounded by relatively dense connective tissues that separates the contents of fibrous plaque from the vessel lumen and hence prevents its contact with the platelets. This cap covers a massive deeper fatty nucleus with atherothrombotic properties consisting of smooth muscle cells and macrophages, many of which contain large accumulation of lipids. Such atheromatous plaques may regress, remain dormant or advance to the most advanced lesions that consist of necrotic debris, cholesterol crystals and deposits of free lipid in the deeper recesses of the plaques (57, 58). When macrophages get activated they release metalloproteinase, a proteolytic enzyme. Thinned and disintegrated fibrous cap gets ruptured by the action of metalloproteinase which is the preliminary step in the formation of occlusive thrombus. Apart from the process of rupture and formation of occlusive thrombus, the atheromatous plaque can also become calcified, ulcerate or bleed (1, 58).

Upon rupture of the atherosclerotic plaque, micro lesions of the vascular lining, thrombogenic matrix and platelets come into contact. This leads to platelet adhesion, activation and aggregation leading to thrombus formation and micro embolization. The thrombi impede blood flow and oxygen supply in the affected arteries resulting in clinical manifestations of atherothrombotic disease (59, 60).

1.3.1 Clinical Implications of Atherosclerosis and Thrombosis

Once atherosclerosis develops, stenosis may occur in the lumens of various arteries of the living body. The spectrum of clinical manifestations depends on the arteries affected and the extent of the luminal obstruction. The three major manifestations of this spectrum include coronary artery disease (CAD), cardiovascular disease (CVD) and PVD. In addition, the

aorta and other systemic blood vessels like the mesenteric arteries can also be involved. The spectrum of CAD includes UA, NSTEMI and STEMI, all three collectively called ACS. Similarly, the carotid and the vertebrobasilar systems can be involved in compromise to the cerebrovasculature causing TIA and stroke. The clinical manifestations of PVD includes intermittent claudication, non-healing ulcerations and infection of the extremities, and impotence (59).

1.4 Antithrombotic Medications

A thrombus consists of both fibrin and platelets. Hence antithrombotic strategies involve anticoagulants and anti-platelets alone or in combination. The European Society of Cardiology (ESC) practice guidelines recommend antithrombotic therapy as a cornerstone of ACS management (61, 62). Despite the abundance of antithrombotic therapies, ACS is tempered by the frequent recurrences of ischaemic events and by the bleeding complications inherent to these therapies (63).

1.5 Antiplatelet Medications

Thrombus formation at the site of atherosclerotic plaque rupture involves three steps, namely platelet adhesion, activation and aggregation. Each of these processes can be potentially targeted for pharmacological treatment to inhibit thrombus formation. Inhibitors of platelet adhesion are still in the investigational stage and are not currently available for clinical use. GP IIb/IIIa inhibitors that inhibit platelet aggregation are reserved for acute phase treatment. Platelet activation inhibitors namely COX -1 inhibitor and P2Y₁₂ antagonists are currently licensed for clinical use. Newer agents, particularly prasugrel has gained acceptance for use in ACS during percutaneous coronary intervention (PCI) in STEMI and ticagrelor has been adopted for use across the ACS spectrum (64, 65).

1.6. Aspirin

Aspirin is derived from acetyl and spirasaure (German word for salicylic acid). A French chemist Charles Frederic Gerhardt first discovered aspirin, but Felix Hoffman first synthesized it in 1897 at the Bayer Pharmacological Research Laboratories. Originally used for its analgesic and antipyretic effect, the effect of aspirin on haemostasis was discovered by Sir John Vane in 1971. The field of antiplatelet therapy was dominated by the monopoly of aspirin for nearly 50 years until the introduction of P2Y₁₂ inhibitors (66).

1.6.1. Mechanism of Action and Pharmacodynamics

TXA₂ is both a potent platelet agonist and a vasoconstrictor. It is produced from prostaglandin, which itself is derived from arachidonic acid. The conversion is catalyzed by COX enzyme that has two isoenzymes namely COX-1 and COX-2. Aspirin permanently inhibits both the COX isoenzymes. However, the antiplatelet properties of aspirin are due to the permanent acetylation of the serine residue (Ser-529) in COX-1 present in platelets. The anti-inflammatory properties are due to its action on COX-2 (67). The action of aspirin on COX-1 is 166-fold higher than its effect on COX-2, which explains why a lower dose of aspirin is sufficient to achieve antiplatelet activity whereas a higher dose is required for anti-inflammatory properties. Aspirin also causes a change in fibrin clot structure by acetylation of fibrinogen (64,68,69). Other mechanisms of action of aspirin include modulation of thrombin generation and inhibition of coagulation factor XIII activation (69).

Aspirin is administered orally in two formulations, namely dispersible and enteric-coated. The bioavailability of dispersible preparations is superior to enteric-coated. Aspirin is predominantly metabolized by the liver and intestinal human carboxyesterase -2 into acetyl and salicylate moieties. It has a half-life of 15 to 20 minutes and peak plasma levels are reached in 40 minutes. The action of aspirin lasts the lifespan of platelets (70).

1.6.2. Aspirin and Primary Prevention

Primary prevention aims to prevent disease or injury before it occurs. This is achieved by avoiding exposure to stimulus that causes disease or injury, making necessary changes in behaviour and eating habits that can lead to disease or injury and increasing the resistance to disease or injury should the exposure occur. In this context, aspirin has long been used for its antithrombotic properties to prevent cardiovascular events in patients with no previous history of cardiovascular disease.

Results from three meta-analyses have consistently shown that aspirin reduces the risk of cardiovascular events by 10% to 12% (table 1.2). This benefit is largely due to reduction in the incidence of non-fatal MI, but not fatal MI, stroke, cardiovascular death, or all-cause mortality. It is also well demonstrated that aspirin significantly increases the incidence of bleeding. Based on these findings, the cardiovascular benefit of aspirin must be weighed against bleeding risk when considered for use in primary prevention.

Table 1.2

Parameters	ATT-2009 (71)	Seshasai –2012 (72)	USPSTF-2015 (73)
No of studies	6	9	10
RR for CV events	12%	10%	11%
Reduction in non-fatal MI	0.18% for Aspirin Vs.0.23% for Placebo P<0.001	RR = 0.80, CI = 0.67-0.96	RR =0.80, CI =0.72-0.88
Reduction in overall mortality	0.19% for Aspirin Vs. 0.19% for placebo P=0.7	OR =0.93, CI =0.84-10.03	No significant reduction in all-cause mortality
Bleeding events	0.10% for Aspirin Vs. 0.07 % for placebo P =0.7	OR 1.70, CI 1.17-2.46	Significant increase in bleeding events

ATT = Antithrombotic Trialists' Collaboration

USPSTF = U.S. Preventive Service Task Force

1.6.3. Aspirin in Secondary Prevention:

Secondary prevention aims to reduce the impact of a disease or injury that has already occurred. This is done by detecting and treating disease or injury as soon as possible to halt or slow its progression, encouraging personal strategies to prevent reinjury or recurrence and implementing programs for people to return to their original health and function in order to prevent long term problems. Unlike the controversy in the use of aspirin in primary prevention, the benefits of aspirin in secondary prevention are well established. Compelling data from hundreds of clinical trials have clearly proved that low-dose aspirin is effective in reducing vascular events (MI, stroke, and vascular death) in patients who have experienced an MI or a stroke, or who are high risk of vascular disease by Framingham risk score. The absolute risk reduction for treatment over 2 years is 36 ± 5 per 1,000 in patients who have had an MI, 36 ± 6 per 1,000 in patients who have had a stroke or transient ischaemic attack, and 22 ± 3 per 1,000 in other high-risk patients. Doses below 162 mg have shown to be beneficial in reducing bleeding events without increasing ischaemic vascular complications (74). Given this information, the American College of Cardiology/American Heart Association (ACC/AHA) guidelines include a level A, class I recommendation for the use of daily aspirin (75–162 mg) in both men and women with known coronary heart disease or atherosclerotic vascular disease (75).

1.6.4. Limitations of Aspirin

1.6.4.1. Aspirin Resistance

The terminology of “aspirin resistance” lacks clear and consensus definition. If at all any exists, it can be classified in to clinical presentation, more appropriately referred to as “treatment failure”, and laboratory phenomenon. The occurrence of ischaemic CV-events whilst on aspirin is termed treatment failure, and the failure of aspirin to inhibit platelet aggregation or platelet TXA₂ release, as proven by a relevant laboratory test, is called laboratory resistance (76-79).

1.6.4.2. Treatment Failure

The prevalence of treatment failure on aspirin is 5% to 45% depending on the risk of the treated population. Laboratory resistance to aspirin is one of the reasons for treatment failure, but the latter could be due to several other clinical reasons. They include poor compliance, use of enteric-coated preparations with low bioavailability compared with dispersible form, concomitant use of NSAIDs which compete with the aspirin for the COX-1 enzyme, diabetes mellitus, raised body mass index (BMI), acute inflammation or infection associated with high C-reactive protein (CRP) levels, smoking and hypertension (76, 78).

1.6.4.3. Laboratory Resistance

Laboratory resistance to aspirin has been defined as $\geq 20\%$ platelet aggregation when using 0.5 to 1.6mg/mL arachidonic acid (AA) as the agonist. The prevalence of aspirin resistance varies between 5 % and 60% depending upon the platelet function test that was used for assessment. The lowest prevalence was found when platelet aggregation is assessed by light transmission aggregometry (LTA) following addition of AA (1-4%) and highest for PFA-100 test (60%) (76, 80). The phenomenon of laboratory resistance can be a dynamic process in an individual, showing poor response to aspirin during the acute phase as compared with findings at a later stable phase.

Again, there may be many reasons why this laboratory finding might have significant clinical implications. Amongst the most disputed ones are increased platelet turnover, genetic pleomorphism to COX-1 or platelet hyper reactivity (81-83). Other possible reasons for aspirin resistance that lacks clinical evidence are increased platelet stimulation with other non-AA-dependent mechanisms like adenosine diphosphate (ADP), collagen and epinephrine.

Non-standardized use of assays and the absence of a formal definition explain the wide range in the prevalence of aspirin resistance. LTA with AA as the agonist typically assess the COX pathway and hence this

method has the lowest prevalence of aspirin resistance. Similarly, AA induced whole blood aggregometry and VerifyNow Aspirin assay are more sensitive in detecting aspirin inhibition and demonstrate lower proportions of aspirin resistance. PFA-100 that is a non-specific PFT shows higher prevalence of aspirin resistance. None of the available PFT either correlates or agrees with the gold standard which is the LTA (84). Hence the reasons for the wide difference in aspirin resistance could be multifactorial including dosage of aspirin, method of defining resistance, type and concentration of the agonist used, baseline platelet reactivity, and the type of population selected (stable or unstable CAD, post CABG, post PCI etc.). In one study, family history of CAD, previous history of MI, history of PCI, were found to be strong predictors of aspirin resistance (85).

1.7. P2Y₁₂ Receptor Antagonists

The P2Y₁₂ receptor plays a key role in platelet activation, aggregation and granule secretion. It is also involved in the amplification of procoagulant and pro-inflammatory responses. Hence, it has become one of the major targets for antiplatelet medications. Thienopyridines (ticlopidine, clopidogrel, prasugrel) were the first P2Y₁₂ receptor antagonists to be developed. Clopidogrel, prasugrel and ticagrelor are the main oral drugs in this category that are in current clinical practice.

1.8. Clopidogrel

1.8.1. Mechanism of Action and Pharmacodynamics of Clopidogrel

Clopidogrel is an oral second generation thienopyridine which has replaced ticlopidine due its better safety margin, tolerability and side effect profile (64, 86). Clopidogrel binds irreversibly to the P2Y₁₂receptor and inhibits its activation by ADP in a non-competitive fashion. Thus it indirectly inhibits the activation of the GP IIb-IIIa receptor which is the final pathway in platelet aggregation (87). Clopidogrel is a prodrug that needs conversion to its active metabolite. 85% of the prodrug is inactivated by

1.8.2. Clinical Trials of Clopidogrel

The two landmark trials that demonstrated the benefit of clopidogrel in the management of ACS were the CAPRIE and CURE trial.

In the CAPRIE (Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events) trial, clopidogrel was found to be superior to aspirin in preventing ischaemic stroke, MI or vascular death in 19,185 patients who were known to have atherosclerotic disease of some form. In patients treated with clopidogrel, the incidence of ischaemic stroke, MI and vascular death was 5.32% as compared with aspirin which was 5.83% ($p=0.0431$)(92).

The CURE Study (Clopidogrel in Unstable Angina to Prevent Recurrent Events) evaluated 12552 patients in total, including patients treated medically-($N=7,985$) and patients treated either by PCI or CABG, ($N=4,577$). Patients with UA or NSTEMI were randomized to receive either 300mg loading plus 75 mg daily maintenance doses of clopidogrel along with standard doses of aspirin or aspirin and placebo. Dual antiplatelet therapy with aspirin and clopidogrel was effective in reducing recurrent events by 20% (RRR, $P<0.001$) compared with aspirin alone. This benefit was observed in all groups of patients regardless of their treatment modality. There was no significant increase in life-threatening bleeding although the incidence of major and minor bleeding was high. The CURE trial established the role of clopidogrel with aspirin in the medical management of ACS in high risk individuals (93).

In the PCI-CURE analysis of a subset of 2,658 patients of the CURE population who underwent PCI, 1313 patients received clopidogrel + aspirin and the rest received aspirin with placebo. There was a significant reduction ($P=0.03$, RR= 0.70) in the incidence of CV death, MI or urgent target vessel revascularization in the clopidogrel group compared with the placebo group (93).

The ARMYDA-2 (Antiplatelet therapy for Reduction of Myocardial Damage during Angioplasty) trial tested the hypothesis that aggressive platelet inhibition might reduce myocardial injury and early cardiac events after

coronary intervention. The rationale behind the hypothesis was the fact that platelet reactivity plays a key role in the pathogenesis of ischaemic complications. Different loading doses of clopidogrel (600mg vs. 300mg) were compared in 255 patients who underwent PCI. The incidence of death, MI and target vessel revascularization at 30 days was 12% in the group treated with 300mg of clopidogrel compared to 4% in the 600mg group ($P = 0.041$) (94). The benefit obtained was largely due to the decreased incidence of periprocedural MI. 600mg of clopidogrel did not cause any increased incidence of bleeding compared to 300 mg.

The CREDO (Clopidogrel for the Reduction of Events during Observation) study was aimed at finding the optimal time for loading and continuation treatment for clopidogrel. One group received 300 mg of clopidogrel 3-24 hours prior to PCI, and continued to receive 75 mg of clopidogrel for 12 months post PCI. The second group received placebo prior to PCI, 75 mg of clopidogrel from day 1 post PCI until 28 days and then continued to receive placebo until 12 months. 85% of the study population underwent PCI in both the groups. There was no statistically significant reduction in death, MI, urgent target vessel revascularization at 28 days compared between the groups (6.8% pretreatment vs. 8.5% no pretreatment $P= 0.23$). However, there was a 38.6% relative reduction in the combined end point in patients who received 300 mg of clopidogrel at least 6 hours prior to PCI reaching a borderline statistical significance ($P= 0.051$). This finding supports the hypothesis that achieving optimal platelet aggregation inhibition prior to PCI is crucial in preventing vascular events post PCI. There was 26.9% reduction in the relative risk of death, MI and stroke at 1 year ($P=0.02$) in patients who continued to receive 75 mg of clopidogrel post procedure compared with those who received placebo. This happened at the expense of a trend towards an increase in major bleeding (8.8% clopidogrel Vs.6.7 placebo $P= 0.07$) (95).

In the CLARITY-TIMI 28 (The Clopidogrel as Adjunctive Reperfusion Therapy-Thrombolysis in Myocardial Infarction 28) trial, clopidogrel (300 mg loading only) followed by 75mg daily was compared with placebo in 3,491 patients with STEMI. All of them received standard treatment for

STEMI including aspirin. A clear benefit in the combined end point of occluded infarct-related artery on angiography, death or recurrent MI before angiography was observed in the clopidogrel group (15% vs. 21.7% P= 0.001) with similar bleeding rates in both groups (96).

PCI-CLARITY-TIMI 28 was a sub-analysis of the CLARITY-TIMI 28 study in which 933 patients with STEMI who were not pre-treated with clopidogrel were compared with 930 patients with STEMI in whom clopidogrel was commenced before the PCI procedure. The incidence of CV death, recurrent MI or stroke at 30 days from the time of PCI was higher (6.2%) in the patients who were not pre-treated compared with those who were pre-treated (3.6%) (P=0.008) (96).

COMMIT (CLOpidogrel and Metoprolol in Myocardial Infarction Trial): This was a very large multicentre trial that established the mortality benefit of clopidogrel. STEMI patients (N=45,852) were randomized to receive 75mg of clopidogrel for 28 days without loading dose and compared with placebo. All patients otherwise received standard treatment for STEMI including aspirin and thrombolytic treatment with or without heparin. The incidence of all-cause mortality in the aspirin plus clopidogrel group was 7.5% compared with aspirin plus placebo 8.1% (P=0.03). There was no significant increase in the risk of major bleeding (97).

The CHARISMA (The Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management, and Avoidance), included 15,000 patients with clinical evidence of CVD or multiple risk factors for CVD who were randomized to receive 75mg of clopidogrel plus aspirin up to 28 months or aspirin plus placebo. This study tested the hypothesis that long-term treatment with a combination of clopidogrel plus aspirin may provide better protection against CV events than aspirin alone. Three quarters of the study population had established CVD and the remaining had multiple atherothrombotic risk factors. There was no statistically significant reduction in the first occurrence of heart attack, stroke or CV death in the DAPT group compared with aspirin plus placebo (6.8 % in the clopidogrel group vs. 7.3% in the placebo group P= 0.22). But the former group had an increased risk of bleeding according to the GUSTO definition (1.7% in

the clopidogrel group Vs. 1.3% in the placebo group P= 0.09). In summary, the combination of clopidogrel plus aspirin was not significantly more effective than aspirin alone in reducing the rate of myocardial infarction, stroke, or death from cardiovascular causes among patients with stable cardiovascular disease or multiple cardiovascular risk factors. Furthermore, the risk of moderate-to-severe bleeding was increased (98).

1.8.3. Limitations of Clopidogrel

1.8.3.1. Interindividual Variability in Response to Clopidogrel

The main caveat for clopidogrel is its broad interindividual response due to unfavourable pharmacokinetic and pharmacogenetic properties among some treated patients. This was reported first by Jaremo et al (99). Different terminologies have been applied to describe this variability, including clopidogrel resistance, clopidogrel treatment failure, clopidogrel hypo-responsiveness and hyper-responsiveness and the recently introduced high on-treatment platelet reactivity (HPR) to ADP. Unfortunately the definition of this clinical entity is yet to be agreed (100, 101), although the recent ACC guidelines have recommended that receiver-operator characteristic (ROC) curve analysis be used to define the HPR to ADP (102). The ROC curve analysis provides the cut-off value of platelet reactivity that is associated with the lowest false negative and false positive rates for ischaemic events (102).

The incidence of clopidogrel hypo-responders ranges from 5%-30% depending on the PFT and the cut-off value. The current ACC guidelines recommends the following cut-off values by ROC curve analysis to define HPR to ADP in the setting of PCI (102).

- 1) PRI 50% by VASP-P analysis
- 2) 235 to 240 P2Y₁₂ reaction units by VerifyNow P2Y₁₂ assay
- 3) 46% maximal 5 µmol/l ADP induced aggregation
- 4) 468 arbitrary aggregation units/min in response to ADP by Multiplate analyzer.

1.8.3.2. Factors Influencing Interindividual Variation

Several reasons are implicated in the aetiology of variable response to clopidogrel. Table 2 summarizes them (101).

1.8.3.3. Genetic Factors

Of the several genetic polymorphisms that have been implicated in the variability of clopidogrel response, the strongest evidence is available for the CYP2C19 isoform of the CYP enzyme, particularly the CYP2C19*2 loss-of-function variant allele. In a study by Simon et al, there was an increased incidence of major adverse cardiovascular events (MACE) at 12 months in patients who underwent PCI who carried any two CYP2C19 loss of function alleles (*2, *3,*4, or *5). Similar results were obtained from a sub study of the TRITON-TIMI 38 trial. Bouman *et al* found that the individuals who developed increased incidence of stent thrombosis following PCI tended to have lower levels of PON1 activity related predominantly to reduced activity of the QQ192 isoenzymes (Table 1.3).

Table 1.3: Factors Associated with Clopidogrel Response Variability

Genetic Factors	Cellular Factors	Clinical Factors
Polymorphisms	Accelerated platelet turnover	Poor compliance
MDR1	Increased ADP exposure	Drug interaction
Cytochrome P450(CYP) Isoform	Reduced CYP activity	Smoking
P2 Y ₁₂	Up regulation of P2Y ₁₂ Pathway P2Y ₁ Pathway	ACS
GP IIb-IIIa	P2Y-Independent	Increased BMI

MDR1 = Multi- Drug Resistance Gene -1
ADP= Adenosine diphosphate
ACS= Acute coronary syndrome
BMI= Body mass index

However, these results have been called into question by other research groups that have not been able to reproduce them (90).

1.8.3.4. Drug–Drug Interaction

Clopidogrel is metabolized by CYP enzymes and hence theoretically any drug that is metabolized by the same enzymes can potentially compete with the enzyme for its metabolism and reduce the conversion of clopidogrel to its active metabolite.

However, this theoretical possibility, at least for statins and calcium channel blockers particularly dihydropyridines, has not been corroborated in any clinical studies or post hoc analysis of large-scale clinical trials or registries. Concomitant use of clopidogrel and proton pump inhibitors (PPI), particularly omeprazole, raised a lot of safety concerns recently (80). This is because at least some PPIs are inhibitors of CYP2C19, which plays a major role in converting clopidogrel to its active metabolite. PPIs differ in their ability to inhibit CYP2C19 and omeprazole is the most potent inhibitor in this class. Hence the antiplatelet activity of clopidogrel can be significantly attenuated by omeprazole.

COGENT study (Clopidogrel and the Optimization of Gastrointestinal Events), which is the only placebo-controlled randomized study, in 3627 patients who received clopidogrel for 12 months aimed to throw light on this controversy. There was no increase in the primary CV outcome of a composite of CV death, nonfatal MI, coronary revascularization and ischaemic stroke (4.9% omeprazole vs. 5.7 % placebo $P= 0.96$). However, this finding of the COGENT study has several limitations. Firstly, the study was stopped prematurely due to funding issues. As the study showed a wide confidence interval in CV outcome (0.68 to 1.44), absence of interaction between clopidogrel and omeprazole cannot be definitively ruled out. The pill used in the study was a combined pill of clopidogrel and omeprazole which might have a different kinetic property compared to the generic omeprazole pill. The study did not compare between various PPIs and finally the follow up time was not of sufficient duration. In view of

these limitations concomitant use of clopidogrel and omeprazole is not recommended in any guidelines and the FDA still issues a safety warning on this combination (103).

1.8.3.5. Clopidogrel Hypo-responders-Clinical Implications

Gurbel *et al* first demonstrated the association of high platelet reactivity despite clopidogrel treatment with increased incidence of recurrent ischaemic events (104). Since then several clinical studies using various platelet function tests have established the increased risk for recurrent ischaemic CV events in clopidogrel hyporesponders (80).

1.8.3.6. Rebound Effect

The phenomenon of rebound effect is characterized by a transient increase in platelet hyper-aggregability upon cessation of drug resulting in increased incidence of thrombotic events (105). Rebound effect following cessation of clopidogrel has conflicting evidence. The PACT (Platelet Activity after Clopidogrel Termination) study did not demonstrate any increase in platelet hyperactivity upon cessation of clopidogrel (106). On the other hand, clustering of clinical events after cessation of clopidogrel has been documented where there was an increased incidence of recurrent CV events in PCI patients following discontinuation (107). However, a subgroup analysis of the CHARISMA trial did not show any increased event rate following discontinuation of clopidogrel. There is some evidence to suggest that this phenomenon might exist in selected high-risk populations, particularly such as those with diabetes or who have a history of ACS or demonstrate HPR on aspirin (108, 109).

The ONSET/OFFSET trial and ISAR–CAUTION trial also did not demonstrate any rebound effect measured by PFT over 10 days after discontinuation of clopidogrel (110, 111).

The DAPT trial (Twelve or 30 Months of Dual Antiplatelet Therapy (DAPT) after Drug-Eluting Stents (DES)) was an international, multicenter, randomized, placebo-controlled trial that was designed to determine the

benefits and risks of continuing dual antiplatelet therapy beyond 1 year after the placement of a coronary stent. A total of 25,682 patients who received drug eluting stent (DES) for treatment of CAD were randomized to receive either clopidogrel 75 mg or prasugrel 10 mg along with 75 mg aspirin following PCI. At the end of 12 months 9961 of these 25,682 patients underwent further randomization to continue to receive aspirin plus a thienopyridine therapy or aspirin plus placebo. Although there was a reduction in the incidence of ischaemic events and this was consistent across stent types, the clinical benefits were tempered by an increase in all-cause mortality in the group that received DAPT compared to those who received aspirin alone. The incidence of bleeding events was also high in the group that received DAPT compared to aspirin alone (112).

1.8.4. Prasugrel

1.8.4.1 Mechanism of Action and Pharmacodynamics

Prasugrel, a third generation thienopyridine, induces irreversible blockade of the P2Y₁₂ receptor. It achieves a better and more consistent inhibition of platelet aggregation (IPA) than clopidogrel. Like clopidogrel this is a prodrug that needs conversion to its active metabolite. However, unlike clopidogrel it has a higher rate of conversion to its active metabolite. In addition, its effects are not so susceptible to variation in the activity of individual CYP isoenzymes. Prasugrel is particularly resistant to inactivation by plasma esterase. Moreover, the activation of prasugrel requires only one oxidative step through CYP isoenzymes for its conversion to active metabolite. These two reasons help the drug to have a better pharmacokinetic profile than clopidogrel and hence achieve a more rapid and effective platelet inhibition. (65, 113, 114)

1.8.4.2 Clinical Trials of Prasugrel

TRITON-TIMI 38 Trial: The TRITON –TIMI 38 trial compared clopidogrel with prasugrel in 13,608 patients treated for ACS. All of them received aspirin. The incidence of primary endpoint of cardiovascular death, MI and stroke was lower in the prasugrel group compared with the clopidogrel

group (12.1% vs. 9.9%). In addition, the incidence of stent thrombosis was half in the prasugrel group compared with clopidogrel group (1.1% vs. 2.4%). However, the incidence of major and fatal bleeding was higher in the prasugrel group compared with the clopidogrel group (2.4% vs 1.8%, 0.4% vs 0.1%). Hence, there was no difference in the overall mortality rate between the two groups. The best balance between safety and efficacy was observed in diabetics and patients who underwent primary percutaneous coronary intervention (PPCI) for STEMI (115).

TRILOGY–ACS: Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes (TRILOGY ACS) study tested the hypothesis that a combination of prasugrel and aspirin is superior to aspirin and clopidogrel in preventing ischaemic events in medically managed ACS (UA/NSTEMI) patients who are less than 75 years of age. 9326 patients were randomized to receive aspirin plus clopidogrel or aspirin plus prasugrel. At a median follow-up of 17 months, the primary end point occurred in 13.9% of the prasugrel group and 16.0% of the clopidogrel group P=0.21. In this large randomized trial, prasugrel was not superior to clopidogrel in preventing primary end points in medically managed ACS patients without STMEI. Although there was no significant increase in major bleed, the more intense platelet inhibition with prasugrel was established by the observation of higher rates of minor or moderate bleeding (116)

1.8.4.3. Limitations of Prasugrel

The risk of bleeding with prasugrel is high in patients who are aged more than 75 years, weigh less than 60 kg and have a previous history of TIA or stroke. Prasugrel is contraindicated in patients with a previous history of stroke or TIA. Limited information is available currently as the benefit of prasugrel is driven from only one trial namely the TRITON TIMI although it is a large trial. The TRITON population included a very small number of African and Asian populations. Therefore, whether the benefits of this trial can be applied to those groups needs further clarification. In addition, the populations with renal impairment and with hepatic dysfunction were

underrepresented in this trial, and as the risk of bleeding with prasugrel is higher, further studies are needed in this high-risk group for bleeding (117, 118).

1.8.5. Ticagrelor

1.8.5.1. Mechanism of Action and Pharmacodynamics

Ticagrelor is a direct-acting P2Y₁₂ antagonist, which does not require liver biotransformation for activation. It reversibly binds to the P2Y₁₂ receptor in all the exposed platelets. The degree of platelet inhibition directly reflects plasma levels. It causes an earlier and more consistent platelet inhibition compared to clopidogrel. Ticagrelor takes 2-3 hours to reach peak plasma levels. The plasma level of the drug directly correlates with the IPA. On cessation of the drug, the platelet function totally reverses to baseline. The IPA achieved is more consistent and greater than clopidogrel. Its plasma half-life is 12 hours and hence twice-daily dosages are needed (63, 119-121).

1.8.5.2. Clinical Trials of Ticagrelor

DISPERSE2: In this trial clopidogrel was compared with ticagrelor at varying doses (75mg of clopidogrel, 90 mg of ticagrelor BD, 180 mg BD) in patients treated for NSTEMI-ACS. A total of 999 patients with NSTEMI-ACS were randomized in a 1:1:1 fashion to receive twice-daily ticagrelor 90 mg, ticagrelor 180 mg, or clopidogrel 300-mg loading dose plus 75 mg once daily for up to 12 weeks. The primary end point, the rate of major or minor bleeding through 4 weeks, was 8.1% in the clopidogrel group, 9.8% in the ticagrelor 90-mg group, and 8.0% in the ticagrelor 180-mg group ($p = 0.43$ and $p = 0.96$, respectively, vs. clopidogrel); the major bleeding rates were 6.9%, 7.1%, and 5.1%, respectively ($p = 0.91$ and $p = 0.35$, respectively, vs. clopidogrel). Although not statistically significant, favorable trends were seen in the rates of myocardial infarction (MI) over the entire study period (MI: 5.6%, 3.8%, and 2.5%, respectively; $p = 0.41$ and $p = 0.06$, respectively, vs. clopidogrel) (120, 122).

PLATO (Platelet Inhibition and Patients Outcome Trial): The study included 18,624 ACS patients treated medically, and by percutaneous and surgical revascularization. Ticagrelor (180 mg loading and 90 mg twice daily maintenance) was compared with clopidogrel (300-600mg loading with 75 mg daily dose). The combined end point of CV death, MI and ischaemic stroke at 12 months was less in the ticagrelor group compared with clopidogrel group. (9.8% vs. 11.7% $P < 0.001$). Overall ticagrelor did not increase major or fatal bleeding risk in comparison to clopidogrel (11.6% vs 11.2% $P = 0.43$), but significantly increased the risk of fatal intracranial bleeding. Significant number of patients were noncompliant with ticagrelor (7.4% vs. 6.0% $P < 0.001$) compared with clopidogrel mainly due to the increased incidence of dyspnoea (13.8 % vs. 7.8% $P < 0.001$) and ventricular pauses ≥ 3 seconds in the ticagrelor group ($p = 0.01$). 79/13339 (5.9%) of patients taking ticagrelor discontinued the medication prematurely due to dyspnoea as compared to 13/798 (1.6%) of patients taking clopidogrel ($P < 0.0001$) (119).

1.8.5.3. Limitations of Ticagrelor

Dyspnoea of mild to moderate severity may lead to discontinuation of drug in a small proportion of patients. Increased incidence of ventricular pauses of > 2.5 seconds was also noted during continuous ECG monitoring. The incidences of these side effects were directly proportional to the dose of ticagrelor. Increased serum level of creatinine and uric acid was also noted in this study population. The dyspnoea and ventricular pauses may be due to an effect of ticagrelor on adenosine metabolism (123).

1.8.6. Indications for Antiplatelets Therapy

- Aspirin should be given at the time of suspected acute MI
- Once MI or ACS is confirmed, a second oral antiplatelet agent such as clopidogrel or ticagrelor can be considered.
- If a coronary stent is deployed then a second antiplatelet agent is mandatory and in ACS patient this can be clopidogrel, prasugrel or ticagrelor.

- A P2Y₁₂ inhibitor is recommended in addition to aspirin, for 12 months following NSTEMI-ACS unless there are contraindications. Clopidogrel (300-600 mg loading dose, 75 mg daily dose) is recommended for patients who cannot receive ticagrelor or prasugrel or who require oral anticoagulation (Class I, Level of Evidence B). Prasugrel is contraindicated in patients with prior stroke/transient ischaemic attack; and there is no apparent benefit with prasugrel in patients >75 years of age or with low bodyweight (60 kg). Previous intracranial haemorrhage is a contraindication to the use of ticagrelor.
- This dual antiplatelet therapy (DAPT) should continue for at least a year unless major bleeding issues arise
- After the first year of an ACS, options include aspirin monotherapy or clopidogrel monotherapy
- In patients with low risk for bleeding and high risk for ischaemic events, DAPT with aspirin plus clopidogrel 75 mg or aspirin plus ticagrelor 60 mg twice daily can be considered
- For patients who undergo elective coronary stenting for stable ischaemic heart disease, current guidelines recommend 6 months of DAPT if a second-generation drug-eluting stent is utilized.
- For bare metal stents, one month of DAPT is the minimum sufficient period per the guidelines
- For patients with stable angina treated medically or who have had coronary artery bypass surgery, aspirin is recommended with stable angina treated medically or who have had coronary artery bypass surgery, aspirin is recommended with stable angina treated medically or who have had coronary artery bypass surgery, aspirin is recommended (124-126).

1.9. Platelet Function Testing (PFT)

The history of PFT dates back 100 years. Traditionally PFT was only used in the management of acquired and inborn bleeding diathesis as opposed to thrombosis (127). Since the role of platelets was established in the

pathogenesis of atherothrombosis, antiplatelet medications have become the cornerstone in the management of CVD (128, 129). However, the novel antiplatelet medication that achieves optimum platelet inhibition without causing bleeding complications is yet to be identified. This has paved the way for the increasing use of already existing and newer point-of-care, PFT tools in the context of monitoring the efficacy and safety of antiplatelet medications both during their developmental stage and in clinical settings.

PFT is used in diverse clinical contexts to study the multitude of functions of platelets. Although an overview of the available tests is briefed, detailed discussion is restricted to those used to analyze the effects of various antiplatelet medications, particularly aspirin, P2Y₁₂ inhibitors (clopidogrel, and GPI).

The two main potential indications for PFT, at least in CV settings, are to predict bleeding and thrombotic events. Bleeding risk may be assessed in patients with platelet defects or postoperative hypocoagulable states, (e.g. Post CABG surgery) and to monitor the safety of antiplatelet medications.

The bleeding risk of an individual, particularly when treatment with antithrombotic medications are considered in case of AF and other conditions, can be calculated using clinical scoring systems like the HAS-BLED scoring system. The HAS-BLED scoring system is well validated and overcomes the limitations of some prior scores. This scoring system has a good predictive value for intracranial bleeding both when warfarin and aspirin is used. The following parameters are taken into consideration: Uncontrolled blood pressure, abnormal renal function, stroke, bleeding tendency, labile INR, age > 65, concomitant use of drugs like aspirin, NSAID and alcohol all score 1 point. Abnormal liver function scores 2 points. A total of 9 points is possible. However, a score ≥ 3 is indicative of the need for regular clinical review and follow up (130). Similarly, thrombotic risk is assessed in high-risk patients, for platelet hyper reactivity and efficacy of antiplatelet medications in medical management, during and after interventional procedures(132). The classification of platelet function tests is presented in table 1.4.

Table 1.4: Classification of Platelet Function Tests (131)

- i) In Vivo
 - (a) Bleeding time (BT)
 - ii) In Vitro
 - (a) Platelet aggregometry
 - 1. Turbidometric aggregometry
 - 2. Light transmission aggregometry (LTA)
 - (b) Impedance aggregometry
 - 1. Multiplate electrode aggregometry (MEA)
 - (c) Particle counting aggregometry
 - 1. Whole blood single platelet counting assay (WBSPCA)
 - 2. PlateletWorks
 - 3. Verify Now Point-of-care (POC) assay
 - (d) Shear dependant platelet function within whole blood
 - 1. Platelet Function Analyser- 100 (PFA- 100)
 - 2. Cone and Plate(let) assay (CPA)
 - (e) Flow cytometry
 - 1. Platelet surface P-selectin, platelet surface-activated GP IIb-IIIa, and leukocytes-platelet aggregates
 - 2. Phosphorylation of vasodilator-stimulated phosphoprotein (VASP)
 - 3. Platelet derived microparticles
 - (f) Global haemostasis tests
 - 1. Thromboelastography
 - 2. Thromboelastography (TEG) platelet mapping system
 - 3. Thromboelastometry
 - 4. Rotational Thromboelastometry (ROTEM)
 - (g) Thromboxane (TX) measurements
 - 1. Serum thromboxane B₂ measurement (TXB₂)
 - (h) Urinary thromboxane measurement
 - 1. Urinary 11- dehydrothromboxane B₂ measurement
-

1.9.1. General Principles

1.9.2. Physiological Vs. Non-Physiological Environments

The major drawback of all the available PFT with the exception of BT is failure to mimic the complex physiological environment in which platelet activation and aggregation takes place. There are several factors that need careful consideration. Firstly, the in vitro tests do not consider the influence that endothelial cells, collagen or vWF have on the process of platelet aggregation. Secondly, in LTA, analysis is carried out using platelet-rich plasma (PRP). The PRP is depleted of WBCs and RBCs. In addition, only 61% to 90% of the whole platelet population is represented in the PRP. Often the giant platelets are removed from the PRP which may be hypo or hyper-active. These factors put together have a great influence on the validity of the results obtained by these tests. Tests performed in whole blood is theoretically assumed to be better than LTA for the above reasons. Thirdly, with the exception of the PFA and the cone and plate(let) method, none of the other tests consider the effect of shear rate on the thrombus formation in CAD. The difference in the velocity of the blood flow between the centre and near the wall of the vessel produces a shearing effect that plays an important role in the formation of platelet aggregation in CAD. The formation of platelet aggregates normally happens in a high shear environment whereas the LTA and impedance aggregometry are done in low shear pressure environments and are therefore less physiological (133).

1.9.3. Other General Considerations

It is recommended that blood is collected via a 19 to 21-gauge needle using a single syringe. Collection of blood in a Vacutainer may cause turbulence with associated platelet activation and hence is not recommended. All samples should be handled gently avoiding any abrupt movement as this can induce platelet aggregation. The tubes used to prepare samples for aggregation studies should ideally be made of plastic or siliconised glass as uncoated glass can induce spontaneous platelet activation. Double pipetting technique is advised to avoid any air bubble in

the analysis tube. A true point-of-care test is one that does not use pipetting. In that respect, VerifyNow and PFA 100 are the only point-of-care tests available as all other tests involve pipetting. Platelet aggregation studies are normally done at 37°C to simulate the in vivo environment. Any diluents added should be at room temperature. When platelets are exposed to cold temperature, then rewarmed and stirred, spontaneous platelet aggregation can take place. Strict thermostatic measures should be in place for impedance aggregometry as the change in impedance between the electrodes is particularly sensitive to change in temperature. Ineffective stirring or non-addition of stir bars will result in a flat curve. Water as a diluent is not recommended as it causes lysis of RBCs and release of ADP. Normal saline or phosphate buffered saline are the commonly used diluents (134-137).

1.9.4. Agonists Used

ADP interacts with P2Y₁ and P2Y₁₂ receptors on the platelet surface. Hence it is used to study the effect of clopidogrel and GP IIb-IIIa inhibitors. Also at lower concentrations in citrate-anticoagulated samples it can stimulate formation of TXA₂ and hence has been used to study the effect of aspirin(138). TXA₂, a potent platelet agonist, is formed as arachidonic acid (AA) interacts with COX. AA is used to evaluate the effect of drugs that acts by inhibiting the production of TXA₂, particularly aspirin (139). Collagen has two type of action depending on the dose used. At low concentration (e.g. 0.2µg/mL), it causes mobilization of AA from the platelet membrane that is converted to TXA₂. This in turn causes release of ADP. At high concentration (e.g.5µg/mL), collagen causes direct release of ADP. Collagen is used to test the antiplatelet effect of aspirin and GP IIb-IIIa inhibitors (140).

Thrombin receptor activating peptide (TRAP) is a synthetic peptide that is consistent with at least the first five N-terminal amino acid sequence generated after the proteolysis of the thrombin receptor PAR1. TRAP (10µmol/L) produces similar strong platelet aggregation response to thrombin. It is used to study the effects of GP IIb/IIIa inhibitors and has

also been used to study the pharmacodynamic effects of the newer PAR1 antagonists (139, 140). Epinephrine is occasionally used to study the effects of aspirin. It is typically used in the concentration of 5-10µmol/L. The other drugs that can influence the aggregation response to epinephrine are nonsteroidal anti-inflammatory drugs, antihistamines, some antibiotics and other over-the-counter medications. Of the agonists for platelet aggregation, epinephrine is the most unpredictable and inaccurate (139, 140). The agonists used in platelet function analysis are summarized in table 1.5.

Table 1.5: Summary of the Agonists Used

Agonist	Concentration	Mechanism of action	Sensitive	Not sensitive
ADP	0.5-20µM	Interacts with the P2Y ₁₂ and P2Y ₁ receptor and causes platelet aggregation via the GP IIb /IIIa pathway	Clopidogrel, GP IIb-IIIa inhibitors	Aspirin
AA	0.5-2.5mM	Converted to TX A ₂	Aspirin	Clopidogrel, GPIIb-IIIa inhibitors
TRAP	1-5µg/mL	Synthetic derivative of thrombin, the most potent platelet aggregator	GP inhibitor IIb/IIIa	Aspirin, Clopidogrel
Collagen	0.2-5µg/mL	Through the action on the collagen receptor causes release of TX A ₂	Aspirin, GP IIb-IIIa inhibitors	Clopidogrel
Epinephrine	10-18µg/mL	Weak and less consistent agonist of COX-1	Aspirin	Clopidogrel, GPIIb-IIIa inhibitors.

1.9.5. Anticoagulants Used (136,141,142)

The choice of *in vitro* anticoagulant used can have an effect on the platelet function. The majority of regulatory events appear to require free calcium. Ionized calcium is the primary bioregulator for platelet activation. A variety

of biochemical mechanisms modulate the level and availability of free cytosolic calcium. Most of the anticoagulants either increase or decrease the blood levels of ionized calcium than the ideal concentration and hence have an indirect effect on the platelet function. Sodium citrate, ethylenediaminetetraacetic acid (EDTA), heparin, hirudin and PPACK (D-Phenyl-alanyl-L-prolyl-L-arginine) are the common anticoagulants in use. EDTA and heparin are less favoured as the former decreases the concentration of calcium ions too low to support platelet aggregation and the latter enhances platelet reactivity and may reduce platelet counts in PRP. Hirudin and PPACK are direct thrombin inhibitors that achieve a more physiological concentration of calcium and hence may be favoured, although they are more expensive than other anticoagulants and are not used on routine basis. Sodium citrate is the most commonly used anticoagulant (9).

1.9.6. Turbidometric Aggregometry

1.9.6.1. Light Transmission Aggregometry (LTA)

This technique was introduced 50 years ago (1962) by Gustav Born and John O'Brien (143). Originally introduced to study primary haemostatic defects and not as a predictor of thrombosis (144, 145), this method measures the GP IIb-IIIa dependent platelet to platelet aggregation (146). It has since been widely used to assess platelet aggregation response to various antiplatelet medications including (aspirin, clopidogrel and GP IIb-IIIa inhibitors) and to predict clinical outcomes. Incorporation of newer technologies like multi-channel capability, computer control and the potential to measure ATP release via luminescence have enabled this technique to still remain a valuable tool to get information about different aspects of platelet function in specialized laboratories (129).

1.9.6.2. Principles of LTA

This is an *in vitro* test. PRP and platelet poor plasma (PPP) are obtained from anticoagulated blood by low force (135-175g) and high force (2700g) centrifugation, respectively. The platelet count in the PRP is standardized

to (250×10^{-9} /l) by addition of PPP. The PRP is in turn stirred in a cuvette at 37°C between a light source and a measuring photocell (147). Addition of a panel of agonist (ADP, AA, collagen and TRAP) at a range of concentration initiates platelet–platelet aggregation and induces a difference in the light transmission. The rate and extent of the increase in light transmission is measured. Typical curves are obtained for an agonist at a particular concentration. Deviation from the normal curve helps in the identification of abnormal platelet function (133).

1.9.6.3. Limitations and Advantages of LTA

When the platelet count in the PRP is less than 100×10^9 /L, then the results are less reliable. Similarly, the test cannot be performed if the plasma is lipaemic (148). It is not a whole blood assay and hence less physiological. It also does not take into account the effect of shear stress and platelet–endothelial cell interaction and hence not sensitive to monitor the early phase of aggregation which is particularly important in studying patients with platelet hyperreactivity (129,133,136). LTA is insensitive to microaggregate formation and hence may not provide a complete picture of platelet reactivity, such as in the assessment of the antiplatelet effect of GP IIb-IIIa inhibitors (148)(141). LTA needs large a relatively large sample volume, is labour-intensive and time consuming, requires technical expertise, and lacks standardization (148). The main advantages include being historical gold standard and proven ability to measure the effects of aspirin, clopidogrel and GP IIb-IIIa inhibitors (131).

1.9.7. Whole Blood Aggregometry

1.9.7.1. Impedance Platelet Aggregometry

The measurement of platelet aggregation by impedance method was introduced by Cardinal and Flower in 1980. This was further refined by Mackie in 1984. Analysis is done in whole blood. This allows the physiological milieu to be preserved and hence the platelets are biologically more sensitive to particular agonist (149, 150).

1.9.7.2. Principles of Impedance Aggregometry

Diluted (to correct the haematocrit to 0.300) anti coagulated blood in a cuvette is stirred at 37°C by placing in the impedance aggregometer. Two palladium electrodes are inserted into the cuvette and alternating current is passed through the blood into the electrodes. The electrodes that are positively charged attract the negatively charged platelets which form a monolayer on the electrode. Upon addition of the agonist, platelet aggregation is induced and aggregates accumulate around the monolayer producing an increase in the impedance between the electrodes. The difference between the impedance in the two electrodes is recorded as a deflection in trace either by a conventional chart recorder or by a computerized system (149-151).

1.9.7.3. Limitations and Advantages of Impedance Aggregometry

Like LTA, this technique also is not sensitive enough to detect platelet microaggregation. Platelets are aggregated at low shear rate. Correction of haematocrit is vital as a high or low haematocrit can cause poor precision of the aggregation values. The aggregometer needs calibration for every new sample either from the same patient or from a different patient which is time consuming. Meticulous measures have to be taken to clean the electrode after every test so that there are no aggregated cells from previous samples left which is a possible source of error (151). The advantages are whole blood assay which can be used when the platelet count is less than $100 \times 10^9/l$ giving good response at a count as low as $50 \times 10^9/l$ and also when the plasma is lipaemic (131). Impedance aggregometry can be used to measure the effect of aspirin, clopidogrel, and GP IIb-IIIa inhibitors. It has also been used to study the effect of dipyridamole (152). Conflicting evidence exists about its correlation with LTA some suggesting good correlation (153), and few suggesting poor correlation (141). Hence conventional impedance aggregometry has been taken over by more modified technique which is user friendly.

1.9.8. Multiplate Electrode Aggregometry

Introduced in 2005 by Orsolya and colleagues, Multiplate electrode aggregometry works on the principle of impedance aggregometry. It is currently one of the most commonly used point-of-care devices at least in Europe, to predict both the efficacy (thrombotic risk) and safety (bleeding risk) of antiplatelet medications (<http://www.multiplate.net>).

1.9.8.1. Principles of MEA

A disposable test cell with four electrodes (two pairs) per cell instead of two makes the multiple electrode aggregometry different from the conventional impedance aggregometry. The device has the potential to analyze five samples at a time. The blood is diluted with warmed saline at 37°C, anticoagulated with hirudin and placed in the test cell. The samples are stirred using a disposable Poly-tetra-fluoro-ethylene (PTFE) coated magnetic stirrer. Addition of agonists induces typical changes in impedance that is detected by individual sensor units. The duplicate sensor acts as an internal quality control. The analysis delivers a kinetic signal which is described as area under the curve (AUC) (154).

1.9.8.2. Limitations and Advantages of MEA

The use of MEA is restricted to patients with normal platelet count as its use in the context of low platelet count or low haematocrit is not validated. The general limitations of any aggregometry technique apply to MEA. Also, there is no option of luminescence available. The advantages are whole blood analysis, fast and convenient. Results correlate well with LTA (131,133,155).

1.9.9. Particle Counting Aggregometry

Whole blood aggregometry using particle counting technique to represent platelet aggregation was introduced by Lumley and Humphrey in 1981. This comprises incubating whole blood and an agonist at 37°C in shaking water bath and withdrawing consecutive aliquots for platelet counting (156).

1.9.9.1. Whole Blood Single Platelet Counting (WBSPC) Assay

This was first introduced by Professor Stan Heptinstall from the University of Nottingham in 1982. Although any automated platelet counter can be employed to assess aggregation, the Ultra-flow 100 whole blood platelet counter was the one originally used in this method. The Ultra-Flo itself has been described in detail by Bacus et al (157).

1.9.9.2. Principles of WBSPCA

This assumes that, as platelets aggregate, the count of single platelets in the sample is reduced. Anticoagulated blood is stirred and incubated at 37°C. It is then allowed to interact with a specific agonist for a particular period of time. Samples are then mixed with a fixative to prevent further interaction with the agonist. This is then processed by the Ultra-flo 100 whole blood platelet counter which uses hydrodynamic focusing to bring the cells into the centre of a counting aperture. As each particle travels through the aperture it evokes a change in the electronic conductance that enables a count based on the size of the cell. In that respect cells at the size of RBC and platelets are counted. The machine then works out the proportion of the platelets to RBC. The real RBC count in the sample is obtained by a different method and when this information is provided, the machine gives an automatic display of the number of the platelets. The fall in platelet count is expressed as percentage of aggregation where a count of zero is equal to 100% aggregation. In further experiments using this technique, the Sysmex K-100 analyzer has been used to count platelets and proved to correlate well with the Ultra-flo 100 counter (148,151,152,157).

1.9.9.3. Limitations and Advantages of WBSPCA

This method is sensitive in detecting micro-aggregation which may be an advantage for analyzing GP IIb-IIIa inhibitor effect. It is also less expensive than most other methods and rapid. It is a whole blood assay and can also be used to study the effect of aspirin and clopidogrel (141, 158). As this method has not been widely studied, limited information is available about its limitations.

1.9.10. Plateletworks

The Plateletworks assay was first devised by Carville and colleagues in 1998. It is based on the standard haematology cell counting principle (159).

1.9.10.1. Principles of Plateletworks

Platelet aggregation to a particular agonist is estimated by the difference in the cell count before and after the addition of an agonist. Standard agonists are used, e.g. collagen for assessment of response to aspirin, and ADP for assessment of clopidogrel. Controls are placed in tubes containing EDTA and test samples in tubes containing citrate. The tubes are simply inverted several times to mix with the anticoagulant and agonist. Exposure to an agonist initiates platelet aggregates that are bigger in size compared to the single platelet and are no longer counted as single platelets. The difference in the platelet count between the control and the sample is represented as platelet aggregation (% maximum) and is calculated using the following formula (159, 160).

$$\% \text{ maximum aggregation} = \frac{(\text{baseline platelet count} - \text{agonist platelet count}) \times 100}{\text{baseline platelet count}}$$

1.9.10.2. Limitations and Advantages of Platelet-Works

With limited data, it does not seem to correlate well with LTA. Counting is the key principle; a good calibrated platelet counting tool is absolutely imperative. The assay is not well standardized as it relies on the user to invert the tubes for mixing of blood and reagents. The main advantages are whole blood assay, simple and easy to perform (131).

1.9.11. VerifyNow Assay (Ultegra Rapid Platelet Function Assay)

Originally devised by Barry S Collier in late 70s and early 80s when he was trying to understand the pathophysiology of thrombasthenic disease and subsequently discovered GPIIb/IIIa receptors. Hence the preliminary assays had the ability to check the effect of GP IIb-IIIa inhibitors only (161-163). The system has since been reconfigured with incorporation of modern technology and was adopted as point-of-care device by Steven R.

Steinhubl and colleagues, having the potential to test not only the GP IIb-IIIa inhibitors but also aspirin and clopidogrel (164).

1.9.11.1. Principles of VerifyNow assay

This assay is an amalgamation of turbidometric principle and whole blood assessment. It relies on the basis that activated platelets in whole blood have the ability to agglutinate with fibrinogen-coated beads. Hence when platelets are exposed to the antiplatelet agent of interest (to be studied) there are receptors that are not inhibited by the drug still available for agglutination. This is achieved by activating the uninhibited receptors by addition of specific agonist and providing fibrinogen coated beads for agglutination. When platelet agglutination occurs, there is gradual increase in light transmission that is measured against time. The rate of agglutination is ultimately expressed as agonist specific units. When assay for clopidogrel is conducted the addition of prostaglandin E₁ suppresses the intracellular calcium levels and augments the inhibitory effects of P2Y₁₂ receptor antagonists, which yields results with higher precision values as it is a more accurate estimate of the inhibition of the P2Y₁₂ receptor. Summary of the three assays available are presented below (162, 164).

1.9.11.2. Limitations and Advantages of VerifyNow Assay

The main advantage of this assay includes whole blood assay, rapid, simple and accurate. Small sample volume is needed for analysis. The system has the potential to measure the effects of aspirin, clopidogrel and GP IIb-IIIa inhibitors individually. The assays correlate well with LTA. It is a true point-of-care device, as no pipetting is involved. On the other hand, the downsides include restrictions to the range of acceptable haematocrit values and platelet counts. The assay is low shear dependent hence does not mimic the actual physiological environment (132). The greatest limitation is high cost of the assay consumables. The details of VerifyNow assay is summarized in table 1.6.

Table 1.6: Summary of the VerifyNow Assay

Name of the assay	Agonist used	Agent tested	Final result
VerifyNow GP IIb-IIIa	TRAP	GP IIb-IIIa inhibitors	PAU
VerifyNow Aspirin	AA	Aspirin	ARU
VerifyNow P2Y ₁₂	ADP + PGE ₁	Clopidogrel	PRU

PAU Platelet aggregation unit

ARU Aspirin reaction units

PRU P₂Y₁₂ reactive units

PGE₁ Prostaglandin E₁

1.9.12. Bleeding Time

This is the only *in vivo* test available to assess platelet function. The first published paper about bleeding time is from a French physician called Milan 1901. However, it was Duke's (1910) description that gained wide acceptance amongst the medical professionals (165). This has been modified to achieve better standardization by the Ivy technique, and further improvement by Mielke et al (166, 167). The test described below is the one adapted by Thiagarajan and Wu.

1.9.12.1. Principles of Bleeding Time

The time taken to arrest bleeding after an incision is made to the skin is called bleeding time. This is measured by inflating a blood pressure cuff to 40mmHg in the upper arm. An incision of 10mm in length and 1mm in depth is made to the volar surface of the forearm. The wound is blotted every 30 seconds until bleeding stops. The normal bleeding time is less than 10 minutes (136).

1.9.12.2. Advantages and Limitations of Bleeding Time

The main merits of BT include assessment of natural haemostasis, less expensive, simple and not prone to anticoagulation artifacts (133). The main limitations are non-specific, insensitive, lack of reliability, scar formation and operator dependence (132). The three major shortcomings of the bleeding time as recognized by The College of American Pathologists and the American Society of Clinical Pathologists includes (168),

- ❖ It performs poorly as a pre-surgical screening test
- ❖ A normal BT does not mean that a patient will not bleed
- ❖ The BT does not reliably identify those subjects who have recently ingested antiplatelet drugs such as aspirin.

1.9.13. Platelet Function Analyzer (PFA 100)

1.9.13.1. Principles of Platelet Function Analyzer- 100

This system basically tests bleeding time in an *in vitro* high shear ambiance ($5000 - 6000 \text{ s}^{-1}$). Citrated blood is warmed at 37°C in a reservoir in the disposable test cartridge. This is sucked into a capillary tube of internal diameter $200 \mu\text{m}$ with a smaller aperture of $150 \mu\text{m}$. The inner aspect of the capillary tube is either coated with collagen and epinephrine (CEPI) or collagen and ADP (CADP). Thus, the platelets are exposed to a combination of agonist and high shear stress at the same time that promotes platelet activation and aggregation. This in turn occludes the blood flow through the aperture which is measured as the closure time (169).

1.9.13.2. Advantages and Limitations of Platelet Function Analyzer-100

The main advantages of this assay are rapid, simple and no sample preparation. It can be used as a point-of-care tool that assesses the platelet function in a high shear climate particularly in whole blood. The closure time is dependent on the von Willebrand factor and the haematocrit and hence could not be used in situations where these factors are abnormal. The PFA 100 system is not sensitive enough to detect the effect of clopidogrel (169).

1.9.14. Cone and Plate (let) Analyzer

Einav S, Dewey C F & Hartenbaum were the first to use the cone and plate(let) device to study platelet function in whole blood under normal flow conditions (169). This method has been further modified by David Varon and Naphtali Savion from Israel into the cone and plate (let) analyzer (170).

1.9.14.1. Principles of Cone and Plate (let) Analyzer

The device consists of a polystyrene coated plate and a rotating cone. The polystyrene plate enables formation of a thrombogenic surface by immobilization of fibrinogen and the vWF. The cone is used to deliver the shear pressure. The citrated blood samples of patients are incubated with specific agonist (depending on the drug of interest to be studied e.g. ADP, TRAP, AA) for one minute. The blood sample is then poured on the plate and subjected to standard shear rate of 1800 s^{-1} for 2 min prior to staining. Platelets in whole blood on exposure to an agonist under shear circumstances get activated and form aggregate that are picked up by staining. The percentage of surface covered (SC) by stained object and the average size (AS) of the object are recorded using an image analyzer (170-172).

1.9.14.2. Advantages and Limitations of Cone and Plate (let) Analyzer

This is a whole blood assay that tests platelet function under high shear conditions. The procedure is simple, rapid and has the potential to be used as a point-of-care instrument. Limited information is available regarding its disadvantages. However, this is not a true point-of-care instrument as pipetting is needed (172).

1.9.15. Flow Cytometry

One of the biggest advances in the quest for the novel PFT tool is the ability to apply whole blood flow cytometry technique for assessment of platelet functions. This was first introduced by Shattil et al. It is a multifaceted tool having the facility to evaluate diverse properties of the platelet. Flow cytometry uses fluorescently labeled antibody and non-

antibody-based probes to determine platelet reactivity or evidence of in vivo platelet activation. A unique advantage of flow cytometry is its ability to assess reliable platelet function in thrombocytopenic samples (173).

1.9.15.1. Vasodilator–Stimulated Phosphorylation Cytometric Analysis

Thienopyridines indirectly promote phosphorylation of vasodilator-stimulated phosphorylation (VASP) and this phosphorylation can be determined by cytometric analysis (174). This is the rationale for measuring the antiplatelet activity of the thienopyridines using the flow cytometric analysis.

1.9.15.2. Principles of VASP Flow Cytometric Analysis

In this assay, the VASP is converted to phosphorylated VASP (VASP-P) by the PGE-1. This is achieved by a multistep process. The initial step involves binding of the PGE-1 to the inositol phosphate receptor on the platelet surface which converts the ATP to cyclic AMP. This conversion is signaled through G stimulatory protein and adenylyl cyclase. The cyclic AMP thus formed converts VASP to VASP-P through protein kinase A. ADP, via P2Y₁₂ receptor activation, inhibits the PGE-1 induced signaling through adenylyl cyclase. Therefore, in the presence of a P2Y₁₂ inhibitor the measure of the VASP-P in the blood is directly proportional to the degree of P2Y₁₂ antagonism.

1.9.15.3. Advantages and Limitations of the VASP Cytometric Analysis

The advantages of this method are that it is a whole blood assay and small sample volume is needed. The limitations includes need to prepare sample and the requirement of a flow cytometer and an experienced technician both of which could be expensive (129). The sample processing and analysis may increase the risk of error.

1.9.16. Thromboelastography

This was first discovered by a German scientist Dr. Hellmut Hartert in the year 1948. Thromboelastography (TEG) is a method of testing the efficiency of blood coagulation. It is a test mainly used in surgery and anesthesiology. More common tests of blood coagulation include prothrombin time (PT, INR) and partial thromboplastin time (aPTT) which measure coagulation factor function, but TEG also can assess platelet function, clot strength, and fibrinolysis which these other tests cannot. Whole blood is inserted in the cup. A torsion wire suspends a pin immersed in the cup and connects with a mechanical–electrical transducer. The cup rotates through 4°45' to imitate sluggish venous flow and activate coagulation. The speed and strength of clot formation is measured in various ways (now usually by computer), and depends on the activity of the plasmatic coagulation system, platelet function and fibrinolysis (175).

The initial application of this system was restricted to study haemostasis as a dynamic process. Many studies were conducted in perioperative settings to predict bleeding using this technique. ROTEM is a modification of thromboelastography (TEG), which also measures the efficiency of coagulation in the blood like the TEG. TEG platelet mapping assay is a further development of the TEG that allows study of the antiplatelet medications by addition of specific agonist that is described further. This was first developed by Robert M Craft and colleagues from Illinois in 2004 (176).

1.9.16.1. Thromboelastography Platelet Mapping System

This method uses clot strength to study platelet function. Heparin annihilates thrombin activity in the blood and hence is used as the anticoagulant. The fibrin's contribution to the clot strength is studied by adding reptilase and factor IIIa (activator F) which generates a cross linked fibrin clot. Similarly, the role of P2Y₁₂ receptor or the COX pathway to clot formation is measured by the addition of ADP and AA respectively.

1.9.16.2. Principles of TEG Mapping System

Blood is added to a rotating sample cup that may or may not be coated with heparin and has a suspended pin that is connected to a chart recorder. As the clot is formed in the cup the motion of the cup is transmitted to the pin by the strengthening clot which is recorded and thus Maximum amplitude (MA) is documented (177, 178).

Three types of MA are obtained (178).

MA_{thrombi} : Thrombin induced clot strength is analyzed by adding neutralized blood to heparin coated sample cup.

MA_{fibrin} : Whole blood fibrin cross linked clot in the absence of thrombin generation or platelet activation is obtained by adding heparinized blood to reptilase and activator F in the rotating sample cup.

MA_{AA} : Whole blood fibrin cross linked clot in the absence of thrombin but presence of platelet activation is achieved by adding heparinized blood to AA and factor F.

Similarly, MA ADP can also be obtained. Platelet aggregation in response to AA is obtained by the formula

$$\% \text{ of aggregation} = [(MA_{AA} - MA_{\text{Fibrin}}) / (MA_{\text{Thrombin}} - MA_{\text{Fibrin}})] / 100.$$

1.9.16.3. Advantages and Limitations of TEG Mapping System

In addition to testing the platelet function it also specifically analyses the platelet contribution to clot strength. This is a whole blood assay which has the ability to monitor all 3 classes of antiplatelet therapy. Limited numbers of literature are available to weigh the merits against demerits (178).

1.9.17: Rotational Thromboelastography & Thromboelastometry

This is an adaptation of the TEG in which the sample cup remains stationary and the suspending pin rotates. The application of this method is largely restricted to estimate blood loss in operative settings and data about its utility in analyzing the effects of antiplatelet medications are limited (177).

1.9.18. Thromboxane generation

Thromboxane $A_2(TXA_2)$ was first described as a biologically active compound by Hambergin the year 1975. Its measurement in serum and urine has been employed to directly study the effect of aspirin on the platelets. TXA_2 is produced by platelets in its active form and its local release recruits' additional platelets to the site of clot formation via activation of platelet TP receptors. It is metabolized from AA by the coordinated action of COX-1 and TX synthase. As AA is derived via the COX-1 pathway, measuring TXA_2 release gives an indirect assessment of the COX-1 pathway and aspirin's effect on the same. TXA_2 is rapidly converted to its active metabolite TXB_2 in the serum. The stable urinary metabolite of TXA_2 is called urinary 11- de-hydro TXB_2 (179).

1.9.18.1. Serum TXB_2

Immuno assay or mass spectrometry can be used to ascertain the level of TXB_2 in the serum. This could either be measured in whole blood or in PRP. When measured in whole blood, serum is obtained by allowing blood to clot at $37^{\circ}C$ for 30 minutes. Similarly, PRP is obtained after standardized procedures to induce platelet activation (179).

1.9.18.2. Limitations of Serum TXB_2

The actual biosynthesis of TXA_2 does not match the estimated biosynthesis and this forms an insurmountable source of artifact. This problem is overcome by measuring the stable metabolite TXB_2 . Two main metabolites that are validated for measurement includes 2, 3 dinor TXB_2 and 11-dehydro TXB_2 . Additionally, TXB_2 levels may not be entirely COX-1 specific. This is because the serum level of TXB_2 is influenced by the prostaglandins that are by products of the leukocyte derived COX-2 (180).

1.9.18.3. Urinary 11-Dehydro TXB_2 Measurements

Estimates of the urinary level of 11-de-hydroxy TXB_2 , a stable metabolite of TXB_2 can also be used to assess the platelet activation in vivo. Neither the serum nor the urinary form is formed in the kidney and hence its

estimation in the urine is predominantly but not solely a non-invasive assessment of the serum TXA₂ level. The plausible effect of renal function is precluded by measuring the ratio of urinary 11- dehydroTXB₂ to creatinine (181).

1.9.18.4. Advantages and Limitations of Urinary 11-Dehydro TXB₂

This is a simple noninvasive measuring tool. It is an indirect way of assessing in vivo thromboxane production and hence may not be platelet specific although it is aspirin specific. It is not suitable to monitor the effects of clopidogrel and GP IIb-IIIa inhibitors. There is a substantial discrepancy in the serum and urinary levels of TXB₂ levels. At least 30% of the urinary TXB₂ is obtained from extra platelet resource whose contribution increases during inflammatory states. The details of all PFT are summarized in table 1.7.

Table 1.7: Summary of the PFT

Principle	PFT	ASA	Clop	GP IIb-IIIa Inhibitor
Platelet- Platelet Aggregation	LTA	Yes	Yes	Yes
	IPA	Yes	Yes	Yes
	Plateletworks	Yes	Yes	Yes
	VerifyNow	Yes	Yes	Yes
Shear Induced Platelet Adhesion	CPA	Yes	Yes	NR
	PFA-100	Yes	NR	NR
Flow Cytometry	VASP P2Y ₁₂	NR	Yes	NR
	P-Selectin	Yes	Yes	Yes
Platelet Contribution to Clot	TEG	Yes	Yes	Yes
COX-1 Dependent	Serum TXB ₂	Yes	No	No
	Urinary TXB ₂	Yes	No	No

ASA= Aspirin
 Clop= clopidogrel
 NR= Non-Reactive

Table 1.8: Summary of the PFT that Measures the Effects of Aspirin(133)

1. Thromboxane as the end point
 - a. Serum TXB₂
 - b. Urinary 11- dehydro TXB₂
 2. Arachidonic acid as the stimulus
 - a. Turbidometric aggregometry
 - b. Impedance aggregometry
 - c. VerifyNow aspirin assay
 - d. Plateletworks
 - e. TEG platelet mapping system
 - f. Impact cone and plate(let) analyser
 - g. Platelet surface-activated glycoprotein IIb-IIIa, platelet surface P-selectin, leukocyte- platelet aggregates
 3. Others
 - a. Platelet function analyser – 100
-

1.9.19. Tests to Monitor the Effects of Aspirin

PFT that can monitor the effects of aspirin belong to three main categories (133).

- 1) Test that can measure thromboxane
- 2) Test using AA as the agonist to stimulate platelet activation
- 3) Tests that do not use AA to stimulate platelet activation, Table 1.8.

1.9.20. Tests to Monitor the Effects of Clopidogrel (133)

The tests to monitor the effects of clopidogrel can be classified in to two

- 1) Tests that specifically measure inhibition of the P2Y₁₂receptor
- 2) Test that uses ADP to activate platelets and hence assess the effects of the drug on the contribution of the P2Y₁₂ receptor to ADP-induced platelet responses

The disadvantage of the second method is that the ADP has two types of receptors namely P2Y₁ and P2Y₁₂. By measuring the overall effect on the ADP receptor, the precision of the test's outcome to truly reflect the effect on P2Y₁₂ receptor blockade is subjected to scrutiny. This is shown in table 1.9.

Table 1.9: Summary of the PFT to Study the Effects of Clopidogrel (133)

-
1. P2Y₁₂ specific test
 - i. VASP phosphorylation test
 2. ADP stimulated tests
 - i. Turbidometric platelet aggregation
 - ii. Impedance platelet aggregation
 - iii. VerifyNow P2Y₁₂ assay
 - iv. TEG platelet mapping system
 - v. Impact cone and plate(let) analyser
 - vi. Platelet surface activated GP IIb/IIIa platelet surface P-selectin
 - vii. Leukocyte platelet aggregates
-

1.9.21. Studies Linking the Clinical Outcomes and Level of Platelet Inhibition

Several studies have been conducted using the above-mentioned platelet function testing tools that are done to see how a newer technique compares with the old conventional methods in the prediction of MACE. PCI related complications like stent thrombosis and periprocedural myonecrosis and bleeding complications. An overview of some of the studies that have been conducted that outlines the utility of the available platelet function testing tools to predict MACE have been briefed in table 1.10 and 1.11.

Table 1.10: Studies Linking Clopidogrel Hypo responsiveness and Clinical Outcomes

Methods	Outcomes assessed	Study	Patient cohort	P. No	Ref
LTA using ADP as agonist to study clopidogrel	MACE up to 30 days	Frere et al	NSTEMI/ACS/PCI	195	(182)
		Geisler et al	CAD/PCI	1092	(183)
		Hochholzer et al	Elective PCI	802	(184)
	MACE within 6 months	Gurbel et al	Elective PCI	192	(185)
		Matetzky et al	PCI/STEMI	60	(186)
		Geisler et al	CAD/PCI	379	(187)
	Late MACE Up to 3 years	Bliden et al	Elective PCI	100	(188)
		Gurbel et al	Elective PCI	297	(189)
		Migliorini et al	PCI/DES/UL MD	215	(190)
	Periprocedural myonecrosis	Gurbel et al	Elective PCI	120	(191)
				200	(192)
		Lev et al	Elective PCI	150	(193)
	Stent thrombosis (ST)	Gurbel et al	Stenting	120	(189)
		Buonamici et al	PCI/DES	804	(195)
	VASP-PRI	One-month MACE	Bonello et al	PCI/Stenting	144
6-month MACE		Bonello et al	PCI/Stenting	162	(197)
Stent Thrombosis		Bonello et al	PCI Stenting	214	(198)
		Barragan et al	PCI	46	(199)
		Blindt et al	High risk ST/PCI	99	(200)
Cuisset et al	NSTEMI/Stenting	598	(201)		
VASP –PRI	Periprocedural Myonecrosis	Cuisset et al	NSTEMI/ACS/PCI	190	(194)
VerifyNow P2Y12 assay	One-month MACE	Patti et al	PCI	160	(202)
	6 MACE including ST	Price et al	PCI	380	(203)
	One-year MACE	Marcucci et al	PCI/ACS	683	(204)
		Breet et al	Elective PCI	1069	(205)
	Post PCI Myonecrosis	Cuisset et al	PCI/SA	120	(206)
Valgimigli et al		Elective PCI	1277	(207)	
Multiplate Platelet analyser	One-month ST	Sibbing et al	PCI/DES	1608	(208)

Table 1.11: Summary of Clinical Studies Correlating Aspirin Hypo responsiveness and Adverse Cardiac Events

Study	Cohort	N	PFT	Events monitored	OR	P
Anderson et al(209)	CAD	71	PFA -100	MI/Stroke/RV	1.8	.28
Eikelboom et al(210)	CVD	488	U TXB ₂	MACE (5y)	1.8	.01
Gum et al(211)	CVD	326	LTA	MACE (2y)	2.9	.09
Cotter et al(212)	CAD	73	Serum TXB ₂	MACE (1y)	6.5	.01
Cheng et al(213)	CAD	422	ASA ARU	MACE	2.9	.002
Pamukcu et al(214)	CAD	105	PFA -100	MACE (1y)	6.1	<.001
Stejskal et al(215)	CAD	103	LTA	MACE (4y)	8.5	<0.001
Chen et al(216)	PCI	151	ASA ARU	Periprocedure Myonecrosis	2.9	<0.004
Lev et al(213)	PCI	150	LTA ASA ARU	Periprocedure Myonecrosis	2.9	.045
Chu et al(217)	ACS+/- PCI	314	VerifyNow	MACE (6mon)	10.0	
Chen et al(218)	Stable CAD	468	VerifyNow ASA	MACE(1Y)	3.12	0.003
Marcucci et al(219)	AMI +/- PCI	146	PFA-100	MACE (1Y)		<0.05

1.9.22. Accuracy of Individual PFT to Predict Clinical Outcomes for Clopidogrel

In a recent single centre prospective study of 1069 consecutive patients undergoing PCI, various platelet function test were conducted to determine their ability to accurately predict both ischaemic and bleeding end points in patients treated with clopidogrel. In this study, only LTA, VerifyNow P2Y₁₂ assay and Plateletworks were significantly associated with the primary endpoint for ischaemic events. However, the predictive accuracies of these tests were only modest. None of the tests provided accurate prognostic information to identify low risk patients at high risk of bleeding following stent implantation. This study did not include the VASP P2Y₁₂ assay and the Multiplate platelet analyzer (220). This is presented in table 1.12.

Table 1.12: Accuracy of Specific Platelet Function Test to Predict Clinical Outcomes for Clopidogrel

Platelet Function Test	P value	AUC-ROC
LTA 20 µmol/L	0.001	0.63
Verify Now P ₂ Y ₁₂	<0.001	0.62
Plateletworks	0.001	0.61
PFA-100 Collagen/ADP	0.15	0.50
Innovance PFA P2Y	0.27	0.56
IMPACT–R ADP Stimulated	0.83	0.53

PFT has only modest ability to predict ischaemic events. The reasons for this are multifactorial and the main issue is the inability of any single PFT to assess platelet function precisely. This is contributed by the fact that in vitro PFT does not reflect what happens in vivo. This can be overcome by using native blood instead of using citrated or anticoagulated (heparin, thrombin inhibitors) blood. Secondly thrombus formation is a complex process that happens in a high shear state augmented by many stimuli including thromboxane, ADP and thrombin. Hence PFT should be done under high shear conditions with the agonist that can stimulate the receptor globally. Currently none of the PFT offers this combination. Also, clear definition of safety/efficacy threshold and evaluation of clinical usefulness is lacking for any test. Timing of the PFT also is crucial. In an ASC setting which is an inflammatory process, patients receive a combination of antiplatelets and anticoagulants at varying dose. Hence PFT performed at this clinical scenario might not reflect the underlying platelet activity of that individual accurately which is not ideal to predict his future ischaemic events. The common shortcoming of all point –of-care PFT is that they use different agonists in separate cartridge to assess different platelet receptor antagonists. In order to monitor DAPT, 4 agonist namely ADP, AA, collagen and thrombin should be measured simultaneously (221)

1.9.23. Accuracy of Individual PFT to Predict Clinical Outcomes for Aspirin

In a recent study conducted on 951 consecutive patients who underwent PCI, PFT specific for inhibition of the COX-1 pathway were used to assess HPR to aspirin and clinical outcomes both ischaemic and bleeding. Only platelet function tests based on aggregation like the LTA and VerifyNow ASA assay correlated well with predicting ischaemic events. Shear-based tests like PFA-100 and Impact-R AA did not show statistically significant results. None of the tests were capable of predicting bleeding events, either major or minor (222, 223). The results are summarized table 1.13.

Table 1.13: Correlation of PFT's Ability to Predict Adverse Cardiac Events for Aspirin

Platelet Function Tests	P value	AUC
LTA	0.03	0.73
VerifyNow ASA Assay	0.02	0.78
IMPACT-R AA	0.94	0.72
PFA-100 Collagen/Epinephrine	0.44	0.72

1.9.24. Risk Stratification Based on PFT

One of the approaches that has been widely recognized to combat the hypo or hyper responsiveness to the available antiplatelet medications particularly clopidogrel and aspirin is an individually tailored approach to assess the risk for ischaemic and bleeding events based on PFT (208). Although this approach seems logical, only very limited data are available to justify such an approach towards better patient care. The preliminary studies conducted by Boneilloet *al* (197),and Fontana et al (224), have shown some promising results. Larger, well designed multicentre trials that address this issue are needed (102). Never-the-less such an approach has some limitations (80).

1.9.25. Limitations of the Risk Stratification Based on PFT

Although several PFT are in existence there is only moderate correlation amongst them which makes their overall use very difficult. For a particular test there has been varying definitions or cut-off values that determine an individual to be poor responder for a specific antiplatelet medication. The recent consensus document published by ACC tries to resolve this issue atleast with clopidogrel. However, their validity in general and in high risk individuals (like the high-risk ACS and diabetics) in particular is still questionable. Moreover this does not address the issue with aspirin (102). Additionally, the relationship between platelet inhibition and bleeding risks needs further investigations. This is particularly important as bleeding has been identified as an independent risk factor for mortality and morbidity in individuals treated with antiplatelet medications for ACS (225). Moreover as discussed before there is no consensus about the best test available and with the cornucopia of the options available choosing the best test still remains an area of great debate and confusion (80). Considering these factors a general implementation of PFT as a standard approach in the treatment algorithms for ACS is not recommended in any international guidelines (ACC,AHA) (226).

1.9.26. PFT- Current Status

The GRAVITAS (Gauging Responsiveness with A VerifyNow assay-Impact on Thrombosis And Safety) study that enrolled 5429 patients on standard dose of clopidogrel underwent PFT with VerifyNow assay 12 to 24 hours post PCI. Patients (41%), who demonstrated high residual platelet activity (PRU \geq 230), were randomized to receive another 600 mg loading of clopidogrel with 150mg maintenance and the rest continued standard treatment. Majority of these patients were relatively at low risk and had stable coronary artery disease. At six months of follow-up, the composite end points of cardiovascular death/MI/stent thrombus were identical in both groups at 2.3%. Stent thrombosis occurred in 0.5% of the high-dose group and 0.7% that was not significant. There was no difference in bleeding. The absolute platelet reactivity was reduced from

an average of about 280 PRU at baseline to 200 PRU in the high-dose clopidogrel group, vs. 240 PRU in the standard-dose group. Despite a reduction in the platelet reactivity, there was no clinical benefit obtained by doubling the dose of clopidogrel in this study. The reasons for this could be that, the study populations were of low risk category. The study was not adequately powered to pick up event rates. The reduction in platelet reactivity obtained was only modest and not sufficient enough to reduce ischaemic events supported by the fact that there was no increased incidence of bleeding in this study. A much aggressive reduction in platelet reactivity can be a strategy that could be adopted in future studies. Also, in this study only one PFT was conducted, may be an alternative PFT could also be employed to see if the outcomes would be consistent. From the GRAVITAS we learned that when a patient is not responding to clopidogrel doubling the dose of clopidogrel although is safe is not efficient enough. Employing another much potent antiplatelet like prasugrel or ticagrelor would be a better choice (227-229).

The TRIGGER-PCI (Testing Platelet Reactivity In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel) study compared prasugrel with clopidogrel in patients with HPR after elective PCI with DES. HPR was assessed with VerifyNow test and a cut-off of < 208 PRU was used to define HPR. Patients were then randomized to receive 10 mg of prasugrel or 75 mg of clopidogrel and were followed up at 3 months and 6 months. In 212 patients who received prasugrel the PRU decreased from a baseline of 245 PRU to 80 PRU at 3 months. In 211 patients who took clopidogrel the HPR reduced to 241 PRU from a baseline of 249 PRU. At 6 months efficacy end point occurred in no patients on prasugrel and happened in one patient on clopidogrel. Major bleeding at 6 months occurred in three patients in prasugrel and in one patient on clopidogrel. The study was terminated early due to less than anticipated efficacy end points (230).

The ARCTIC Study aimed at the evaluation of PFT guided dose adjustment in sub optimal responders compared to a more conventional strategy without monitoring and without dose adjustment to reduce primary end points after 1 year in patients who underwent PCI and had a DES implanted. 2466 patients with stable angina/ ischaemia or NSTEMI undergoing PCI with DES were enrolled. PFT with VerifyNow assay was performed at the time of PCI and repeated 2 to 4 weeks after PCI. 600 mg of loading clopidogrel with 150 mg of maintenance was compared to 10 mg of prasugrel. After one-year primary efficacy end point were 31.6% in clopidogrel vs. 31.1% in prasugrel group $P= 0.10$. Major bleeding happened in 2.3% in clopidogrel compared to 3.3% in prasugrel ($P= 0.12$) (231)

The ANTARCTIC study enrolled 877 patients aged 75 years or more. All underwent coronary stenting with DES. All patients received 5 mg of prasugrel. 442 patients were randomized to no dose adjustment and the remaining 435 to monitoring and treatment adjustment. VerifyNow assay was used to define HPR (208 PRU). PFT was performed on day 14 in the monitoring arm. Depending on their platelet reactivity they were either left on 5 mg of prasugrel, or changed to 75 mg of clopidogrel if showed low platelet reactivity below the range or the dose of prasugrel was increased to 10 mg if they demonstrated high platelet reactivity. For patients who had dose adjustment made, PFT was repeated again on day 28. The primary end point of the trial was the composite of cardiovascular death, MI, stroke, stent thrombosis, urgent revascularization, and further bleeding complication at 1 year. This end point occurred at a similar rate in both arms of the study: 27.6% in the monitoring group and 27.8% in the conventional group (HR 1.003, 95% CI 0.78–1.29; $P=0.98$). There was similarly, no significant difference in rates of the main secondary end point (a composite of cardiovascular death, MI, stent thrombosis, or urgent revascularization), which occurred in 9.9% and 9.3%, respectively (HR 1.06, 95% CI, 0.69 to 1.62; $P=0.80$). Rates of major bleeding, minor bleeding, or either type of bleed also did not differ between the two groups (232).

A class IIb recommendation with level-C evidence is given for potentially high-risk situations, including suspicion of resistance to treatment or high bleeding risk. After the ANTARCTIC study whether this recommendation would be modified needs to be watched for. A class IIa recommendation with level C evidence was issued for platelet-function monitoring to guide antiplatelet interruption before coronary arterial bypass graft surgery. This recommendation would also deserve a specific randomized study (233). The ARCTIC demonstrated that there no benefit in PFT monitored modification of antiplatelet agent in low risk ACS patients. In the ANTARCTIC a similar strategy in high risk individuals is also not beneficial. The latest 2014 and 2016 ACC guidelines do not recommend the routine use of platelet-function test before or after stenting (class III LOE A) (234).

1.9.27. Cost Effectiveness of PFT

The cost saving potential of individualizing antiplatelet therapy remains to be determined. Cost-effectiveness in such situations must include both cost saving driven by the use of generic clopidogrel and the cost associated with adverse events including bleeding and thrombotic complications (235). Straub et al. studied the economic impact of personalized antiplatelet treatment based on PFT with Multiplate analyzer as opposed to unrestricted use of prasugrel and ticagrelor ACS patients undergoing PCI. He found that PFT guided therapy with clopidogrel is cost effective compared to the routine use of prasugrel or ticagrelor (generic clopidogrel is 20 times less expensive compared to prasugrel and ticagrelor) and reduces the incidence of adverse events in clopidogrel treated patients (236). This concept is now being tested in a large multicenter trial the TROPICAL-ACS study. This aims to switch patients between clopidogrel and prasugrel based on the Multiplate analyzer test. If this study meets its end point the, the tailored antiplatelet therapy will carry a significant cost saving potential alongside the benefit to the patients (237).

1.9.28. Summary of PFT

The main aim of PFT is to indicate bleeding and thrombotic risk both in natural and iatrogenic settings. Despite the field's advancement the quest for the ideal test continues. No particular test has gained wide acceptance and been recommended for routine use in clinical practice. LTA is no longer considered to be a gold standard test at least to test for HPR to ADP (102). Traditional impedance aggregometry has been subjected to very limited studies to assess its utility to foresee such conditions. The new point-of-care tests look promising; however, large multicentre randomized trials are highly needed to support its routine use. Flow cytometry techniques are definitely a major revolution that has the potential of being turned into the ideal test-but it is labour intensive and can be at best a research tool rather than a clinical tool.

1.10. Cardiac Troponins

Cardiac specific troponins play a crucial role in the diagnosis and risk stratification of ACS patients. Troponin is a protein complex of three subunits (T, I, C) that is involved in the contractile process of skeletal and cardiac muscle. Troponin C is expressed both in cardiac and skeletal muscle. Troponin T (cTnT) and I (cTnI) are generally considered to be cardiac specific. Indeed, cTnT has not been identified outside the myocardium. cTnT and cTnI are cardiac regulatory proteins that promote the calcium-mediated interaction between actin and myosin.

Cardiac troponins are detected in the serum by the use of monoclonal antibodies to epitopes of cTnI and cTnT. When cardiac injury occurs, troponins are released from cardiomyocytes. Troponin levels increases within 3 to 4 hours after the onset of damage and remain high for up to 4 - 7 days in case of cTnI or 10 to 14 days for cTnT.

Analysis of troponin provides diagnostic and prognostic information in patients with ACS. Cardiac troponins also offer clinicians a valuable tool for therapeutic decision making. ACS patients who are troponin positive are more likely than troponin negative patients to have more complex

lesions with greater thrombus burden, a greater propensity for platelet embolization and distal microvascular obstruction as well as depressed LV function.

For diagnosis of ACS, a stipulated rise in cardiac troponin is necessary. Previous guidelines recommended that a change of 20% at 3 to 6 hours from a previous sample is necessary for diagnosis with sampling at baseline, approximately 6 to 9 hours later and again between 12 and 24 hours from the baseline. Newer studies have supported more rapid algorithms even employing a change between baseline and 1 hour later. An elevated troponin level that is relatively constant over an appropriate sampling interval is more likely to be caused by chronic diseases like renal failure, heart failure etc. Although the recommended change in troponin is suggestive of acute myocardial injury, it does not discriminate acute injury as a result of ACS from other causes of acute myocardial injury (pulmonary embolism or myocarditis). Hence troponin evaluation should be performed only if clinically indicated and elevated troponin must be interpreted in the context of the clinical presentation.

1.11. Study aims

- ❖ To describe the variability in response to clopidogrel between individuals and its potential clinical relevance using WBSPCA
- ❖ To describe the variability in response to aspirin between individuals and its potential clinical relevance using WBSPCA.
- ❖ To assess the inhibition of thromboxane A₂ generation by aspirin in patients with ACS and obtain pilot data for the relative clinical importance in terms of safety and efficacy.
- ❖ To validate further the utility of WBSPCA in patients with ACS.
- ❖ To assess the relationship with post PCI myonecrosis and level of inhibition of platelet function at the time of the intervention in patients with ACS
- ❖ To assess the relationship between levels of inhibition of platelet function and changes in inflammatory markers over time in patients with ACS.

Chapter 2: Methods and Materials

2.1. Preface

This chapter has been structured in such a way that all necessary information regarding experiments conducted, other tests performed and statistical analysis for the whole document are all explained in this chapter in a sequential manner. No repetition of the information is provided in other chapters unless it is relevant to do so.

2.2. Patients

This is a prospective single centre study. Patients admitted to Northern General Hospital, Sheffield from November 2005 until November 2007 with a diagnosis of ACS including UA, NSTEMI and STEMI were included in this study. The following baseline data were recorded for all patients: age; sex; risk factors for CAD, including family history; hypertension; diabetes mellitus; smoking history; antiplatelet therapy used prior to admission; previous history of CAD; previous history of CVD; previous history of haemorrhage or peptic ulcer disease. History and duration of chest pain, cardiac enzyme levels including troponin and CK-MB, electrocardiogram (ECG) changes, chest X-ray findings and echocardiogram findings if available were collected for all patients. Co-medications administered to the patients other than antiplatelets and other antithrombotics were also collected.

Written informed consent was obtained from all patients according to a protocol approved by the North Sheffield Research Ethics Committee (REC), reference number NS2003 11 1800 (Appendix 1). As the period of the study was extended for follow up to 12 months, the appropriate amendments to the consent form were made in agreement with the Central Office of Research Ethics Committee (Appendix2).

2.3. Inclusion Criteria

- All patients who were able to give written informed consent
- Patients who were admitted to hospital for ACS and received both aspirin and clopidogrel as part of their standard treatment.

2.4. Exclusion Criteria

- Patients who had active infection, inflammatory or autoimmune conditions.
- Patients who were on steroids or immunosuppressive treatment.
- Platelet count of $< 125,000/\text{mm}^3$.

2.5. Study Design

The study was designed as two phases. In the first phase patients with ACS who were treated with aspirin and clopidogrel were selected. They underwent PCI if that was the treating physician's decision, but there were a substantial number of patients who were managed medically without intervention. For the phase one patients' only platelet aggregation studies with WBSPCA was performed. In phase two, patients with ACS who received aspirin and clopidogrel and were planned for PCI were included. For these patients both WBSPCA and LTA were performed and blood was collected for analysis of inflammatory markers. The results of troponin measurements done as part of their routine treatment were simultaneously collected. All patients were followed up for 12 months.

2.5.1. My Role in this Project

The study protocol was written by Professor Storey and request for ethical approval was obtained by him. My role involved identifying the suitable patients for the study, performing the study procedures and collating and analyzing the results. Firstly, this included screening patients on presentation in the casualty and patients admitted to the cardiology ward at the Northern General Hospital including coronary care unit.

Once the right patient was identified who met the inclusion and exclusion criteria, they were approached and written informed consent was obtained. Then blood was collected from these patients according to the study protocol.

I then conducted platelet aggregation studies in the lab. In the research lab, my role involved preparing the agonist at the specified dilution and storing them in the freezer for later use. Conduction of WBSPCA assay on the samples and of course cleaning the lab equipment used to enable other candidates to perform their study. The data obtained from the experiments were entered electronically on the same day for analysis at a later date. Any spurious results were discussed with Professor Storey, or a member of his laboratory team for any error in the conduction of the experiment.

For the second phase of the study, blood was collected from patients who underwent coronary stenting. It involved waiting in the cath lab for the diagnostic angiogram to be over. Once the patient was found to have a lesion requiring stenting, blood was collected before the administration of heparin. Hence all patients undergoing angiogram were consented, but only those who had a stenting procedure done were taken for the study. I conducted both WBSPCA and LTA on these patients. Blood samples collected for inflammatory markers were centrifuged and stored in the freezer for later analysis. 24 hours later from the time of PCI, blood was collected for troponin analysis, and inflammatory markers. This involved stepping in over the weekend. A pot was given to the patient to collect their early morning urine sample, and these samples were collected from them and stored in the freezer for later analysis

Clinical details about the patients were collected from the medical records. Information regarding their intervention was obtained from the hospital cathlab database Infoflex. Follow-up of the patients involved telephoning them at one month, three months and 12 months for any ischaemic and bleeding complications. Their compliance to medications was also checked during these phone calls. All steps were taken to ensure

that the patients were alive before calling them which involved, checking the hospital database for their last attendance and calling their respective general practitioners for any reported/documentated death. All the information collected was entered into an electronic database.

Statistical analysis was done by me, taking advice from Professor Storey about the type of statistical test to be used. I am also thankful to Dr. Saiganesh, biostatistician and epidemiologist for his input in advising the best analysis to be performed. I spent a lot of time learning basic statistics, and attended tutorials about the usage of SPSS and PRISM software. I also enhanced my experience with Microsoft Word and Excel software

To summarize, my role in this work was substantial that involved recruiting patients, collection of samples, conduction of experiments, collection of data in hard copy, follow up through telephone calls, data entry into Excel sheets, data analysis and interpretation of results and writing up of the work done.

2.6. Sample Collection

2.6.1. Blood Collection for Assessing the Effects of Aspirin and Clopidogrel by WBSPCA

At baseline, 10 ml venous blood was collected from the antecubital fossa using a 19G - 21 G needle and syringe and anticoagulated with hirudin for platelet function testing analysis. The timing of this sample collection was dependent on the dose of clopidogrel given. Blood samples were collected at least 24 hours, 48 hours and 5 days following 600mg loading dose, 300 mg loading dose and 75 mg daily maintenance dose respectively. These time points reflect the time taken for the drug to reach steady state inhibition of platelets (33). Hence samples may have been taken at later time points but not less than the time points mentioned above. According to the study protocol it was mandatory to ensure that samples were collected once steady state of platelet aggregation inhibition was achieved. This was accomplished by ensuring, that samples were taken at least 24 hours later if 600 mg loading dose of clopidogrel was given, and at least 48 hours later if 300 mg loading dose of clopidogrel was given.

Care was taken that all continued with 75 mg of maintenance dose. However, no emphasis was given about the duration of exposure to study medications (aspirin/clopidogrel) particularly whether exposure was long-term or short term. Moreover, as the sample size was small and patients received various dose of medications for varying period it was difficult to categorize them into group.

Careful precaution was taken to ensure that the effect of other antiplatelets/anticoagulants (glycoprotein inhibitors (GPI), thrombolytic, intravenous (IV) heparin) did not influence the PFT done for clopidogrel. IV heparin has a modest effect on platelet function and the effects wear off in 2 hours from stopping the infusion. Thrombolytic agents may have some weak effect for 24 hours. Abciximab is a monoclonal antibody that binds noncompetitively with high affinity to the GP IIb/IIIa receptor. The biological half-life of this agent is 2 hours and it takes 8-12 hours for restoration of normal haemostatic function. Eptifibatide is a peptide that competitively inhibits the GP IIb/IIIa receptor. The plasma elimination half-life is approximately 2.5 hours. 3-4 hours is needed for normalization of haemostatic function. Tirofiban is a nonpeptide that competitively inhibits the GP IIb/IIIa receptor with high specificity and low affinity. The plasma half-life of tirofiban is short (1.6 hours). After 4 hours the haemostatic function returns to normal (238). Samples were collected keeping these time points as reference. Another 3ml of blood was collected into a serum tube and allowed to clot at room temperature. The serum was separated and stored at - 20°C for analysis of serum TXB₂.

2.6.2. Blood Collection for Assessing the Effects of Clopidogrel by LTA and WBSPCA

After informed consent, 20 ml of blood was collected from the arterial sheath before any interventional procedure and before heparin was given. The first 3ml of blood was always discarded. 8ml of blood in a polystyrene tube (Sarstedt) was anticoagulated with 80µl of hirudin for WBSPCA and another 8ml of blood in a separate polystyrene tube was anticoagulated with 80µl of hirudin for LTA analysis.

2.6.3. Blood Collection for Assessment of Inflammatory Markers

Blood for assessment of plasma levels of sCD40L and CRP was collected in a blue top Vacutainer (BD biosciences) tubes. These tubes contained 0.109M sodium citrate; an inhibitor of platelet aggregation. Within an hour of collection, the samples were centrifuged at 1500 g for 15 minutes and the plasma removed. The separated plasma was stored at -80° C for analysis of CRP and sCD40L at a later stage. The samples were collected at baseline before PCI and 2-4 hours and 18-24 hours following PCI. Another 5ml of blood was collected in a serum tube and allowed to clot at room temperature. The serum was separated using a pipette and stored at -80°C for analysis of inflammatory markers, namely tumour necrosis factor- α (TNF- α) and IL-6.

2.6.4. Blood Collection for Troponin-T Analysis

No blood was collected for analysis of cTnT. As part of the hospital protocol cTnT was routinely measured in all patients who underwent PCI. Assays were done at baseline at the time of presentation and at 18-24 hours post PCI. The available information was utilized with the consent of the treating physician and the patient.

2.6.5. Collection of Urine Sample

Early morning urine samples were collected in sterile sample pots. Aliquots of urine were frozen at -80°C within an hour of collection for analysis of 11-dehydro TXB₂.

2.7. Materials

Hirudin, a potent thrombin inhibitor used as an anticoagulant for the study, was a gift from Novartis. This was a recombinant desulphato hirudin (Revasc). 5 μ g of hirudin was diluted with 3ml of 0.9% saline. 10 mL of whole blood was anticoagulated with 100 μ l of hirudin. Hirudin was preferred over citrate as it prevents artefactualTXA₂ formation that happens with citrate(239). ADP and AA were from Sigma. AA is from Sigma. Fixing solution (Solution A) consisted of saline with 4.6 mM sodium EDTA, 4.5 mM Na₂HPO₄, 1.6mM KH₂PO₄ and 0.16% (w/v)

formaldehyde, pH 7.4. Other materials used included polystyrene tubes, magnetic stir bars, water bath at 37°C. Normal saline was used as diluents for the reagents. A Sysmex KX21 haematology analyzer was used for platelet counting. Materials used for LTA includes Biodata-platelet aggregator profiler (PAP)-4 optical aggregometry, chart recorder, LP3 tubes, large magnetic stir- bars and centrifuge.

2.8. Methods

Concentrations of 0.3,1,3,10 and 30µmol/L ADP were prepared by diluting, 100µmol/L ADP with 0.9% saline at the required proportions. Similarly, concentrations of 0.1, 0.2, 0.4, 0.8, 1mM of AA were prepared by diluting AA with the required volume of normal saline.

2.8.1. Whole Blood Single Platelet Counting Assay for Clopidogrel

- ❖ 10 mL of whole blood was transferred into a polypropylene tube containing 100µL of hirudin and mixed by gentle inversion and transferred to the lab, carefully avoiding any physical stimulation of the platelets
- ❖ The blood was incubated in a preheated water bath at 37 °C
- ❖ 6 LP3 polystyrene tubes were filled with 20µL of increasing concentrations of ADP (0.3, 1, 3, 10, 30,100µmol/L) along with small magnetic stir bars and placed in a stirring water bath (stirring speed 1000rpm)
- ❖ Aliquots (480µL) of whole blood taken from the anticoagulated sample (by using a double pipetting technique) was added to each tube containing ADP and magnetic stir bar and allowed to interact with the agonist at set time points.
- ❖ At 30 seconds, 20µL of blood from each concentration of ADP was removed and added with 40µL of solution A which arrests the interaction of blood with the agonist
- ❖ At 2 minutes 20µL of 30ADP was removed and fixed with 40µL of solution A
- ❖ At 4 minutes 20µL of the blood from each concentration of ADP was removed and fixed with 40µL of solution A

❖ The samples thus obtained are added with 460µL of cell pack buffer to make the volume of the sample to 520µL enabling analysis in the Sysmex analyser.

❖ The number of platelet and RBC in the whole blood sample is obtained by adding 20µL of whole blood with 500µL of cell pack buffer and processing in the Sysmex analyser.

❖ The RBC count is used to adjust the platelet count for variations between samples (e.g. irregular mixing) by the following formula, Adjusted platelet = sample platelet x (Whole Blood RBC/ sample RBC). Aggregation can then be expressed as a percentage.

$$\% \text{ Aggregation} = \frac{100 \times (\text{Whole Blood Platelet} - \text{Adjusted Platelet})}{\text{Whole Blood Platelet}}$$

RBC = Red Blood Count.

The percentage of aggregation for each concentration of ADP (0.3, 1, 3, 10, 30, 100µmol/L of ADP) was thus determined after 30 seconds and 4 minutes.

2.8.2. Whole Blood Single Platelet Counting Assay for Aspirin

The same protocol as above was followed but except for step 3, where increasing concentrations of AA (0.1, 0.2, 0.4, 0.8, 1mM) were added instead of ADP. Percentage of platelet aggregation in response to aspirin was thus determined.

2.8.3. Light transmission aggregometry

- PRP was prepared by centrifuging whole blood at 200g for 12 minutes at room temperature. The PRP thus made was stored in a capped polystyrene tube at room temperature.
- Platelet poor plasma (PPP) was prepared by centrifuging the residual blood at 1500g for 10 minutes.
- The platelet count in the PRP was analysed using a platelet analyser. A platelet count of 300 000 platelets/µL was acceptable. If the platelet count was more than 400 000 platelets/µL it was diluted to approximate a count

of 300,000/mm³ by adding PPP. Samples with a PRP platelet count of < 200,000/mm³ were not considered for analysis.

- 2, 5 and 20 µmol/L concentrations of ADP were prepared by mixing 100µmol/L of ADP in normal saline at required dilutions.
- The optical aggregometer is warmed at 37°C and set to stir at a speed of 900 rpm
- The chart recorder is set at 10 mv and 3cm/minute speed
- LP3 tube containing 500µl of PPP is placed in one well
- Three LP3 tube with 480µl of PRP along with magnetic stir bars were placed in three consecutive wells.
- All the tubes containing PRP and PPP were warmed for 1 mint.
- At 15 second intervals the tubes were transferred to the front wells and baseline tracing of LTA before adding the reagent was recorded for one minute and observed for any oscillations and stability.
- 30µl of 2, 5, 20µmol/L concentration of ADP were added to the three tubes with PRP
- Platelet aggregation was recorded for 5 minutes
- Maximum percentage of platelet aggregation and the percentage of platelet aggregation at the end of the monitoring period (5th minute) were recorded
- Care was taken that all aggregation studies were performed within 2 hours of the preparation of the PRP.

Note: One patient had a platelet count of 598 in the PRP. 1.7ml of PPP was added to the PRP to make the platelet count 300,000/mm³. Three samples had a platelet count less than 300,000/mm³ but more than 200,000/mm³ hence considered for analysis.

2.8.4. Measurement of Serum TXB₂

Serum TXB₂ was measured using a commercially available ELISA kit (Cayman Chemical Company, Michigan, and USA). Serum samples were centrifuged at 2000 g for 10 minutes and stored at -20°C for analysis at a later stage. The manufacturer's guidelines for assessment were adhered to.

2.8.5. Measurement of cTnI

cTnI was measured by the hospital laboratory according to its standard clinical protocol. The assay used was the ADVIA Centaur cTnI assay and were carried out using serum samples. The assays were able to measure cTnI concentrations up to 50ng/ml with lower limit of detection of 0.1ng/ml.

2.8.6. Enzyme Linked Immunosorbent Assay Test

Blood for inflammatory markers were collected 30 minutes before PCI and, 2-4hours and 18-24 hours after PCI. These time points were selected on the basis of previous evidence (240). Inflammatory markers sCD40L, IL-6 and TNF- α were measured with enzyme linked immunosorbent assay (ELISA) kit from Bender MedSystem (Burlingame, California). Plasma samples were used to measure IL-6 (BMS213), TNF- α (BMS223/3) and sCD40L (BMS293) with ELISA. Assays were performed in accordance with manufacturer's guidelines.

2.8.7. Measurement of Urine 11-Dehydro TXB₂ Levels

Urine samples were analyzed by commercially available ELISA kit (Cayman Chemical Company, Michigan, USA) for estimation of 11-dehydro TXB₂ levels.

2.9. Definitions

2.9.1. Clopidogrel Responders and Non-Responders

At the beginning of 2005 when this study was started, there was a lack of consensus regarding clear definition about clopidogrel resistance. In fact, the terminology of clopidogrel resistance by itself was not universally accepted and alternative terminologies were used like clopidogrel hyper-responders, hypo-responders and clopidogrel resistance. However, by 2010 a clearer definition on the terminology namely high on therapy platelet reactivity (HPR) was postulated and accepted. Similarly, a clear cut of value for individual test to define HPR for P2Y₁₂ blockade was proposed by Bonello et al based on numerous studies using receiver operating characteristic (ROC). The consensus values for HPR for various

PFT are (1) >46% maximal for a 5- μ mol/L ADP-induced aggregation; (2) > 50 % PRI using the platelet VASP test; (3) 230-240 PRU by VerifyNow P2Y12 assay (241).

However, defining clopidogrel hypo- and hyper responders based on WBSPCA was difficult given the fact that there was no agreed upon cut-off value, and this tool has been studied less compared to other PFT. For WBSPCA, the principal measurement for all analyses was percentage platelet aggregation at 4 minutes after the addition 10 μ mol/L of ADP to whole blood. Clopidogrel responders were defined as those who had a percentage of platelet aggregation < 60%. Non-responders were defined as those whose percentage of platelet aggregation was \geq 60%.

For LTA, the principal measurements for all analyses were the final platelet aggregation response at 6 minutes after the addition of ADP 5 μ mol/L, and the maximum aggregation response after the addition of ADP 20 μ mol/L. For ADP 5 μ mol/L, clopidogrel responders were defined as percentage of platelet aggregation < 14% and non-responders as those whose percentage of platelet aggregation was \geq 14%. For 20 μ mol/L of ADP, patients with a percentage of platelet aggregation < 50% were defined as responders and \geq 50% were defined as non-responders.

2.9.2. Aspirin Responders and Low-Responders

Aspirin responders were defined as patients whose percentage of platelet aggregation was < 30% measured at 4 minutes with 0.8mM AA assessed by WBSPCA. Likewise, low-responders were defined as percentage of platelet aggregation \geq 30%.

2.9.3. PCI Related Myonecrosis

The 2012 Third Universal Definition of MI formulated by a joint ESC/ACC/AHA/World Health Federation task force arrived at the following definition. MI associated with PCI is arbitrarily defined by elevation of cTn values >5 x 99th percentile upper reference limit (URL) in patients with normal baseline values (\leq 99th percentile URL) or a rise of cTn values >20 % if the baseline values are elevated and are stable or falling. In addition,

either (i) symptoms suggestive of myocardial ischaemia, or (ii) new ischaemic ECG changes or new LBBB or (iii) angiographic loss of patency of a major coronary artery or a side branch or persistent slow- or no-flow or embolization, or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required. The incidence of periprocedural MI using this new definition has not yet been well described (242).

The above-mentioned definition was clearly not in existent when the study was conducted and hence no information regarding the symptoms, ECG findings and angiographic findings of the study population is available. For the study purpose periprocedural MI is defined as elevation of cTn values $>5 \times 99^{\text{th}}$ percentile upper reference limit (URL) in patients with normal baseline values ($\leq 99^{\text{th}}$ percentile URL) or a rise of cTn values $>20\%$ if the baseline values are elevated and are stable or falling.

2.9.4. Ischaemic Events

An Ischaemic event for the study is defined as death due to cardiovascular cause, non-fatal MI including NSTEMI and STEMI and ischaemic stroke. Procedure related complications like in-stent restenosis and stent thrombosis were also included. Troponin elevation following PCI was not taken as ischaemic events unless patient developed clinical symptoms of ACS supported by ECG and angiographic findings.

2.9.5. TIMI Major and Minor Bleed

Thrombolysis in Myocardial Infarction (TIMI) major bleeding is defined as a drop in haemoglobin $>5\text{g/dL}$ (with or without an identified site), intracranial haemorrhage or cardiac tamponade. TIMI minor bleeding is defined as a haemoglobin drop of $> 3 \text{ g/dL}$ but $\leq 5\text{g/dL}$, with bleeding from a known site or spontaneous gross haematuria, haemoptysis or haematemesis. For study purposes, bleeding was entirely assessed using the TIMI criteria. However, more up-to-date bleeding classifications are now being adopted.

In 2011, the Bleeding Academic Research Consortium (BARC) published a consensus classification for bleeding. BARC has been prospectively validated and BARC type 2, 3, or 5 bleeding is associated with increased one- and two-year mortality. In addition, BARC has a comparable predictive ability to the TIMI and GUSTO (Global Utilization of Streptokinase and TPA for Occluded Arteries), scales. While not universally accepted, BARC provides a contemporary standard and has been commonly used in clinical trials published after 2013 (243). However, BARC bleeding classification refers to procedure related bleeding complications when antithrombotic medications are given.

2.10. Telephone Interview

Patients were followed up for a total of 12 months (at 30 days, 3 months and 12 months) through telephone interviews and routine outpatient visits. Information regarding recurrent ischaemic events and bleeding complications were collected and compliance with clopidogrel was established through these telephone interviews. When telephone interview was not possible, information was collected from medical records and hospital database. All efforts were taken to confirm that the patient was alive before contacting them through telephone by checking in the hospital database and confirming with their respective general practitioners. If, during the telephone interview, it was found that the patient was not taking clopidogrel any more, no further phone calls were made to them. The details of the phone call protocol is attached (Appendix 3).

2.11. Statistical Analysis

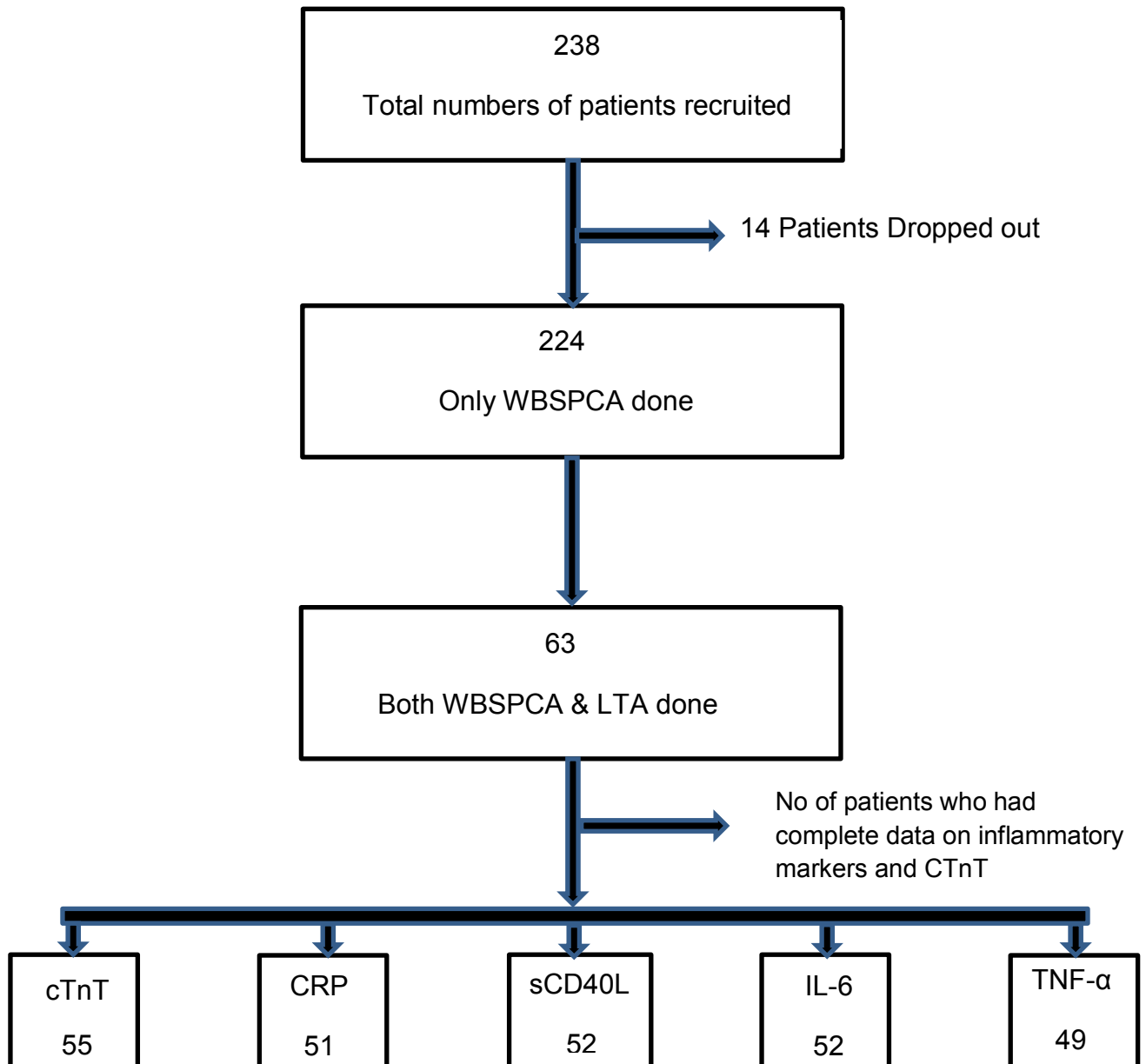
As this study was a pilot study, a sample size calculation to address outcome objective was not done. Normality test was performed with Shapiro-Walk test. Continuous variables of normally distributed data are presented as mean. For non-normally distributed data median with intergroup (IQ) range is presented. Categorical variables are presented as percentage and frequencies. Comparison between two means was done with student's t test if the variables were normally distributed and with

Mann Whitney U test or, Wilcoxon- Signed rank test for non-normally distributed variables. When comparing more than two means, analysis of variance (ANOVA) was used. Student's t test was used to estimate odds ratio for normally distributed data and Fishers exact test was used for data that was not normally distributed.

Linear correlation was performed using Pearson's correlation for normally distributed data and Spearman's non-linear correlation test for non-normally distributed data. A correlation co-efficient of 0.00-0.19 was classed as very weak correlation, 0.20-0.39 as weak, 0.40-0.59 as moderate, 0.60-0.79 as strong and 0.80-1.0 as very strong. Agreement between the two tests was calculated using the kappa statistics. A kappa statistic value of < 0.40 typify poor-to-fair agreement, a value of 0.41-0.60 match moderate agreement, a value of 0.61 – 0.81 is treated as strong agreement, and a kappa value of 0.81-1.00 is taken as excellent agreement.

Receiver Operating Characteristics (ROC) curve analyses using LTA as comparison was performed to identify the cut- off in WBSPCA that is most sensitive and specific in identifying responders and non-responders. The AUC for each concentration of ADP namely, 2, 5 and 20 μ mol/L using LTA was compared with 10 μ mol/L ADP in WBSPCA. An AUC of 0.91-1 was considered to be excellent. 0.80-0.90 was classed as good and 0.70-0.80, 0.60-0.70, 0.50-0.60 were categorized as fair, poor, and fail respectively. A two tailed P value of < 0.05 was considered to be significant. All statistical analysis was carried out either with Statistical Package for Social Sciences (SPSS, version 20, IBM New York, NY) or with Prism (version 6, GraphPad, SanDiego, CA).

2.12. Details of Patients Distribution



Chapter 3: Utility of Whole Blood Single Platelet Counting Assay in Predicting the Clinical Outcomes in Acute Coronary Syndrome Patients Treated with Clopidogrel

3.1. Background and Aim

Patients admitted to the hospital for ACS receive a combination of antiplatelets and anticoagulant medications. The oral antiplatelets include aspirin and clopidogrel or more recently aspirin and newer P2Y₁₂ inhibitors like prasugrel and ticagrelor. The anticoagulants include fondaparinux, LMWH and unfractionated heparin. Some patients may also receive an intravenous GP IIb/IIIa inhibitor for additional antiplatelet effect. The optimal strategy for combining these agents is yet to be defined. Interindividual variability in the effect of clopidogrel is a well-documented phenomenon. HPR has clearly been linked to increased incidence of MACE in patients undergoing PCI. Similarly, hyperresponders to the medication have increased incidence of bleeding complications. This has led to the concept of individually tailored treatment as decided by the PFT results.

Mixed evidence exists regarding adoption of this strategy with some limitations. This has led to the invention and use of newer antiplatelet agents like prasugrel and ticagrelor, but due to cost factors and concerns about bleeding complications, at least in the elderly population, clopidogrel still remains one of the commonly used P2Y₁₂ inhibitors in conjunction with aspirin globally. Still there is no routine method used in clinical practice for monitoring the effect of these medications and more research is required to establish whether there is a cost-effective way of assessing response to clopidogrel and adapting therapy accordingly. The low cost of WBSPCA and ready availability of materials for performing this assay make it potentially suitable for assessing clopidogrel response, particularly in health care systems with very restricted budgets or those that require patients to pay for treatment.

We aimed to study the utility of WBSPCA in predicting the risk of both ischaemic and bleeding events in patients treated with clopidogrel for ACS.

Results

3.2. Patients and Demographics

A total of 238 patients were recruited for this study. Patients were treated either medically or by intervention. 14 patients were found to be ineligible for analysis either because they had received other antiplatelet medications that would interfere with the platelet function test performed for clopidogrel, or they dropped out of the study after the collection of the initial sample. 224 patients had whole blood single platelet counting assay performed and were followed up by telephone interviews. Only those patients who were proven to be taking clopidogrel at the time of occurrence of complications (ischaemic and bleeding) were considered for final analysis in determining the utility of the WBSPCA in assessing the response to clopidogrel. In that respect data for clinical outcome was assessed in 189 patients out of 224 patients. This is represented in table 3.1.

Table 3.1: Study Design and Details of Patients Studied

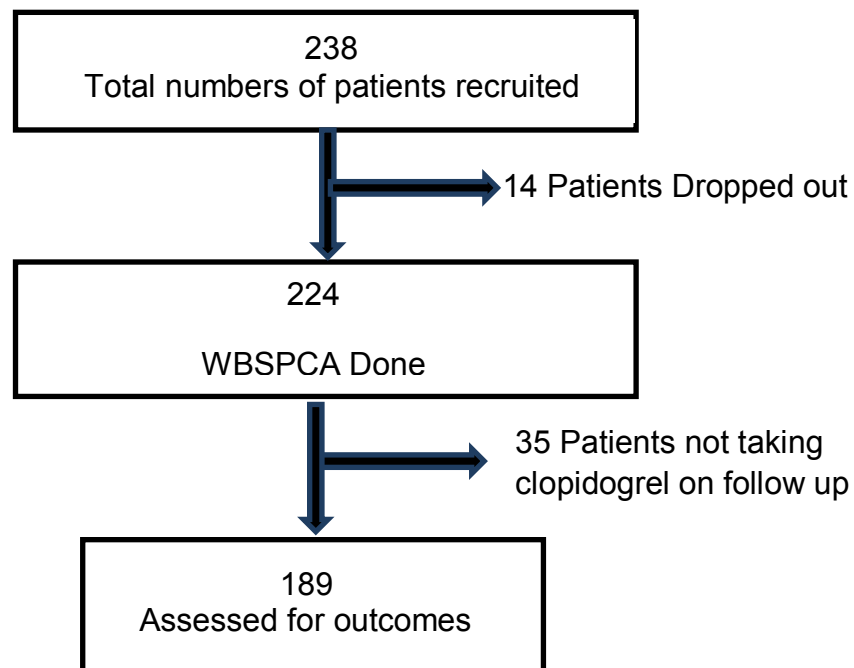


Table 3.2: Demographic Data/ Investigations and Treatment Given for All Assessed Patients and in the Subgroup, who sustained an Ischaemic Event or a Bleeding Event

Demographic data	N = 189	Ischaemia N= 20/189	Bleed N= 21/189
Age (Median + range)	60(31-87)	57(45-80)	62(37-83)
Risk Factors			
Diabetes Mellitus	17%	25%	19%
Hypertension	52%	45%	57%
Smoking	61%	50%	66%
Dyslipidaemia	60%	50%	57%
Family History	41%	30%	48%
Previous H/o IHD	21.2%	35%	29%
Investigations			
^a Positive ECG	83%	93%	66%
^b CXR evidence of LVF	34%	13%	14%
^c ECHO - LV dysfunction	23%	40%	14%
^d Troponin positive	97%	100%	76%
Medications on Discharge			
B blockers	91%	70%	100%
ACE – I	85%	75%	85%
Statins	94%	100%	100%
CCB	21%	15%	14%
Diuretics	7%	1%	0%
ARB	6%	1%	0%

^a Positive ECG finding include ST elevation, ST depression and significant T wave inversion

^b Pulmonary venous congestion or florid pulmonary edema

^c Left ventricular dysfunction (LVF) proved by assessment of ejection fraction

^d Troponin > 0.04ng/ml

Of the 189 patients on whom complete demographic details are available (Table 3.2), more than three-quarters were male (163 out of 189). 50 patients were more than 65 years of age. Mean age was 60 years (range 31-87). Smoking and hypercholesterolaemia were the two major risk factors identified. 61% of the study groups were smokers and 60% of the patients were found to have raised cholesterol. 17%, 52% and 41% of the patients had diabetes, hypertension, and positive family history respectively. A minor proportion of them had suffered previous cerebrovascular or CV events, 8 patients had suffered previous stroke/TIA and 44 patients had suffered previous MI.

83% of the study population had significant ECG changes (ST elevation, ST depression and pathological T wave changes). 23 % of the patients had echocardiographic evidence of left ventricular dysfunction out of which 34 % had clinical and/or CXR evidence of left ventricular failure. 3% of the patients had a negative troponin result and the rest (97%) had raised levels of troponin either at the time of admission or 12 hours after the onset of symptoms.

91% of patients received β blockers. 85 % of the patients were treated with angiotensin converting enzyme inhibitors (ACE-I) whereas 6% were given angiotensin receptor blockers (ARB). 94% of the patients were established on statins. 21% of the patients were treated with a calcium channel blocker (CCB) and 7% had received diuretic. 64% of the patients had received 300 mg loading dose of clopidogrel followed by 75 mg of maintenance dose. 22% received 600 mg loading and 75 mg maintenance. 14% received 75 mg of maintenance dose only. The demographic data are presented in table 3.2.

3.3. Descriptive Statistics of Aggregation Data in Response to Clopidogrel Using WBSPCA

Platelet aggregation data was assessed by WBSPCA in 224 patients to increasing concentrations of ADP (0.3, 1, 3, 10, 30, and 100 μ mol/L) at different time points, namely 30 seconds and 4 minutes. The mean

percentage of platelet aggregation (\pm SD) in response to 10 μ mol/L of ADP in patients receiving clopidogrel at 4 minutes was 46 \pm 28% (figures 3.1 and 3.2).

Figure 3.1: Platelet Aggregation Assessed by WBSPCA 30 seconds

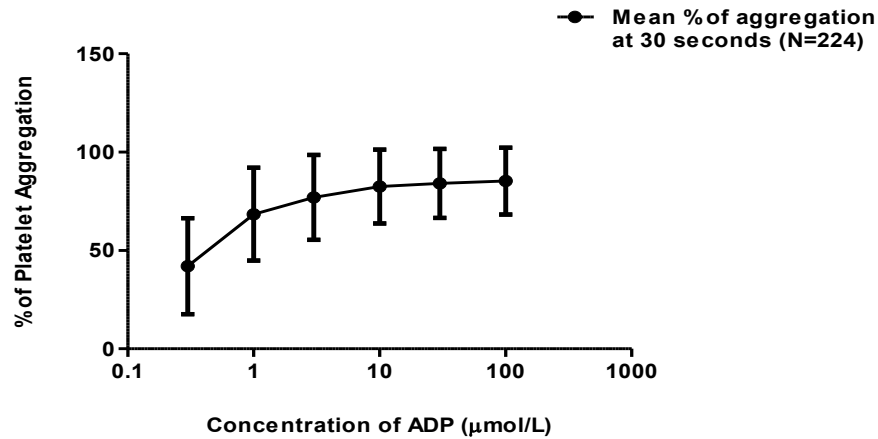


Figure 3.1: Mean % of platelet aggregation in response to increasing concentration of ADP (0.3, 1, 3, 10, 30 and 100 μ mol/L) at 30 seconds after addition of ADP assessed by WBSPCA in patients taking clopidogrel, (N = 224).

Figure 3.2: Platelet aggregation assessed by WBSPCA 4 minutes

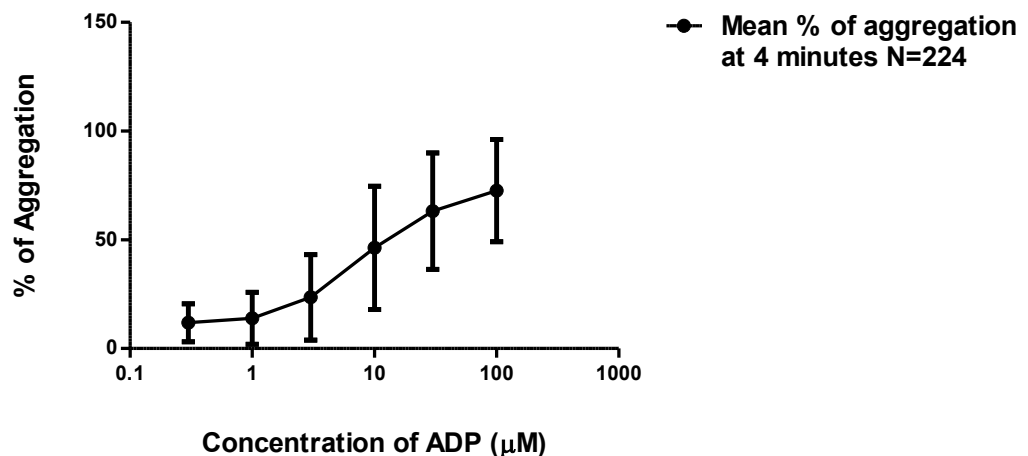


Figure 5: Mean percentage of platelet aggregation in response to increasing concentration of ADP (0.3, 1, 3, 10, 30 and 100 μ mol/L) at 4 minutes after addition of ADP assessed by WBSPCA in patients receiving clopidogrel, (N= 224).

3.4. Prevalence of Clopidogrel Responders and Non- Responders Assessed by WBSPCA

The overall prevalence of clopidogrel responders in this study is 63% (N=142/224). The remaining 37% (N=82/224) were classified as non-responders (t test) (figure 3.3). Demographic profiles of these patients are presented in table 3.3

Table 3.3

Parameters	Clopl non-resp (N=82)	Clopl resp (N=142)
Age	60 (32-87)	63 (39-86)
Sex	58 (M)	94 (M)
Risk factors		
Hypertension	50%	52%
Diabetes mellitus	24%	21%
Dyslipidaemia	68%	60%
Previous h/o CAD	32%	30%
Smoking	48%	60%

Figure 3.3: Prevalence of Clopidogrel Responders and Non- Responders (N=224)

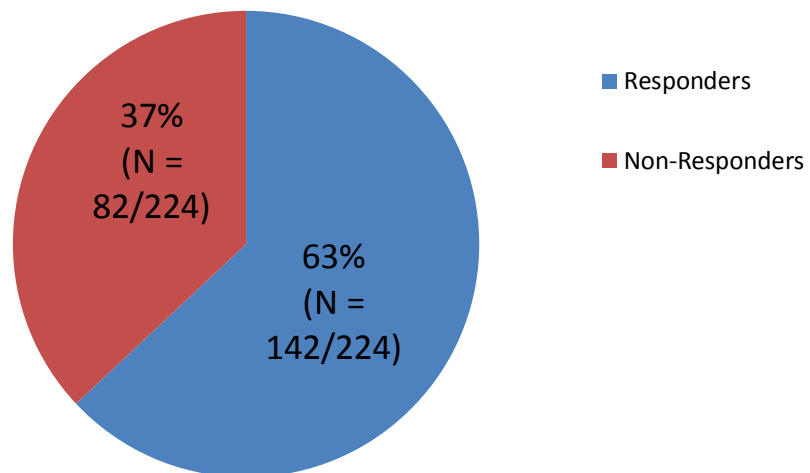


Figure 3.3: Prevalence of clopidogrel responders and non- responders in the study population (N=224). Responders (N = 142/224, 63%) Non-responders (N = 82/224, 37%).

3.5. Interindividual Variation in the Platelet Aggregation Response to Clopidogrel

The percentage of platelet aggregation in clopidogrel-treated patients shows marked interindividual variability as illustrated in figure 3.4. Again, the value of platelet aggregation induced by 10µmol/L of ADP after 4 minutes was found to be most discriminating in demonstrating this. The maximum number of patients lay in the frequency of 15-30% and the least number of patients were found to have less than 15% of aggregation. However, our data did not demonstrate the normal distribution frequency found in other studies that used different methodologies (101).

Figure 3.4: Interindividual Variation in Clopidogrel Response (N=224)

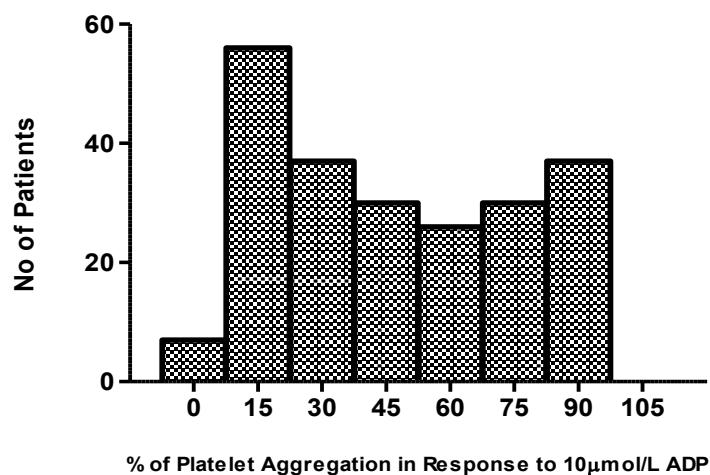


Figure 3.4: Interindividual variability in platelet aggregation to 10µmol/L of ADP measured at 4 minutes in patients treated with clopidogrel assessed by WBSPCA. (N=224)

3.6. Ischaemic Outcomes in Clopidogrel Treated Patients Assessed by WBSPCA

3.6.1. Demographic Details

The median age of the patients who suffered an ischaemic event during follow-up was 57(45-80) similar to the overall cohort (Table 3.2). One quarter of the cohort was diabetic (25%), a numerically higher proportion

than the overall cohort, and half of them were smokers (50%). Dyslipidaemia was the most common risk factor in this population (70%) and less than half were hypertensive (45%). 35% of the cohort had a previous history of MI. 93% of the study group had positive ECG findings and almost all of them were troponin positive. Beta blockers (70%) and ACE-I (75 %) were underused in this group compared with the whole study population.

3.6.2. Platelet Aggregation Details

The aggregation data for 189 patients who have been assessed for clinical outcomes is presented in figure 3.5. The mean % of platelet aggregation (\pm SD) to $10\mu\text{mol/L}$ of ADP measured at 4 minutes is $46 \pm 28\%$. The mean % of aggregation (\pm SD) to $10\mu\text{mol/L}$ of ADP measured at 30 seconds is $81 \pm 19\%$. The platelet micro aggregation in response to ADP occurs in early stages as demonstrated by the high mean % of aggregation at 30 seconds. The reversal of this micro aggregation explains the lower mean % of aggregation obtained at 4 minutes (239).

Figure 3.5: Platelet Aggregation Assessed by WBSPCA (N=189)

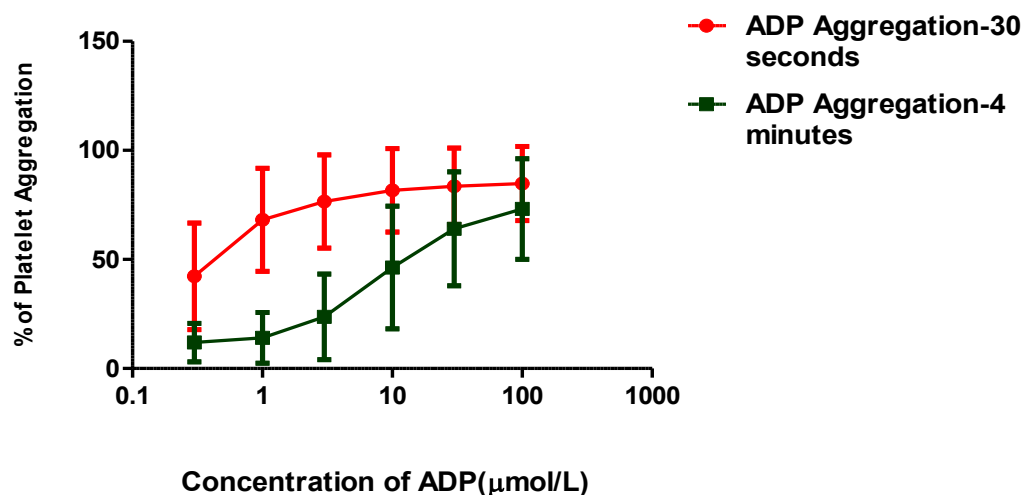


Figure 3.5: The mean % of aggregation (\pm SD) to increasing concentration of ADP (0.3, 1, 3, 10, and 30 and 100 $\mu\text{mol/L}$) measured at 30 seconds and 4 minutes using WBSPCA (N=189).

3.6.3. Incidence of Ischaemic Complications in Responders and Non-Responders

Out of the 189 patients assessed for ischaemic outcomes, 120 patients were found to be responders ($120/189 = 63\%$) and the remaining 69 patients were found to be non-responders ($69/189 = 37\%$), as assessed by the WBSPCA using $10\mu\text{mol/L}$ ADP as agonist measured at 4 minutes. The overall incidence of ischaemic complications was 20 out of 189. Among this 20, 11 events occurred in responders ($11/120=9\%$) and 9 events occurred in non-responders ($9/69=14\%$). There were no significant differences in their demographic characteristics. There were 5 ischaemic events related to interventions. Out of this, 3 were angiographically proven stent thrombosis and 2 were angiographically proven in-stent restenosis. The remaining patients either had NSTEMI with no angiographically proven lesions or with new lesions needing interventions including PCI with stenting or CABG. Of the three stent thrombosis events, 2 events occurred in non-responders and the remaining 1 in a responder. Although there was a marginal increase in the number of recurrent ischaemic events in non-responders (14%) compared with responders (9%), this was not statistically significant by Chi-Square test ($P= 0.404$, $OR = 0.673$, 95% $CI = 0.306$ to 1.612) (figure 3.6).

Figure 3.6: Ischaemic Events in Responders and Non-Responders (N=20)

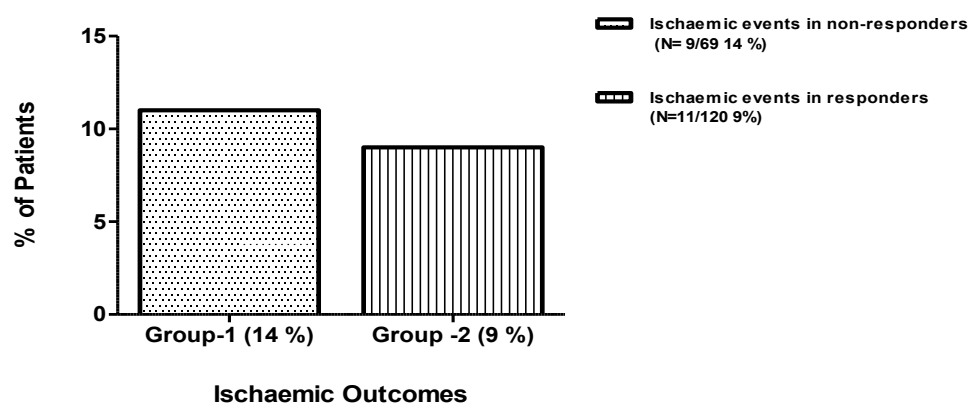


Figure 3.6: Ischaemic outcomes in responders and non-responders. Group 1 non-responders (N=9/69 14%). Group 2 responders (N=11/120 9%) ($P= 0.404$).

Figure 3.7: Platelet Aggregation Measured at 30 Seconds

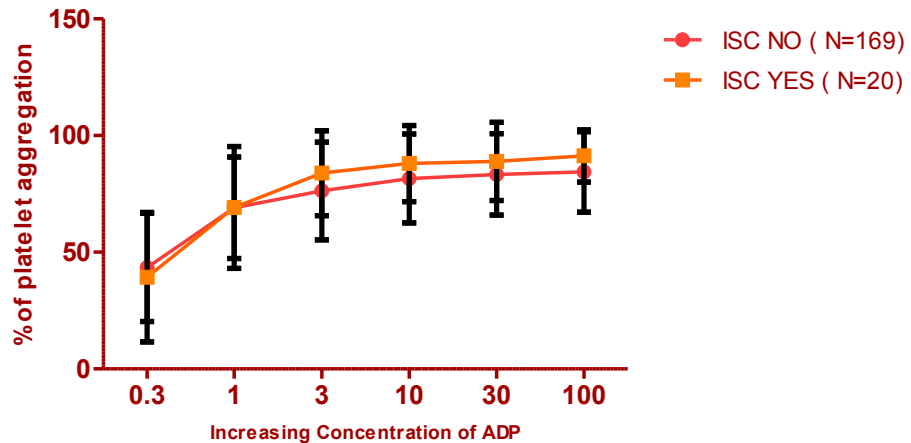


Figure 3.7: The mean (\pm SD) percentage of platelet aggregation in patients who developed ischaemic complications compared with those who did not measured at 30 seconds by WBSPCA.

3.6.4. Platelet Aggregation in Patients with and without Ischaemic Complications (20/189 vs.169/189)

The mean (\pm SD) of the percentage of platelet aggregation measured at 30 seconds and 4 minutes by WBSPCA in patients who did or did not develop ischaemic complications is presented in figure 3.7 and 3.8. The mean percentage of platelet aggregation measured at 30 seconds in patients who sustained ischaemic events was $88 \pm 19\%$ and the same value for 4 minutes was $47 \pm 28\%$. Likewise, the percentage of platelet aggregation in those who did not develop an ischaemic complication was $82 \pm 19\%$ and $45 \pm 33\%$ measured at 30 seconds and 4 minutes, respectively.

The mean percentage of platelet aggregation measured at 4 minutes to $10\mu\text{mol/L}$ of ADP in those who developed an ischaemic event was $45 \pm 33\%$. Similar data for those who did not develop an ischaemic event was $43 \pm 28\%$. There was no statistically significant difference in the median compared between the groups, (Wilcoxon- Signed rank test $P= 0.808$). This is demonstrated in figures 3.9.

Figure 3.8: Platelet Aggregation Measured at 4 Minutes (N=189)

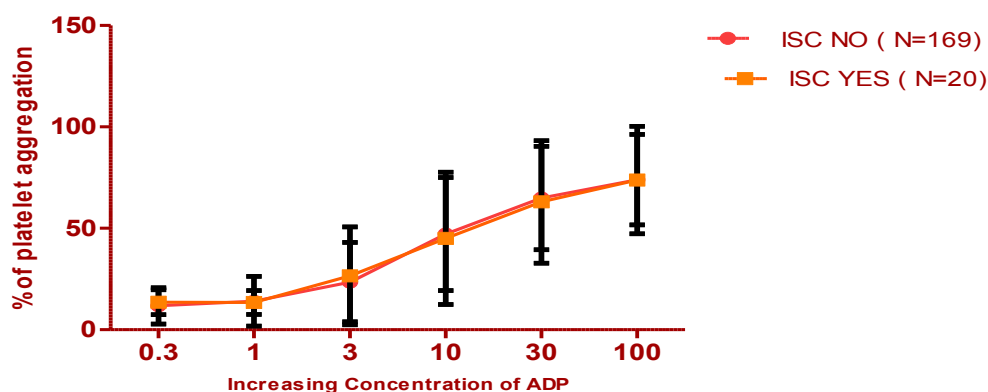


Figure 3.8: Mean percentage of platelet aggregation (\pm SD) to increasing concentration of ADP (0.3, 1, 3, 10, 30 and 100 μ mol/L) measured at 4 minutes in patients who did or did not develop ischaemic complications on treatment with clopidogrel measured by WBSPCA in hirudin anticoagulated whole blood(N=189)

Figure 3.9: Ischaemic Outcomes

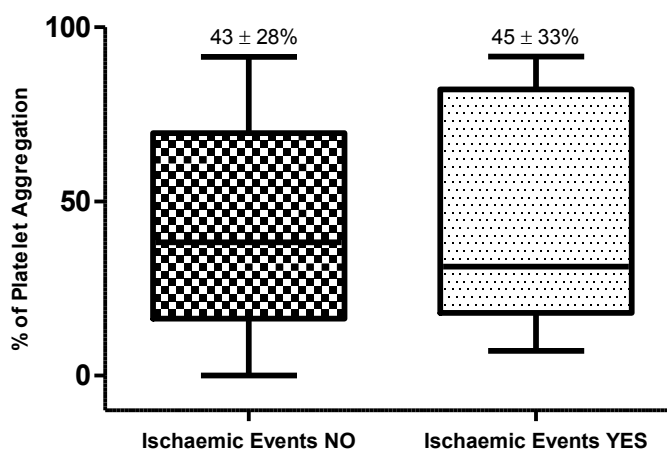


Figure 3.9: Box and whiskers plot demonstrating the range of platelet aggregation to 10 μ mol/L of ADP measured at 4 minutes in two groups. Group 1- Occurrence of ischaemic events (N=20), Group 2- Nonoccurrence of ischaemic events (N= 169) (P=0.808)

3.7. Bleeding Complications – Details of Bleeding Events and Demographics of the Patients

The total number of bleeding events in this study was 33 out of 224 patients. Out of these 33 events, 21 bleeds happened in the 189 patients taking clopidogrel and were considered for statistical analysis. The remaining 12 happened in patients either not taking clopidogrel at the time of bleed or were related to coronary artery bypass grafting (CABG). Of the 21 bleeds, 2 were TIMI major bleeds consisting of one gastrointestinal bleeding (GI) bleed needing blood transfusion and one retroperitoneal bleed dropping haemoglobin to 6g/dl needing blood transfusion, 5 were right femoral artery haematoma following angiogram not needing blood transfusion or intervention, 14 were nuisance bleeding including 1 gum bleeding, 11 epistaxis (one needed nose packing) and 2 spontaneous bruises. This is summarized in table 3.4.

Amongst the 21 patients with bleeding events, more than 85% of them were less than 75 years of age and the rest were more than 75 years. It was interesting to note that the majority of the bleeds occurred in non-diabetic patients (17/21)

Table 3.4

Classification	Interventional Related N=6	Non-Interventional Related N=15
TIMI Major	1 (Retroperitoneal Bleed)	1 (GI Bleed)
TIMI Minor	5 (Femoral haematoma)	<ul style="list-style-type: none"> • 11-Epistaxis • 1 - Gum bleed • 2 - Spontaneous bruises

3.7.1. Incidence of Bleeding Complications in Responders and Non-Responders

The overall incidence of bleeding was numerically higher in responders (N = 15/120, 12.5%) compared with non-responders (N = 6/69, 9%). However, this was not statistically significant (N = 15/120 vs. 6/69, P = 0.536, OR=1.368 95% CI= 0.506–3.698) (figure 3.10).

Figure 3.10: Bleeding Complications in Responders and Non-Responders (21/189)

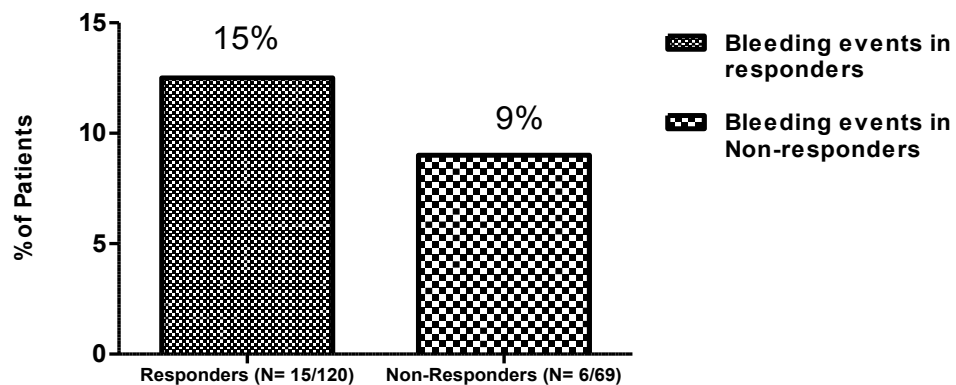


Figure 3.10: There was no significant difference in the incidence of bleeding between responders (N=15/129 15%) and non-responders (N=6/69 9%) (P=0.536)

3.7.2. Platelet Aggregation in Bleeders and Non-Bleeders

The mean percentage of platelet aggregation in response to 10 μ mol/L of ADP measured at 30 seconds and 4 minutes between those with, and without bleeding events are 82 \pm 19% vs.87 \pm 13% and 39 \pm 24% vs. 46 \pm 28% respectively. Figure 3.11 and 3.12

Figure 3.11: Platelet Aggregation Measured at 30 Seconds N=189

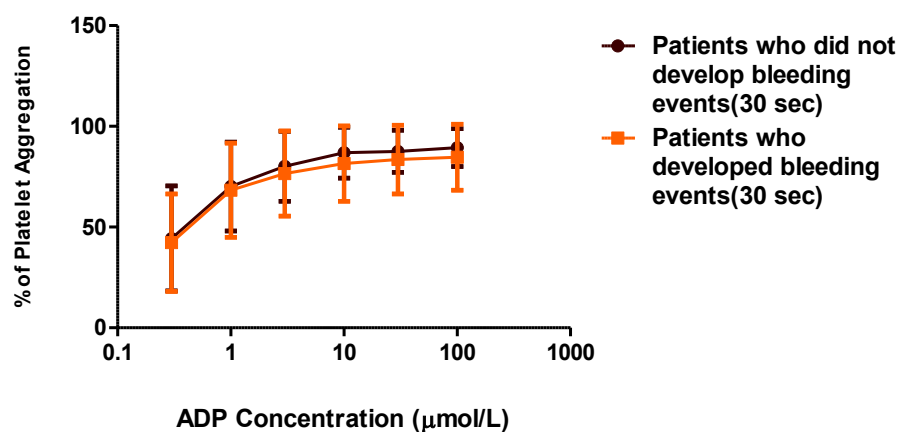


Figure 3.11: Mean percentage of platelet aggregation (\pm SD) to increasing concentrations of ADP (0.3, 1, 3, 10, and 30 and, 100 μ mol/L) measured at 30 seconds in two groups (bleeders (N=21) and non-bleeders (N= 168) by WBSPCA in hirudin anticoagulated whole blood (N= 189).

Figure 3.12: Platelet Aggregation Measured at 4 Minutes N=189

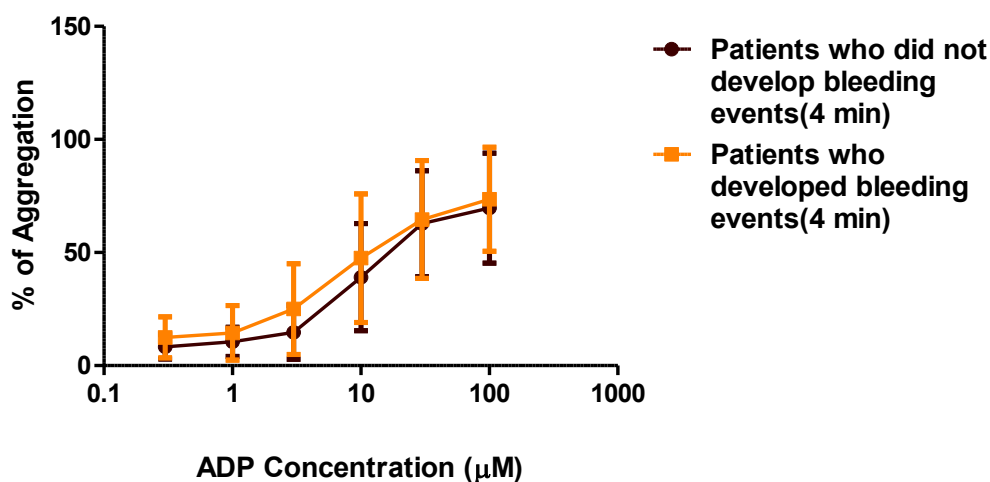


Figure 3.12: Mean percentage of platelet aggregation induced by increasing concentrations of ADP (0.3, 1, 3, 10, and 30 and 100 μmol/L) measured at 4 minutes between non-bleeders (B=168) and bleeders (N=21) by WBSPCA (N=189).

Figure 3.13: Platelet Aggregation in Ischaemic (N=20) and Bleeding Outcomes (N=21)

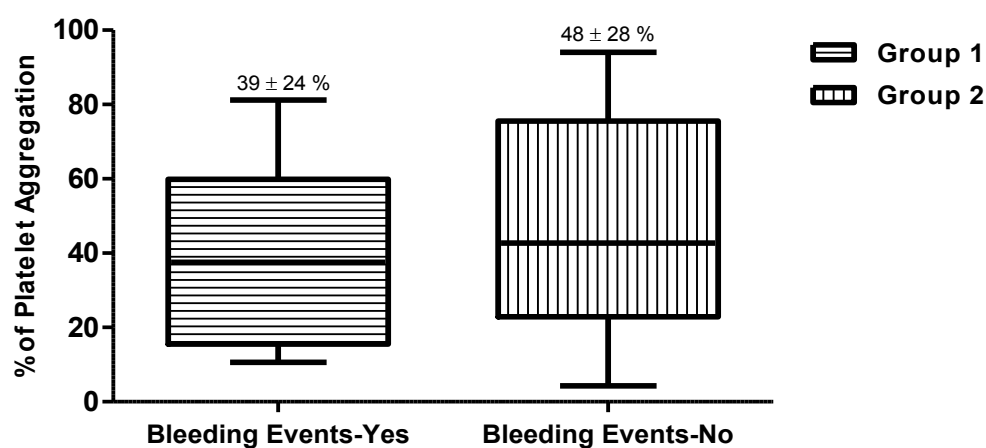


Figure 3.13: Platelet aggregation in response to 10 μmol/L of ADP measured at 4 minutes in two groups. Group 1-Patients who developed bleeding complications (N=21), Group 2-Patients who did not develop bleeding complications (N= 168) (P=0.791).

3.7.3. Platelet Aggregation Compared between Bleeders and Non-Bleeders

The mean (\pm SD) percentage of platelet aggregation in patients who bled (N=21/189) and did not (N=168/189) was $39 \pm 24\%$ and $48 \pm 28\%$ respectively. This was not statistically significant (Paired t test $P = 0.791$) (Figure 3.13).

3.7.4. Platelet Aggregation Compared between Ischaemic and Bleeding Complications

The mean (\pm SD) percentage of platelet aggregation in patients who developed an ischaemic complication (n=20) was $46 \pm 31\%$. The respective value in patients who developed bleeding complications (N=21) was $39 \pm 24\%$. The difference was not statistically significant (paired t test $P= 0.496$) (figure 3.14).

Figure 3.14: Platelet Aggregation in Patients with Bleeding and Ischaemic Complications

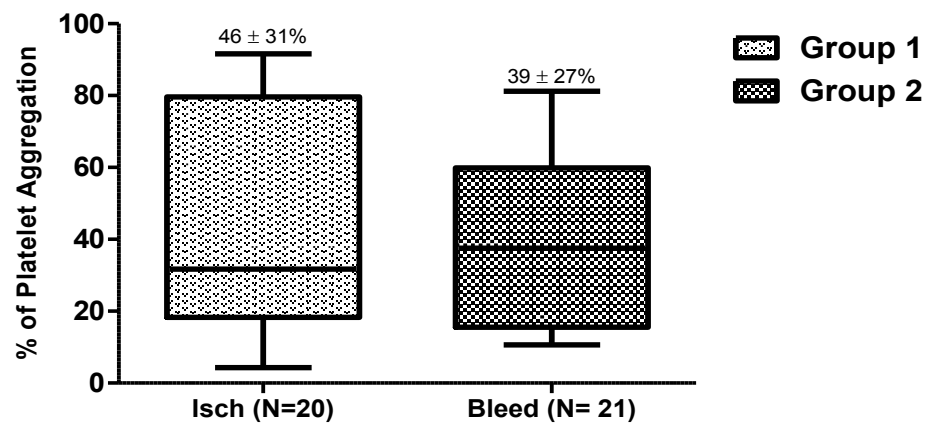


Figure 3.14: Platelet aggregation in patients who developed an ischaemic and bleeding complications. Group 1- Ischaemic events positive (N= 20), Group 2- Bleeding events positive (N=21) ($P= 0.496$).

3.8. Discussion

Our study assessed the utility of WBSPCA in determining the risk of ischaemic and bleeding events in patients treated with clopidogrel for ACS. Ischaemic events include CV related death, STEMI, NSTEMI, and stent stenosis or stent thrombosis needing revascularization. PCI-related increase in troponin alone was not considered as ischaemic event unless there were significant ECG findings/clinical symptoms or angiogram findings. Ischaemic events occurred in 20 out of 189 patients and bleeding events occurred in 21 out of 189 patients. There were no significant differences in the demographics of patients who developed ischaemic and bleeding complications compared to those who did not. The incidence of ischaemic event was numerically higher in non-responders compared to responders (14 % vs.9% P= 0.404). Similarly, the incidence of bleeding was higher in responders compared to non-responders (12.5 % Vs.9% P= 0.536). The mean percentage of platelet aggregation measured at 4 minutes of ADP interaction with the sample assessed by WBSPCA was $39 \pm 24\%$ in patients who had a bleeding event and $48 \pm 28\%$ in those who developed ischaemic complications. Despite numerical differences, neither of these values was significantly discriminating from those who did not sustain bleeding or ischaemic events.

WBSPCA measures the fall in single platelet count that occurs with aggregation. When hirudin anticoagulation is used, aspirin has no effect on platelet aggregation induced by the standard platelet agonist ADP so that ADP induced aggregation can be used to selectively define the inhibitory effects of clopidogrel. ADP is a key platelet agonist which acts via subtypes P2 receptors namely P2Y₁ and P2Y₁₂ receptors. As clopidogrel is a P2Y₁₂ antagonist, ADP is used to study the effect of clopidogrel. Addition of increasing concentrations of agonist will give increasing percentage of aggregation. Aggregation can be measured by the fall in single platelet count in the sample blood relative to the unstimulated whole blood sample (which acts as the control). A single platelet count of zero gives 100% aggregation (239).

The mean percentage of platelet aggregation (\pm SD) to 10 μ mol/L of ADP was 46 \pm 28%. As with other studies(101, 244, 245), the platelet aggregation inhibition by clopidogrel as assessed by the WBSPA shows marked inter individual variations (101). The prevalence of non-responders in our study was 37%. Different studies have used various cut-off to define non-responders. One of the commonly used cut-off value is <10% decrease in aggregation values obtained at baseline and after treatment with clopidogrel using various platelet function testing assays most commonly LTA (101). In one study, \leq 10% absolute reduction in 10 μ mol/L ADP-induced WBSPC aggregation from baseline to post clopidogrel time point was used. The prevalence of non-responders assessed by this definition was 37% for 300mgs of clopidogrel when the PFT was performed 4 hours post drug intake (245). In another study patients have been grouped as responders, semi responders and non-responders based on absolute platelet inhibition cut-off points of < 10%,10%-30%,>30% respectively (246). The validity of this definition is questionable given the interindividual variability in baseline ADP induced platelet aggregation. It has been postulated that this method can potentially overestimate the ischaemic risk and underestimate bleeding risk (247,248). Hence the platelet reactivity on treatment has been proposed as a better measure of thrombotic risk which we have adopted (241).

Several studies have consistently shown that the HPR is an independent risk factor for occurrence of ischaemic events following PCI (241). In our study, although there is no statistically significant difference in the occurrence of ischaemic events between the responders and the non-responders, the mean percentage of platelet aggregation in those who developed ischaemic complications was higher than those who did not. In our study, patients were managed both medically and by interventions. In the future, a study that truly evaluates the utility of this tool in patients who are exclusively managed by interventions would be ideal to determine its precise utility that would suit the current clinical practice.

The sensitivity of WBSPCA in predicting the bleeding risk assessed by this study appears to be slightly better compared with the test's ability to predict ischaemic events. There were two incidences of TIMI major bleed unrelated to CABG in this study. Both of them occurred in patients who were responders with the percentage of platelet aggregation value in the lowest group (12%, 29%). Similarly, although there was no statistically significant difference in the occurrences of bleeding events between the responders and non-responders, the mean percentage of platelet aggregation in patients who developed bleeding complications is less compared with those who did not. However, a larger study is required with more events to accurately determine the prognostic relevance of WBSPCA results in clopidogrel-treated patients.

Clopidogrel has proven benefit in the treatment of thrombotic events in patients with ACS. The platelet aggregation inhibition achieved by clopidogrel is not consistent and subjected to interindividual variations. This has been referred to as clopidogrel resistance or more precisely HPR. HPR has been found to have an increased risk for MACE. The reason for this variable antiplatelet effect of clopidogrel is multifactorial. It includes noncompliance, absorption problem, smoking, drug-drug interaction, intrinsic variation in platelet function before exposure to medication, obesity, renal dysfunction, and DM. This is also the reasons why PFT is poor in predicting ischaemic events in PCI treated ACS patients. A 600 mg loading dose of clopidogrel as opposed to a 300 mg loading dose only partially overcomes some of these issues (249)

Patients who are at increased risk for cardiac ischaemic events whilst on short term and long-term treatment with clopidogrel, as demonstrated by HPR has been identified by a number of ADP-dependent PFT. These include VerifyNow, Multiplate, TEG PlateletMapping system, PFA-100System and VASP. Of these, the VerifyNow assay is the most widely used as it has the advantage of being a point-of-care tool (POCT) and very user friendly. In fact, large observational studies have shown an independent relationship between HPR as measured by POCT PFT and

ischaemic events in patients undergoing PCI. However, there are no robust data on the relationship between HPR and ischaemic events in ACS patients managed medically. Initial small studies suggested that individualizing the antiplatelet therapy as guided by the PFT might reduce ischaemic events. These data resulted in low-level recommendation for use of PFT in clinical guidelines from the AHA/ACC and ESC. However, three large prospective randomized controlled trials, GRAVITAS (2,800 patients), ARCTIC (2,440 patients), and ANTARCTIC (877 patients), showed that personalized antiplatelet therapy based on point-of-care assessment of platelet function is not effective in reducing ischaemic events. Accepting the limitations of these trials, the final evidence does not support the concept of altering antiplatelet therapy based on PFT. Thus HPR may, at least partly, be a non-modifiable clinical risk factor in clopidogrel treated patients (249)

Some but not all studies of clopidogrel-treated patients suggest a relationship between low platelet reactivity and bleeding events. However, the link is weaker than that between HPR and thrombotic events and there is no convincing evidence that changing clopidogrel therapy based on PFT can result in a reduction in bleeding events.

3.9. Conclusion

In summary, we have investigated the usefulness of WBSPCA which has not been studied before in the context of its clinical utility to predict ischaemic and bleeding complications in patients treated with clopidogrel. Ischaemic events tended to occur in individuals with higher level of platelet aggregation and bleeding complications in patients with lower level of platelet aggregation. The incidence of ischaemic events was numerically higher in non-responders and the incidence of bleeding complications was numerically higher in responders although not statistically significant. With the increasing use of point-of-care test in assessing platelet function in diverse clinical scenarios, there is a promising scope for WBSPCA to become one potential tool in the future, although more work is needed to validate its utility in specific clinical conditions.

Chapter 4: Utility of Whole Blood Single Platelet Counting Assay in Assessing the Efficacy of Aspirin

4.1. Background and Aim

Aspirin has been in existence now for nearly 125 years and is one of the commonly used cardiovascular medications worldwide. Aspirin has gained an established role in the active treatment of ACS and in secondary prevention of CVD conditions. However aspirin alone was inferior in preventing MACE in patients with established CAD compared with a combination of aspirin and other antiplatelets like clopidogrel, prasugrel or ticagrelor (250). The reasons for the failure of aspirin to prevent vascular events solely is multifactorial and one of the aspects being the inability of aspirin to fight against the thrombus formation itself. These groups of patients are often referred to as aspirin low-responders (251). There is an increased incidence of MACE and other vascular complications in aspirin low-responders (252). Despite this wealth of information, monitoring the antiplatelet effects of aspirin is not recommended in routine clinical practice. The reason for this is largely due to lack of a single tool that can readily assess the antiplatelet effects of aspirin consistently. If that test is available, individually tailored antiplatelet therapy might gain wider acceptance.

The aim of this study is to assess the utility of WBSPCA monitoring the efficacy of aspirin in patients treated for ACS. We also aim to obtain data regarding the association between platelet aggregation inhibition measured by WBSPCA and serum TXB₂ in patients treated with aspirin.

Results

Platelet aggregation data is presented in 236 patients. Platelet aggregation data related to aspirin responders and low- responders and the incidence of ischaemic and bleeding complications are presented in 189 patients. Platelet aggregation in response to AA and its relationship to urine 11- dehydro TBX₂ is presented in 62 patients. Platelet aggregation in response to AA and its relationship with serum TBX₂ is presented in 98

patients. Demographic details about the whole study (N= 236) and patients who were assessed for ischaemic and bleeding complication (N=189) are presented in chapter 3. Demographic details of the patients assessed for urinary TBX₂ and serum TBX₂ are presented in table 4.1

Table 4.1

Parameters	Urine-TBX ₂ N=65	Serum –TBX ₂ N=98
Age	60	60
Sex	54/65 (Male)	80/98 (Male)
Risk Factors		
Diabetes Mellitus	10/65 (16%)	16/98 (18 %)
Hypertension	34/65 (55%)	47/98 (52%)
Smoking	39/65 (63%)	63/98 (69 %)
Dyslipidaemia	41/65 (66 %)	62/98 (69%)
Previous H/O of IHD	16/65 (26 %)	21/98 (84 %)

4.2. Descriptive statistical Data

The median percentage of platelet aggregation with their intergroup range (IQ) in response to increasing concentrations of AA (0.1, 0.2, 0.4, 0.8,1 mM) measured by WBSPCA at 30 seconds and 4 minutes are represented in figure 4.1 and 4.2 respectively. Detailed descriptive data presented in table 4.2

Table 4.2: Descriptive Statistics of the Platelet Aggregation at 30 Seconds and 4 Minutes by WBSPCA (N=236)

N=236	0.1AA 30S	0.2AA 30S	0.4AA 30S	0.8 AA 30S	1 AA 30S
Mean %	7	7	9	28	39
Median %	6	6	7	23	35
IQ range %	8	8	9	31	43
N=236	0.1AA 4M	0.2AA 4M	0.4AA 4M	0.8AA4M	1AA 4M
Mean %	14	15	17	25	34
Median %	15	15	16	20	23
IQ range%	11	11	13	17	36

Figure 4.1: Platelet Aggregation in Response to Increasing Concentrations of AA Measured at 30 Seconds by WBSPCA (N=236)

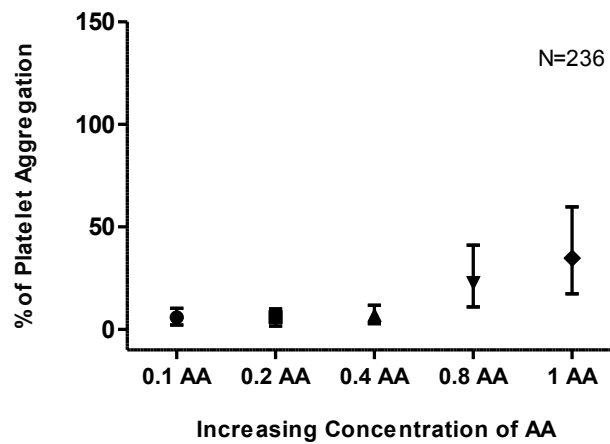


Figure 4.1: Percentage of platelet aggregation measured by increasing concentration of AA (0.1, 0.2, 0.4, 0.8, 1mM) measured at 30 seconds by WBSPCA. Data is represented as median with their IQ range N=236.

Figure 4.2: Platelet Aggregation to Increasing Concentration of AA Measured at 4 Minutes by WBSPCA (N=236)

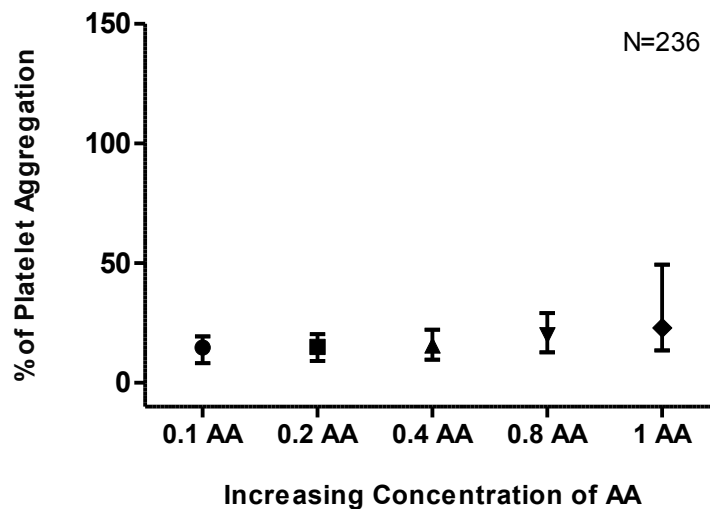


Figure 4.2: Percentage of platelet aggregation in response to increasing concentrations of AA (0.1, 0.2, 0.4, 0.8,1 mM) measured by WBSPCA at 4 minutes. Data is represented as median with (IQ) range. N=236

4.3. Interindividual Variation of Platelet Aggregation in Patients Treated with Aspirin

Platelet aggregation in response to 0.8mM AA was taken into consideration to assess the interindividual variation to the antiplatelet effect of aspirin. Patients were categorized according to their percentage of platelet aggregation at intervals of 10%. Predominantly the percentage of aggregation was found to be less than 50% with maximum frequency in the range of 20% followed by 10%. The data was not normally distributed (Shapiro–Wilk normality test $W=0.792$), (figure 4.3).

Figure 4.3: Interindividual Variation of Platelet Aggregation in Patients Treated with Aspirin

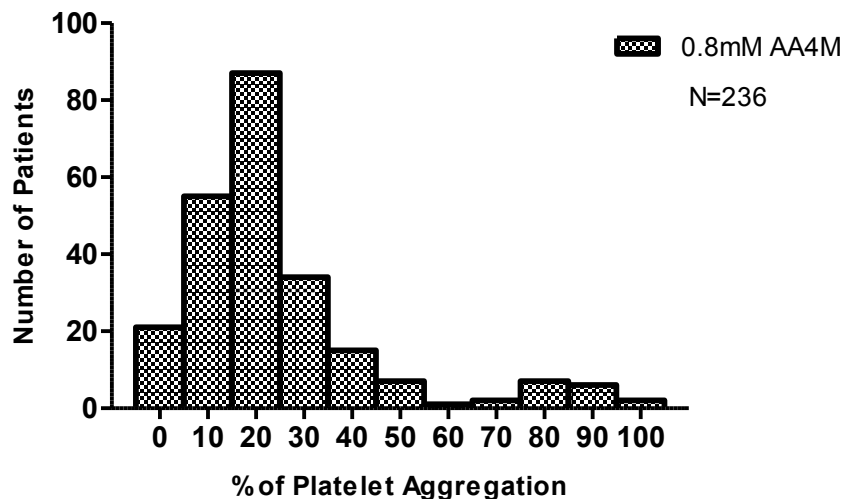


Figure 4.3: Interindividual variation in the percentage platelet aggregation in patients treated with aspirin. (N=236).

4.4. Prevalence of Aspirin Responders and Low Responders

The prevalence of aspirin responsiveness as assessed by this study is 76% (N=180/236). The rest of the patients, 24% were defined as slow-responders to the effect of aspirin (N=56/236). Considering the overlap between both aspirin and clopidogrel response, 52% of patients were responders to both aspirin and clopidogrel, (N=122/236), 24% (N=58/236)

responded only to aspirin but not to clopidogrel, 11% (N=26/236) responded only to clopidogrel but not aspirin, and 13% of patients did not show adequate platelet aggregation inhibition to both aspirin and clopidogrel (N= 30/236). The data is presented in figure 4.4. Their demographic profile is presented in table 4.3

Table 4.3

Parameters	Asp Resp Clop Resp N=122	Asp Low-Resp Clop Resp N=26	Asp Resp Clop Non- Resp N=58	Asp Low-Resp Clop Non-Resp N=30
Age	60 (32-88)	60 (37-80)	63 (40-86)	64 (39-83)
Male	62%	69%	55%	87%
Risk Factors				
Hypertension	52%	52%	58%	54%
Diabetes Mellitus	20%	26%	25%	21%
Dyslipidaemia	60%	52%	72%	61%
Previous H/O IHD	27%	43%	32%	36%
Smoking	58%	65%	58%	39%

Figure 4.4: Prevalence of Aspirin and Clopidogrel Non-Responders

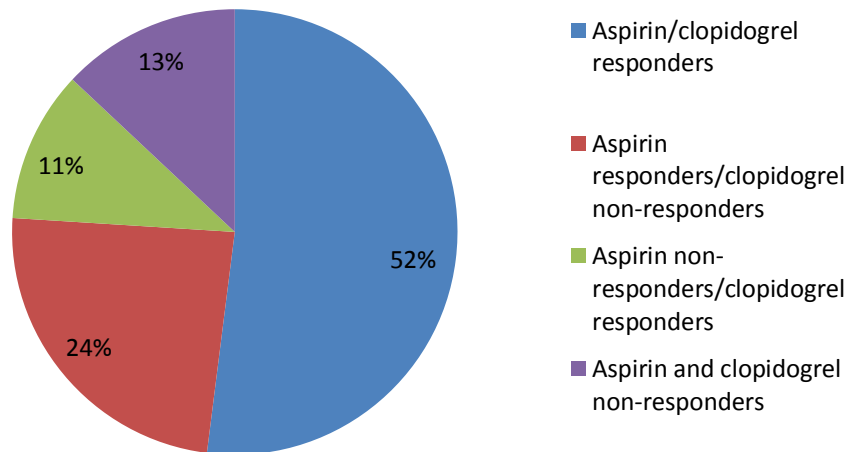


Figure 4.4: Prevalence of aspirin and clopidogrel responsiveness and non-responsiveness as assessed by WBSPCA, (N=236).

4.5. Incidence of Ischaemic and Bleeding Events

The incidence of ischaemic events in patients taking aspirin was 11% (N= 20/189) and the incidence of bleeding complications was 11% (N=21/189). The incidence of aspirin responders among the 189 patients was 75% (N = 142/189). The incidence of aspirin low-responders were 25% (N= 47/189).

4.6. Ischaemic Events – Platelet Aggregation

The median percentage of platelet aggregation in patients who developed ischaemic events was 23 % with an IQ range of 15 % in response to 0.8mM AA measured at 4 minutes. The median percentage of platelet aggregation in patients who did not develop ischaemic events was 20% with an IQ range of 17%. The mean percentage of platelet aggregation (\pm SD), between both the groups is presented in figure 4.5. There were no statistically significant differences in the median between both the groups, (Wilcoxon signed rank test, P=0.715).

Figure 4.5: Platelet Aggregation in Patients who Developed Ischaemic Events and Not

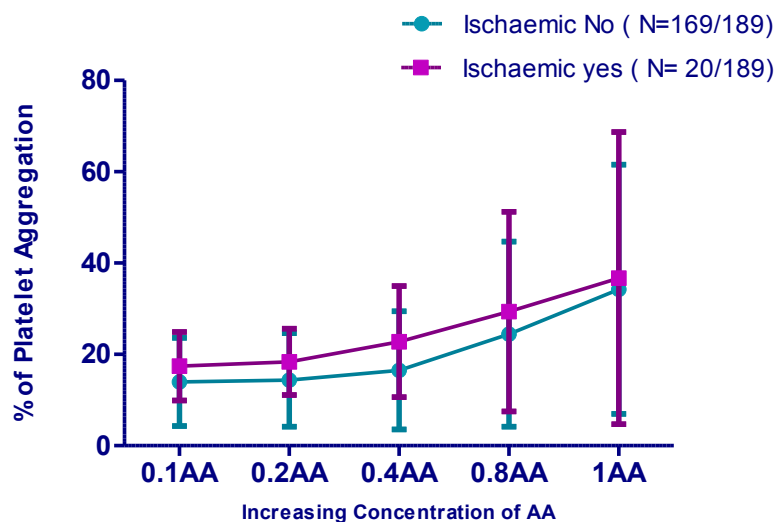


Figure 4.5: Mean percentage of platelet aggregation in response to 0.8mM AA measured at 4 minutes is 29% in patients who developed ischaemic events and 24% in patients who did not develop ischaemic events,(P= 0.715).

4.7. Incidence of Ischaemic Events in Aspirin Responders and Low-Responders

20 out of 189 patients developed ischaemic events whilst on aspirin. Of these 13 events occurred in patients who were responders to aspirin (N=13/142, 9%) and 7 events occurred in patients who were low-responders to aspirin (N=7/47, 15%). The incidence of ischaemic events were higher in aspirin low-responders compared with those who were aspirin responders. However, the difference was not statistically significant, Chi Square test (P=0.268, OR=0.576 and 95% CI=0.261-1.449) (figure 4.6).

Figure 4.6: Incidence of Ischaemic Events in Responders and Low-Responders

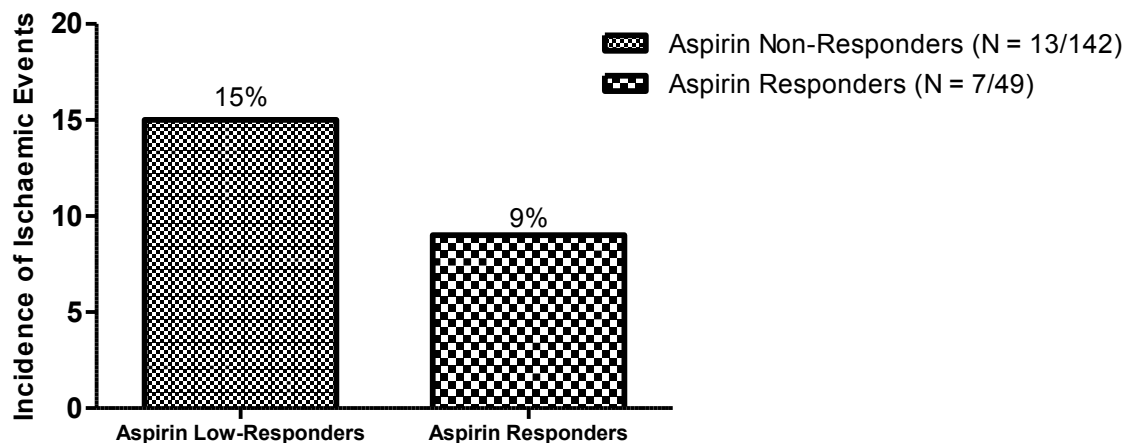


Figure 4.6: Ischaemic events occurred in 9% of responders and 15% of low-responders to aspirin (P= 0.268).

4.8. Bleeding Complications in Responders and Low-Responders to Aspirin

The median percentage of platelet aggregation in response to 0.8mM of AA measured by WBSPCA at 4 minutes in patients who developed bleeding complications was 16% with an IQ range of 8%. The median percentage of platelet aggregation in response to 0.8mM of AA measured by WBSPCA at 4 minutes in patients who did not develop bleeding complications was 21% with an IQ range of 17%. There was no

statistically significant difference in the median, (Mann Whitney U test, P= 0.076) (Figure 4.7).

Figure 4.7: Platelet Aggregation in Bleeding Complications

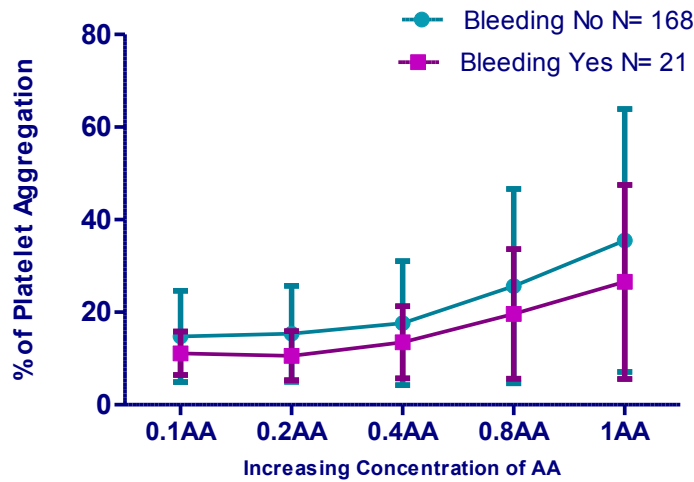


Figure 4.7: Mean percentage of platelet aggregation in bleeders is 20% and in non-bleeders is 26% (P= 0.076). (Total number = 189. Bleeding happened in 21/189).

4.9. Incidence of Bleeding Events in Responders and Low-Responders

Bleeding complications occurred in 21 patients (N= 21/189, 11%). Of the 21 events 19 happened in responders and the remaining 2 occurred in low-responders (N= 19/142, 13% vs. 2/47, 4%). Although the incidence was higher in aspirin responders, this was not statistically significant, (Chi Square test P=0.085, OR = 3.476 and 95% CI = 0.778-15.53) (Figure 4.8).

4.10. Platelet Aggregation -Ischaemic and Bleeding Complications

The mean percentage of platelet aggregation in patients who developed ischaemic complications was higher than those who developed bleeding complications (29±22% vs. 20 ±14%). There was a statistically significant difference in the median between the two groups (P= 0.017) (figure 4.9).

Figure 4.8: Bleeding Complications in Aspirin Responders and Low-Responders to Aspirin

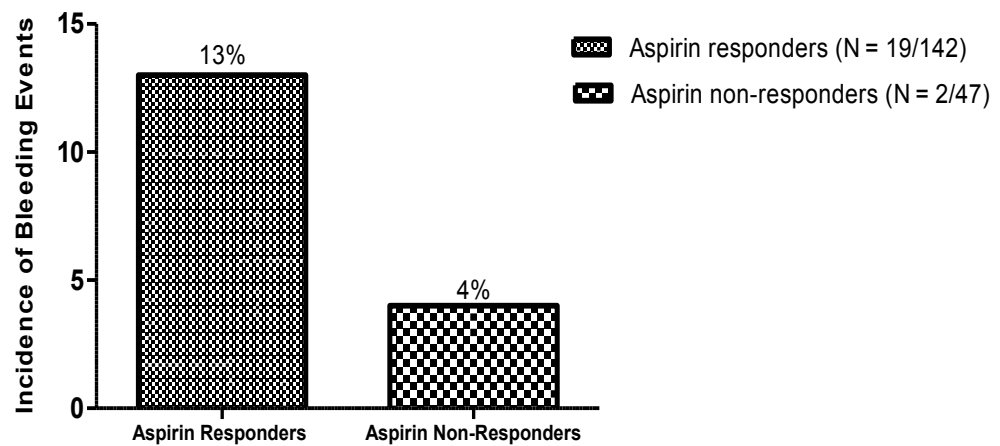


Figure 4.8: Bleeding complications occurred in 13% of responders and 4% of low-responders ($P= 0.085$). ($N=21/189$)

Figure 4.9: Platelet Aggregation in Ischaemic and Bleeding Events

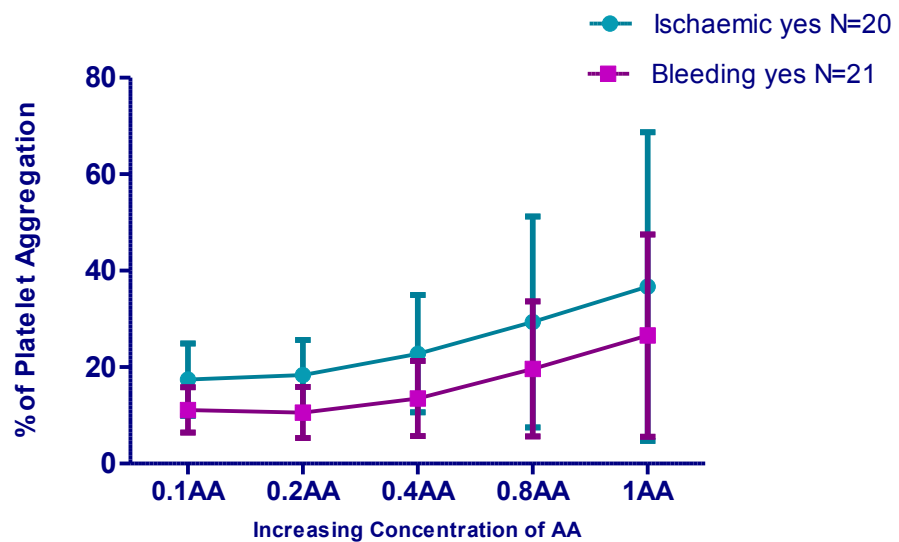


Figure 4.9: The mean percentage of platelet aggregation in response to 0.8mM AA measured at 4 minutes is $29 \pm 22\%$ for ischaemic complications and $20 \pm 14\%$ for bleeding complications ($P= 0.017$).

4.11. Correlation of Platelet Aggregation Assessed by WBSPCA and Urine 11- dehydro TXB₂ Levels.

In 62 patients urine 11-dehydro TXB₂ levels were measured. The median increase in urine TXB₂ was 406pg/ml with an IQ range of 621pg/ml. There was no correlation between the urine 11-dehydro TXB₂ levels and the percentage of platelet aggregation in response to 0.8mM of AA measured by WBSPCA at 4 minutes, (Spearman's correlation co-efficient $r=0.030$, $P= 0.814$). Among the 62 patients 39 (63%) were found to be responders and the remaining 23 (37%) were low-responders. The median of the urine 11-dehydro TXB₂ in responders is 406pg/ml with an IQ range of 625pg/ml. The median of the same in low-responders is 420pg/ml with an IQ of 582 pg/ml. There was no significant difference in the median between the two groups, (Wilcoxon Signed–rank test, $P=0.466$) (Figure 4.10)

Figure 4.10: Correlation between Platelet Aggregations in Response to AA and Urine 11-dehydro TXB₂ Levels (N=62)

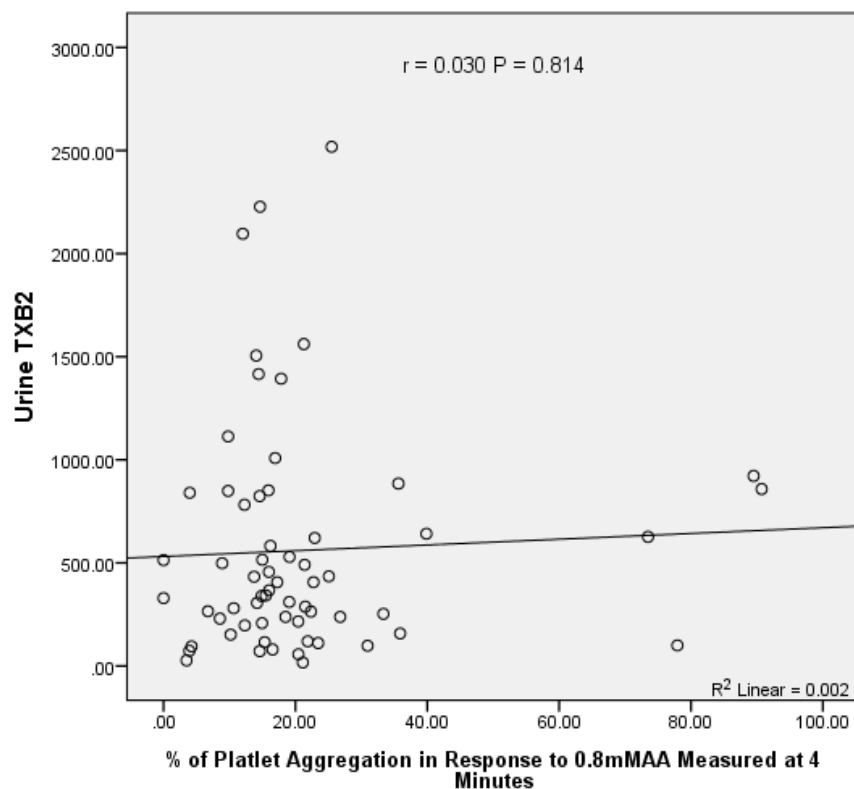


Figure 4.10: Correlation between platelet aggregations in response to 0.8mM AA and urine TXB₂ levels ($r= 0.030$, $P= 0.814$). (N=62)

4.12. Correlation between Platelet Aggregations Assessed by WBSPCA and Serum TXB₂ Levels

Serum TXB₂ levels were measured in 98 patients. The median value of the serum TXB₂ is 14pg/ml with an IQ range of 51-206pg/ml. There was a moderate, but significant correlation between the serum TXB₂ levels and platelet aggregation in response to 0.8 mM AA assessed by WBSPCA, (Spearman's correlation co-efficient $r = 0.368$, $P=0.000$) (Figure 4.11).

Figure 4.11: Correlation between Platelet Aggregations Assessed by WBSPCA at 4 Minutes and Serum TBX₂ Levels

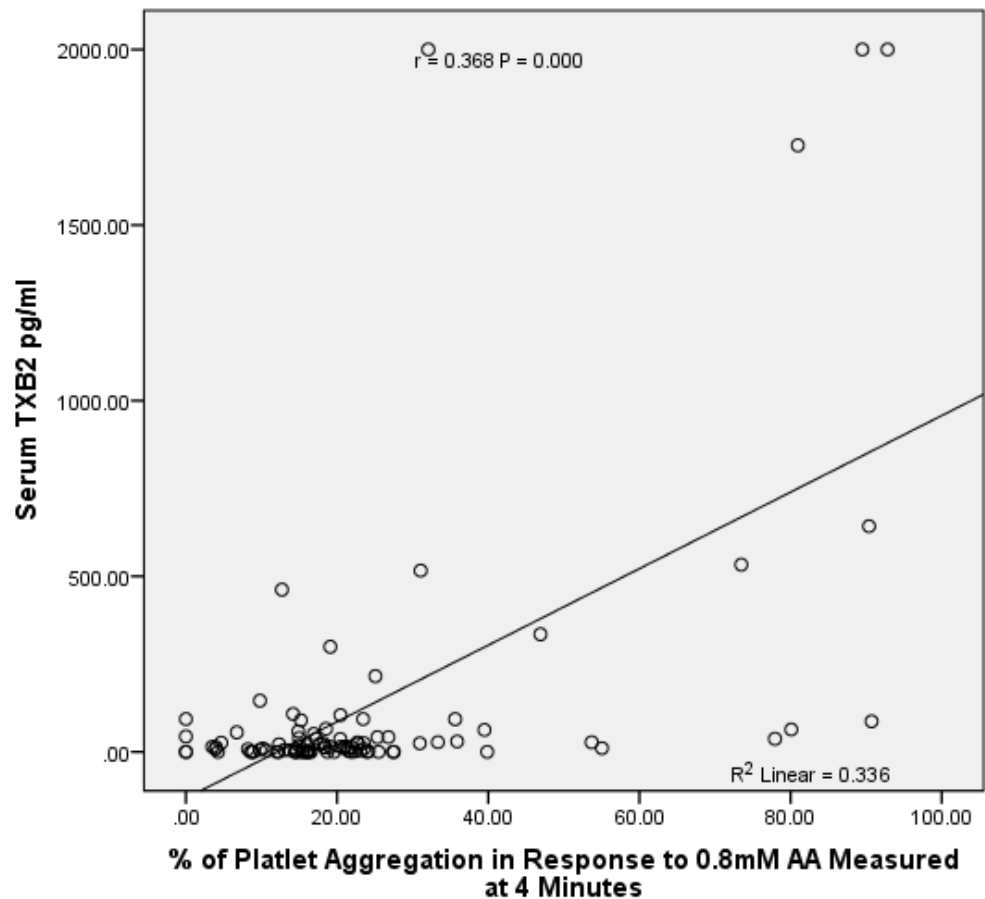


Figure 4.11: Correlation between serum TBX₂ and percentage of platelet aggregation in response to 0.8mM of AA ($r = 0.368$, $P = 0.000$).

55 out of 98 (56%) patients were aspirin responders and 43 out of 98 (44%) were aspirin low responders. The median increase in serum TXB₂ levels in responders against aspirin low responders were 8pg/ml and 27pg/ml respectively. There was a significant difference in the median between the two groups, (Wilcoxon-signed rank test, P=0.015). The ROC analysis was not effective enough to discriminate a cut-off value to identify responders from low responders (AUC=0.550, upper bound 0.637, lower bound 0.463 and P=0.225).

Serum TXB₂ levels and ischaemic/bleeding outcomes were available in 61 patients. The median serum TXB₂ level in bleeders (11/61 18%) was 8pg/ml with an IQ range of (0-146pg/ml). The respective data for those who did not develop bleeding complications (N = 50/61 82%) was 12pg/ml with an IQ range of (0- 42pg/ml). The difference in the median was not significant (Wilcoxon–Signed rank test P=0.465). Likewise, ischaemic events occurred in (N = 12/61 20%) of the patients. There was no difference in the median between those who developed ischaemic complication and did not (371± 13-2000pg/ml vs. 8± 0-2000pg/ml, P= 0.301).

4.13. Correlation between Urine 11-dehydro TBX₂ and Serum TBX₂

63 patients had their serum and urine TBX₂ levels measured. There was no correlation between both the parameters, (Spearman's correlation coefficient $r = -0.017$, P= 0.96) (figure4.12).

Figure 4.12: Correlation between Serum and Urine TBX₂ (N=63)

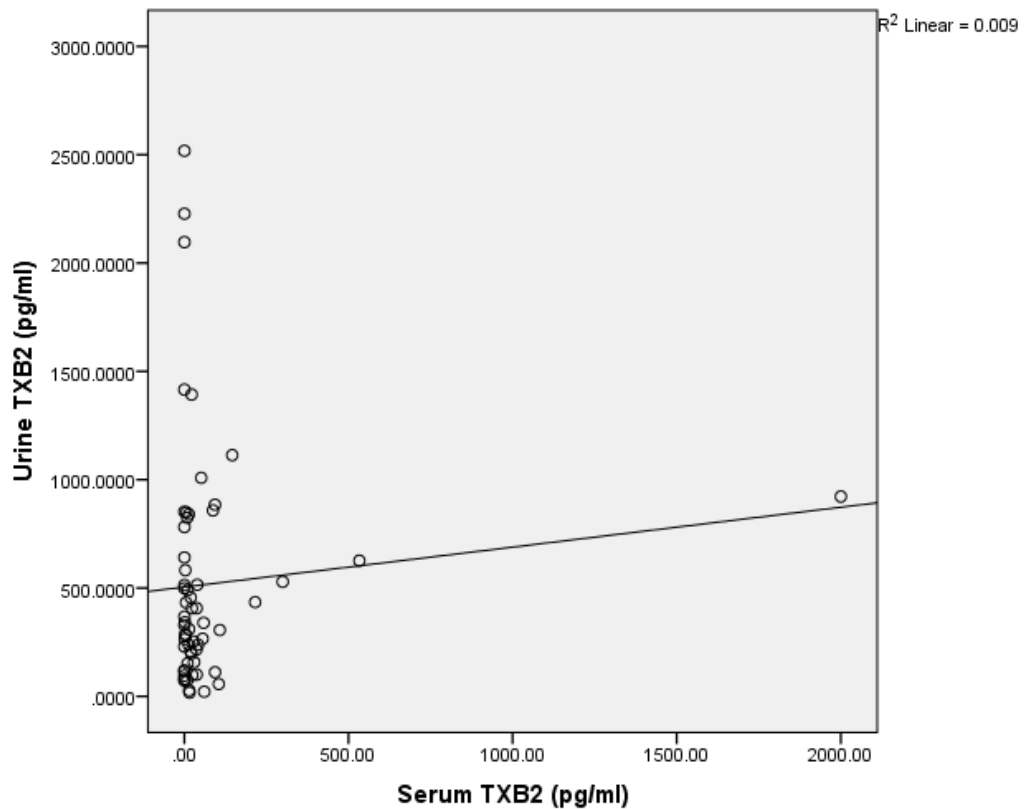


Figure 4.12: Correlation between Serum and Urine TBX₂($r=-0.017$, $P=0.96$) (N=63).

4.14. Discussion

This study aimed to glean information regarding the utility of WBSPCA in assessing the efficacy of aspirin in patients with ACS. This assay as a tool to measure the platelet aggregation in relation to aspirin has not been widely studied and hence defining aspirin low responders was difficult. Low response to aspirin has been defined as platelet aggregation $\geq 30\%$ and those with a value of $<30\%$ were considered to be aspirin responders using LTA in response to 0.5mM of AA (253). In one study, aspirin low responders were defined as platelet aggregation induced by AA $\geq 30\%$ (254) which is adopted in our study.

The prevalence of aspirin low-responders in our study was 24%. The prevalence of aspirin low-responders in other studies varies between 5 to 40%. Clinical, demographical, genetic factors, and physiology of platelet

aggregation all have been attributed to this phenomenon (253). There is no single PFT that has been advocated as a standard to measure aspirin low-response which by itself can be a dynamic process (255). In one meta-analysis the prevalence of aspirin low- responders was higher when whole blood counting assay such as platelet function analyzer 100 assay was used, compared with LTA (256).

The incidence of ischaemic events was higher in the low-responders group compared to the responders, but failed to gain statistical significance ($P=0.268$, $OR =0.576$). The median percentage of platelet aggregation was higher in patients who developed ischaemic complication compared with those who did not, but not statistically significant ($P=0.715$). In one study with a sample size of 120 patients with peripheral artery disease (PAD), the prevalence of aspirin resistance as assessed by VerifyNow assay was 26%. Aspirin resistance was associated with significantly higher rates of MACE. In a meta-analysis conducted by Krasopoulos and colleagues, which included 2,930 patients with CV disease from 20 studies, the prevalence of aspirin resistance was 28% (810 patients). CV-related events occurred in 41% of these patients (256, 257).

The ISAR-ASPI (Intracoronary Stenting and Antithrombotic Regimen-Aspirin and Platelet Inhibition) assessed the utility of high on aspirin platelet reactivity (HAPR) as a possible prognostic biomarker in PCI treated patients. This is a large study that included 7,090 consecutive PCI treated patients. Platelet function was assessed with Multiplate analyzer before PCI. Death and stent thrombosis at one year were measured. 1,414 patients were found to have HAPR as measured by the Multiplate measurement. There was a significant risk of death and stent thrombosis at one year in patients who had HAPR (6.2% vs.3.7% $P= <0.0001$). On the other hand, the ADAPT-DES trial that included 8,665 PCI treated patients did not show any significant association between HAPR and ischaemic outcomes including death and stent thrombosis at one year. The precise reason for this wide variation in outcome is unknown. Possible explanations include timing of blood sampling, type of PFT employed, and different cut-off point to define HAPR. To be more specific,

in the ADAPT-DES study blood testing was performed day one post-PCI and VerifyNow PFT was used to assess platelet function. The prevalence of HAPR in the ISAR-ASPI study was 20 % and 5.6% for the ADAPT-DES study (258)

Similarly, the incidence of bleeding complications tended to be higher in patient who responded well to aspirin compared with those who were low-responders though this was not statistically significant ($P=0.085$, $OR=3.476$). The median percentage of platelet aggregation is lower in patients who developed bleeding complications compared with those who did not, but not significant ($P=0.076$). The importance of aspirin and clopidogrel hyper responders and its clinical implications in causing bleeding complications is now being increasingly recognized(254). In one study, the incidence of CABG related bleeding was higher in aspirin hyper responders compared to normal responders and hypo responders (259). This study used PFA-100 to assess aspirin response. The incidence of bleeding was found to be higher in hyper responders to aspirin in Japanese patients (260). The incidence of minor bleeding was found to be high in patients treated with aspirin and who were found to be hyper responders to the same having the need for discontinuation of treatment with aspirin (261).

There was no correlation between the urinary 11-dehydro TXB_2 and platelet aggregation in response to 0.8mM AA measured by WBSPCA at 4 minutes ($P=0.466$). A recent study in Chinese patients have failed to establish any correlation or agreement between the urine 11-dehydro TXB_2 levels and platelet aggregation measured by LTA (262). Moreover it has been well acknowledged that the measurement of urinary 11-dehydro TXB_2 is not a reliable tool to estimate the effect of aspirin on platelet aggregation (253).

The serum TXB_2 levels were significantly lower in responders compared with patients who were low-responders to aspirin. There was a statistically significant positive correlation between the serum TXB_2 levels and platelet aggregation in response to 0.8mM AA measured by WBSPCA at 4 minutes ($r=0.368$, $P=0.000$). In one study Gerber *et al* showed a significant correlation between serum TXB_2 and five other PFT namely

VerifyNow point-of-care system, Cone and Plate analyzer, Whole blood aggregometry using electrical impedance and with PFA-100. In whole blood aggregometry AA, ADP and collagen were used as agonist (263). To the best of our knowledge our study is the first to demonstrate a correlation between serum TXB₂ and WBSPCA.

In summary, we observed a numerically higher incidence of ischaemic events in aspirin low responders as assessed by WBSPCA and an increased incidence of bleeding complications in responders. There was a statistically significant correlation found between serum TXB₂ levels and platelet aggregation in aspirin treated patients assessed by WBSPCA. No difference was found in the levels of serum TXB₂ in patients who developed ischaemic and bleeding complications although numerically the values were higher in the ischaemic group and lower in the bleeding groups. There was no correlation between the urine 11-dehydro TXB₂ and serum TXB₂ as well as the platelet aggregation indicating the non-specific nature of urinary measurement of TXB₂.

4.15. Conclusion

The platelet aggregation assessed by WBSPCA in patients treated with aspirin correlated well with serum TXB₂ levels considered to be the gold standard to measure aspirin response. The incidence of ischaemic events was numerically higher in aspirin low-responders and the incidence of bleeding events was numerically higher in aspirin responders although the difference was not statistically significant. The median percentage of platelet aggregation was higher in patients with ischaemic complications and lower in patients with bleeding complications, again not statistically significant. HAPR as a biomarker to predict clinical outcomes in aspirin treated patients although not well accepted cannot be dismissed as an option just as yet given the fact that there is no universal PFT that has been accepted to test this parameter. As long as the quest for the ideal PFT continues, WBSPCA can always be a potential option that can be considered if more information is available through large scale studies.

Chapter 5: Comparison of Whole Blood Single Platelet Counting Assay and Light Transmission Aggregometry in Assessing Platelet Aggregation in Patients Treated with Clopidogrel

5.1. Background and Aim

Clopidogrel, a P2Y₁₂ inhibitor plays a crucial role in the treatment of ACS both in medical management and by intervention. Despite the introduction of newer P2Y₁₂inhibitors like prasugrel and ticagrelor, clopidogrel still remains the most common drug used globally in this class of medications due to its wide availability and cost effectiveness. However, the clinical benefits obtained through clopidogrel are relatively limited. One among the various reasons attributed to this failure of clopidogrel to prevent thrombotic events despite treatment is the significant interindividual variation in the degree of platelet inhibition achieved by the drug (101). At the same time increasing evidence suggest that bleeding complications are common in patients who are hyper responders to P2Y₁₂ inhibitors particularly clopidogrel (264). This raises important safety issues as patients with ACS are more likely to receive a cocktail of antiplatelets and anticoagulants, and currently these are unmonitored. It has been increasingly acknowledged that future research is warranted in order to assess an individual's risk for bleeding, to guide the use of antiplatelet and antithrombotic medications (265).

None of the PFT is considered to be gold standard to monitor the effects of clopidogrel although a few points-of-care test (POCT) have been recommended accepting their limitations (265). We have shown in the previous two chapters that the incidence of ischaemic events were numerically higher in clopidogrel and aspirin non-responders and the incidence of bleeding events were numerically higher in responders to clopidogrel and aspirin assessed by WBSPCA. Previous studies have shown the effectiveness WBSPCA to monitor the efficacy of GP IIb/IIIa inhibitors (244). We aim to determine the correlation between WBSPCA and LTA, the gold standard for measuring platelet aggregation, in

assessing the platelet aggregation inhibition in response to ADP in clopidogrel treated patients.

5.2. Results-Demographics

Platelet testing was performed in 63 patients. Demographic data is available in 60 patients which are summarized in Table 5.1. Dyslipidaemia and hypertension were identified as the most common risk factor in this study, 62% and 60 % respectively. Half of the patients were smokers. 13 % of the patients had diabetes. 2/3rd of the patients were treated for NSTEMI (68%) and the remaining for either STEMI or UA. All patients received aspirin, clopidogrel, LMWH for treatment of ACS and unfractionated heparin in the lab before proceeding to PCI.

Table 5.1: Demographic and Clinical Characteristics (N=60)

Age	62 ± 22
Men	71%
Risk Factors	
Hypertension	60%
Diabetes	13%
Dyslipidaemia	62%
Smoking	50%
Previous coronary artery disease	35%
Diagnosis	
NSTEMI	68%
STEMI	15%
Unstable Angina	17%
Medications	
Aspirin	100%
Clopidogrel	100%
LMWH	100%
Heparin	100%
GP IIb-IIIa Inhibitor (Reopro)	10%
β Blocker	91%
ACEI/ARB	94%
Statins	100%
Angiogram Findings	
Single vessel disease	55%
Double vessel disease	32%
Triple vessel disease	13%

Figure 5.1: Mean Percentage of Platelet Aggregation Assessed by WBSPCA (N= 63)

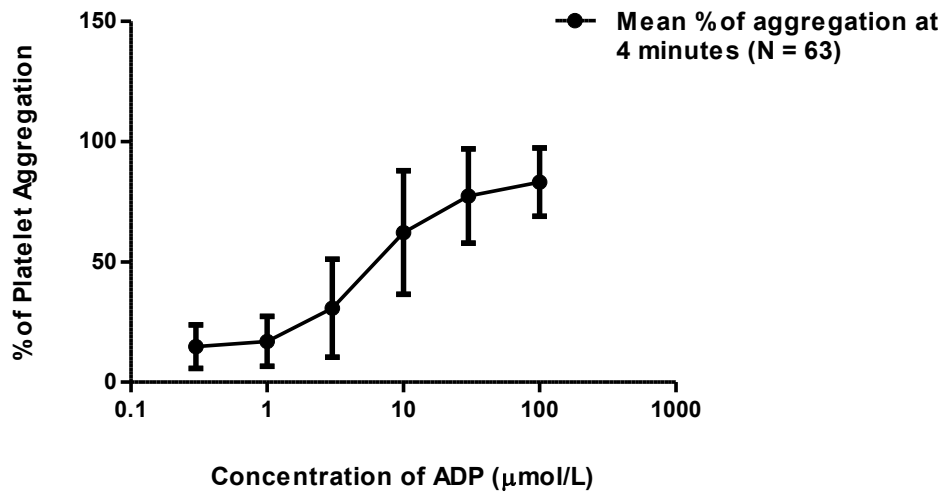


Figure 5.2: Mean percentage of Platelet Aggregation Assessed by LTA (N=63)

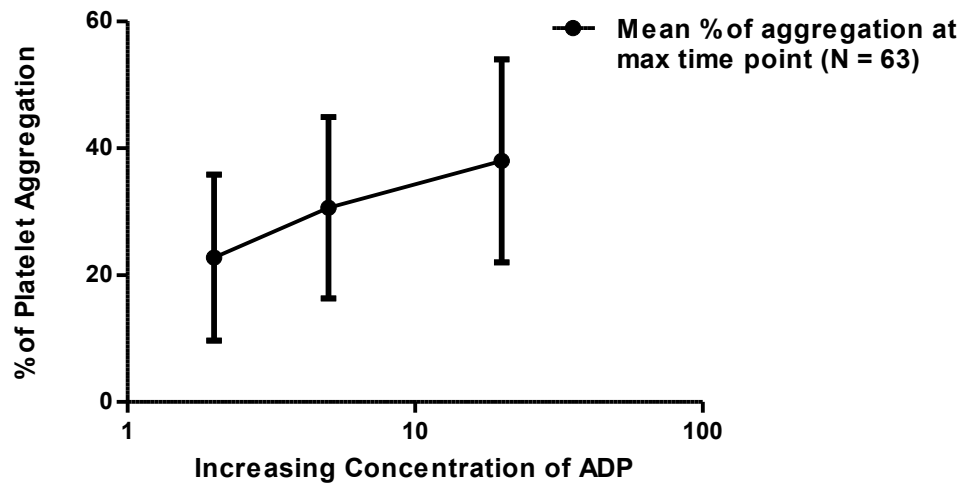


Figure 5.1: Mean (\pm SD) percentage of platelet aggregation in response to increasing concentrations of ADP (0.1, 0.3, 1, 3, 10, 30, and 100 $\mu\text{mol/L}$) assessed by WBSPCA.

Figure 5.2: Mean (\pm SD) percentage of platelet aggregation in response to 2, 5 and 20 $\mu\text{mol/L}$ of ADP assessed by LTA.

5.3. Platelet Aggregation Data

The mean percentage of platelet aggregation in response to 10µmol/L ADP at 4 minutes assessed by WBSPCA was 61 ± 15%. Similar values for 5µmol/L ADP and 20µmol/L ADP measured at maximum time point assessed by LTA were 31 ± 15% and 37 ± 17% respectively, presented in figure 5.1 & 5.2. Descriptive data is presented, (Appendix 4).

5.4. Correlation between WBSPCA and LTA

Increasing concentrations of ADP (2, 5, and 20 µmol/L) using LTA assay was correlated with 10µmol/L of ADP measured at 4 minutes using WBSPCA. Platelet aggregation in response to 2, 5, 20µmol/L of ADP at maximum time points assessed by LTA was normally distributed so was platelet aggregation in response to 10µmol/L of ADP measured at 4 minutes assessed by WBSPCA.

5.4.1. Correlation between 10µmol/L-ADP-WBSPCA and 2µmol/L-ADP-LTA

The percentage of platelet aggregation in response to 10µmol/L of ADP assessed by WBSPCA was correlated with platelet aggregation in response to 2µmol/L of ADP measured as maximum and final response (end of 5 minutes after the addition of agonist ADP) assessed by LTA. Spearman's correlation for 2µmol/L of ADP at final and Pearson's correlation for 2µmol/L of ADP at maximum time point were used. Moderate but statistically significant correlation existed between 2µmol/L of ADP measured at maximum time point in LTA and 10µmol/L of ADP measured at 4 minutes in WBSPCA ($r= 0.503$ $P<0.0001$). However, there was no correlation between 2µmol/L of ADP measured at final time point assessed by LTA and 10µmol/L of ADP measured at 4 minutes assessed by WBSPCA ($r= 0.253$, $P= 0.045$).

5.4.2. Correlation between 5µmol-ADP-LTA and 10µmol/L- ADP - WBSPCA

Using Pearson's correlation, the platelet aggregation in response to 5µmol/L of ADP measured at maximum time point assessed by LTA was

correlated with platelet aggregation in response to 10µmol/L of ADP measured at 4 minutes assessed by WBSPCA. There was a strong positive correlation between platelet aggregation assessed by WBSPCA and LTA which was statistically significant ($r=0.611$ $P < 0.0001$) (figure 5.3).

Figure 5.3: Correlation between 10µmol/L-ADP-WBSPCA and 5µmol/L- ADP-LTA (N=63)

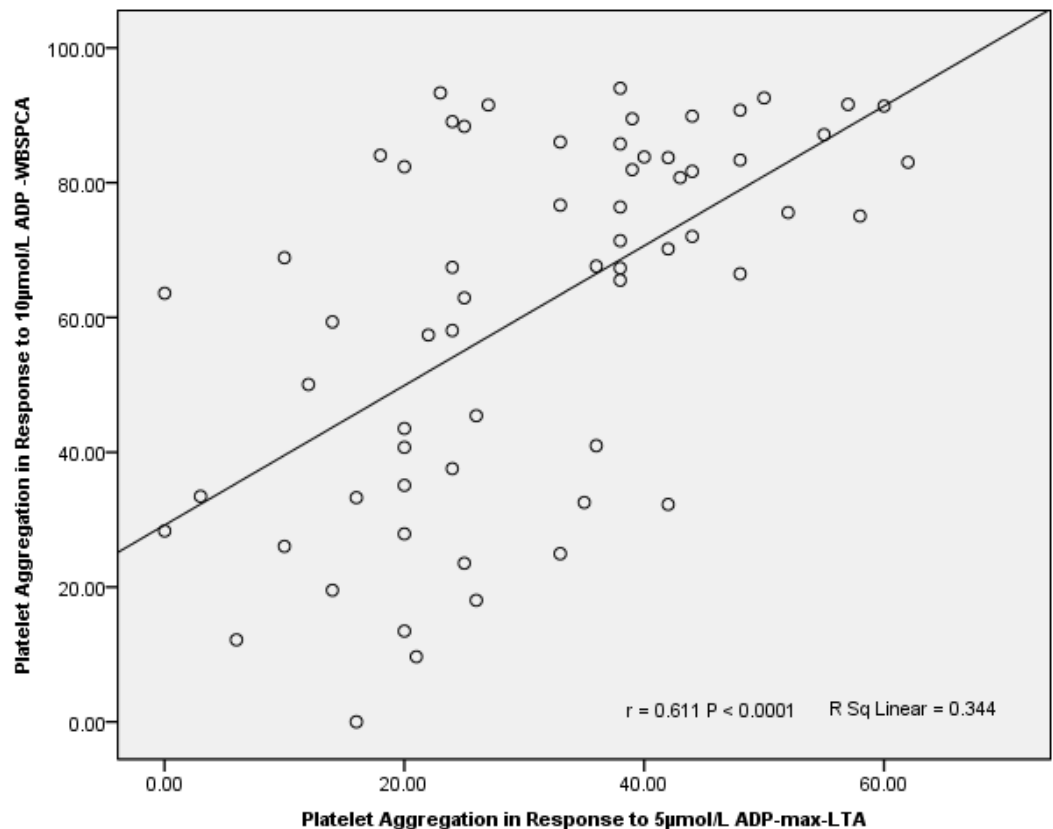


Figure 5.3: Strong positive linear correlation which was statistically significant ($r = 0.611$, $P < 0.0001$, $R^2 = 0.373$) (N=63)

Similarly, Spearman's rank correlation was employed to study the correlation between the platelet aggregation in response to 10µmol/L of ADP measured by WBSPCA after 4 minutes of the agonist contact with blood (data normally distributed) and platelet aggregation in response to 5µmol/L of ADP assessed by LTA after 5 minutes of agonist contact (data

not normally distributed). There was a moderate positive, non-linear correlation between both the parameters that was statistically significant, but with a weak co-efficient of determination ($r = 0.567$, $P < 0.001$, $R^2 = 0.206$) (figure 5.4).

Figure 5.4: Correlation between 10 μ mol/L-ADP-WBSPCA and 5 μ mol/L ADP-LTA (N=63)

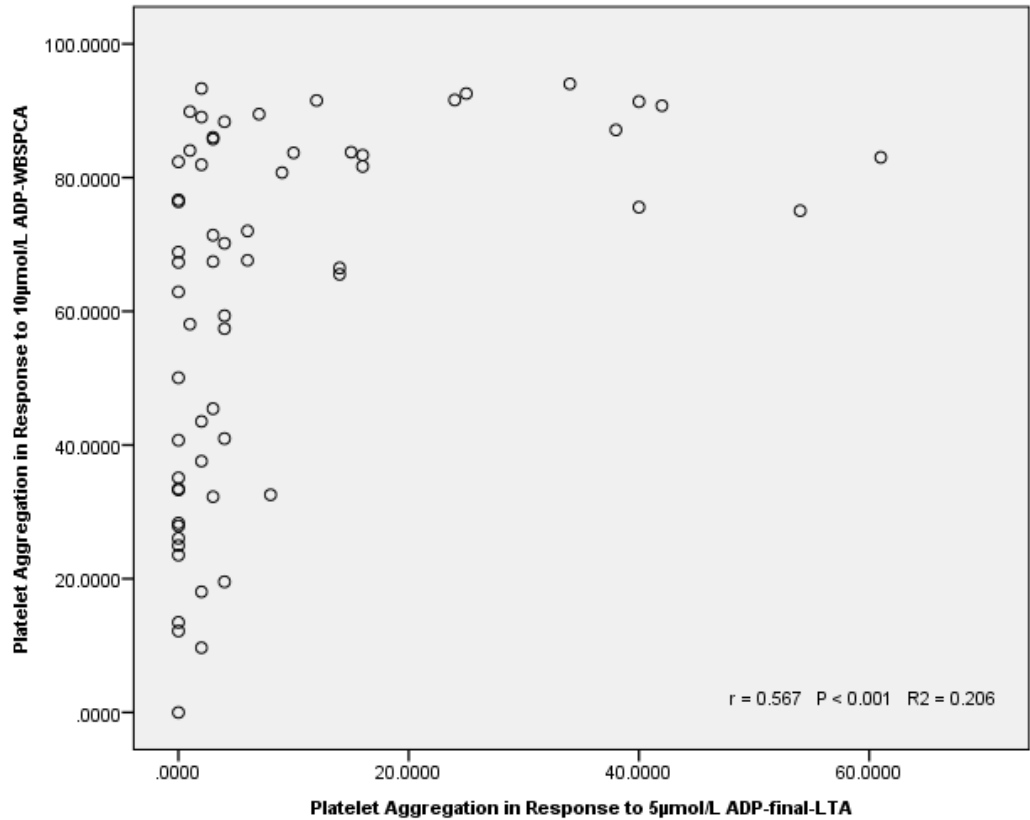


Figure 5.4: There was a moderate non-linear positive correlation ($r = 0.567$) that was statistically significant, ($P < 0.001$) with weak co-efficient of determination ($R^2 = 0.206$) (N=63).

5.4.3. Correlation between 20 μ mol/L-ADP-LTA and 10 μ mol/L- ADP-WBSPCA

Pearson's correlation showed a moderate positive correlation between the platelet aggregation in response to 20 μ mol/L of ADP measured at maximum time point of the agonist contact with the blood assessed by

LTA and platelet aggregation in response to 10µmol/L of ADP assessed by WBSPCA ($r= 0.507$) which was statistically significant ($P < 0.0001$). However, the co- efficient of determination was weak (R^2 of 0.257) (figure5.5).

Figure 5.5: Correlation between 10µmol/L-ADP-WBSPCA and 20µmol/L-ADP-LTA (N=63)

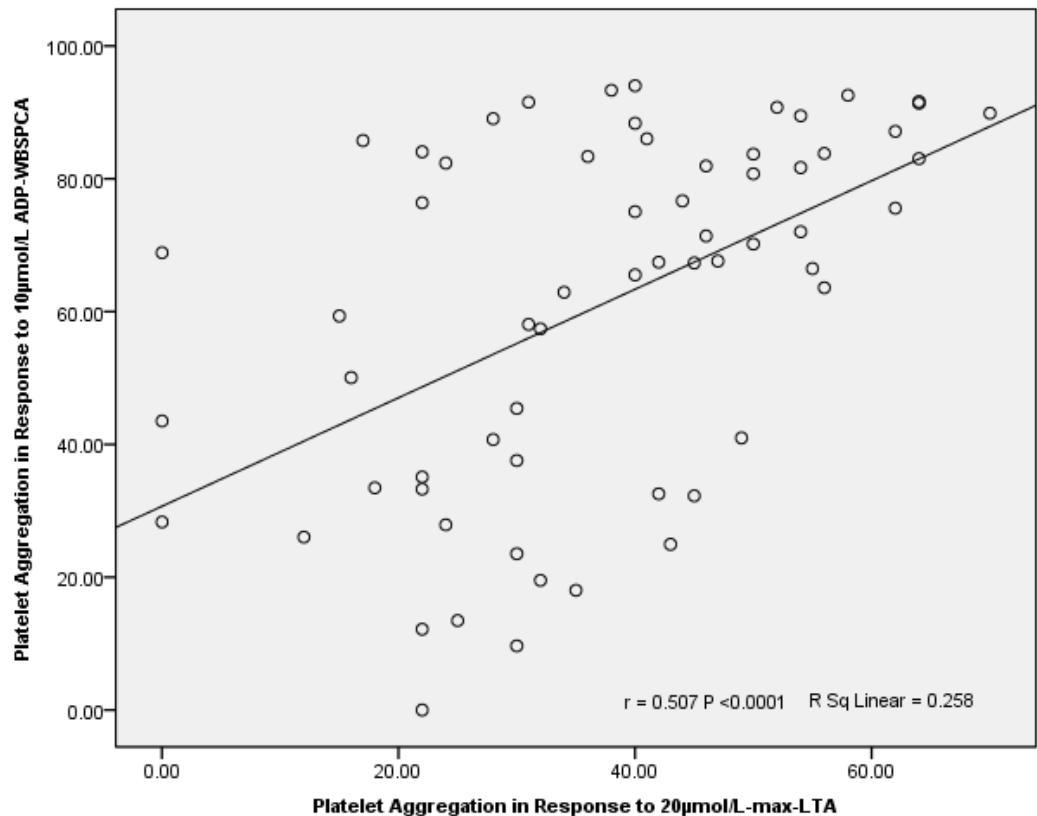


Figure 5.5: Moderate positive correlation was seen ($r=0.507$) that was statistically significant ($P < 0.0001$).

There was a moderate positive correlation in platelet aggregation in response to 20µmol/L of ADP measured at final time point assessed by LTA and platelet aggregation in response to 10µmol/L of ADP measured at 4 minutes assessed by WBSPCA ($r=0.599$) that was statistically significant ($P < 0.0001$)(figure 5.6). A summary of all the correlation coefficient (r) and their statistical significance values P are presented in table 5.2.

Figure 5.6: Correlation between 10µmol/L-ADP-WBSPCA and 20µmol/L- ADP-LTA (N=63)

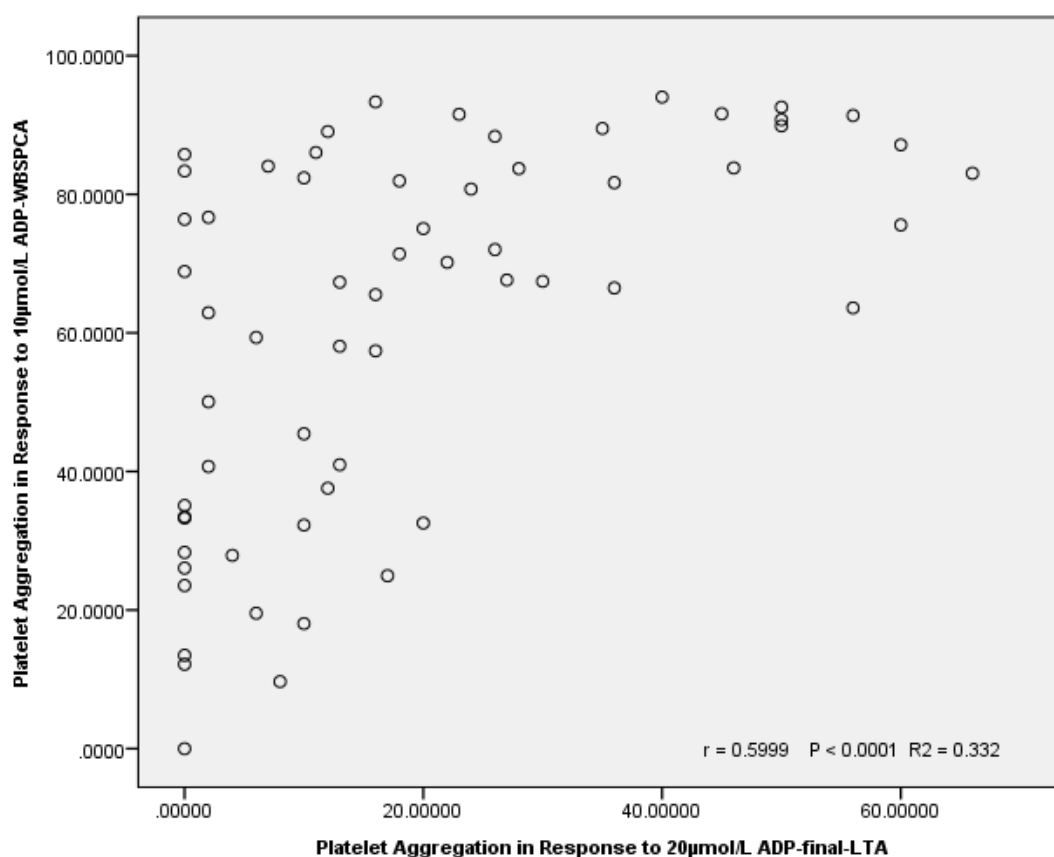


Figure 5.6: A moderate positive correlation ($r = 0.599$) which was statistically significant ($P < 0.0001$) with a weak co-efficient of disintegration ($R^2 = 0.332$) (N=63).

Table 5.2: Correlation co-efficient r and P between 10µmol/L-ADP-WBSPCA and Varying Concentration of ADP-LTA

Time of contact	ADP	r	P
Max	2µmol/L	0.503	<0.0001
Final	2µmol/L	0.253	0.045
Max	5µmol/L	0.611	<0.0001
Final	5µmol/L	0.567	<0.0001
Max	20µmol/L	0.507	<0.0001
Final	20µmol/L	0.599	<0.0001

Max = Maximum time point of contact with the agonist
 Final = Final time point of contact with the agonist

5.5. Agreement between WBSPCA and LTA – The Kappa Statistics

Although a moderate to strong correlation was demonstrated between WBSPCA and LTA they failed to agree significantly. Using the defined cut-off, many patients who were identified as non-responders by WBSPCA were identified as responders by LTA.

5.5.1. Agreement between 10 μ mol/L-ADP-WBSPCA and 5 μ mol/L-ADP-LTA

The incidence of responders and non-responders as identified by WBSPCA using 10 μ mol of ADP as agonist in clopidogrel treated patients was 40%(N=25/62) and 60 % (N = 37/62) respectively. Similar incidences as assessed by LTA with 5 μ mol/L of ADP as agonist measured at final time points was 77% (N= 48/62) and 23% (N=14/62) (figure 5.7).

Figure 5.7: Incidence of Responders and Non-Responders Assessed by WBSPCA and LTA

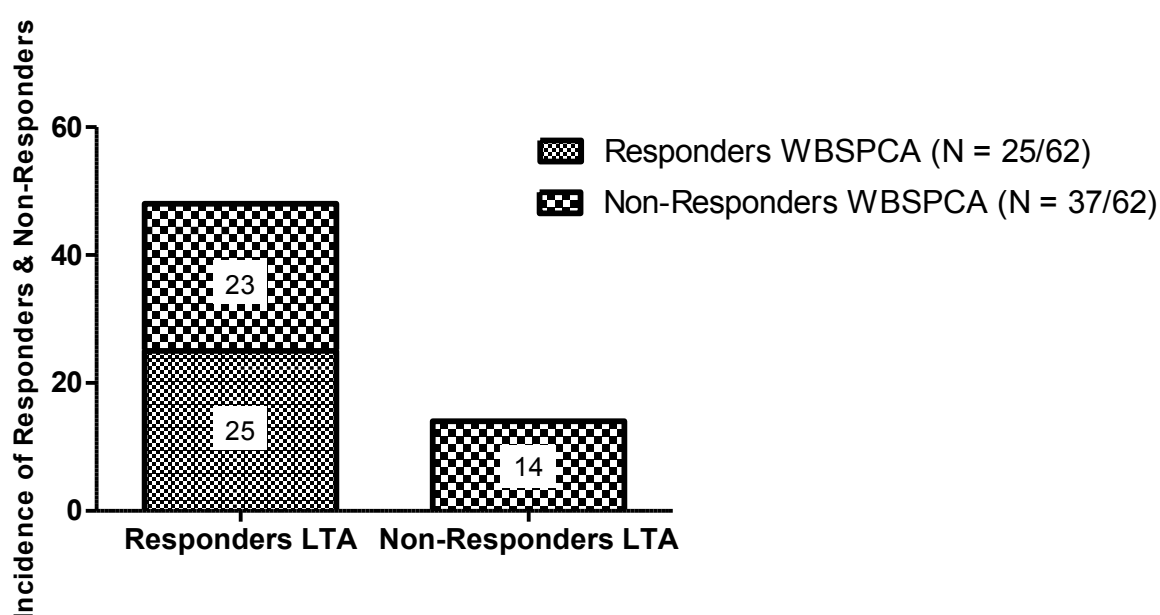


Figure 5.7:All patients who were identified as responders by LTA were also identified as responders by WBSPCA (N=25). 23 patients who were defined as non-responders in WBSPCA were categorized as responders in LTA.

All the patients identified as responders by WBSPCA were also categorized as responders by LTA (N = 25/48). The remaining 14 patients who were classed as non-responders by LTA were categorized as responders by WBSPCA. There was a fair agreement between both the test (kappa co-efficient = 0.279)(Figure 5.8).

Figure 5.8 : Agreement between 5µmol/L-ADP-LTA and 10µmol/L-ADP-LTA

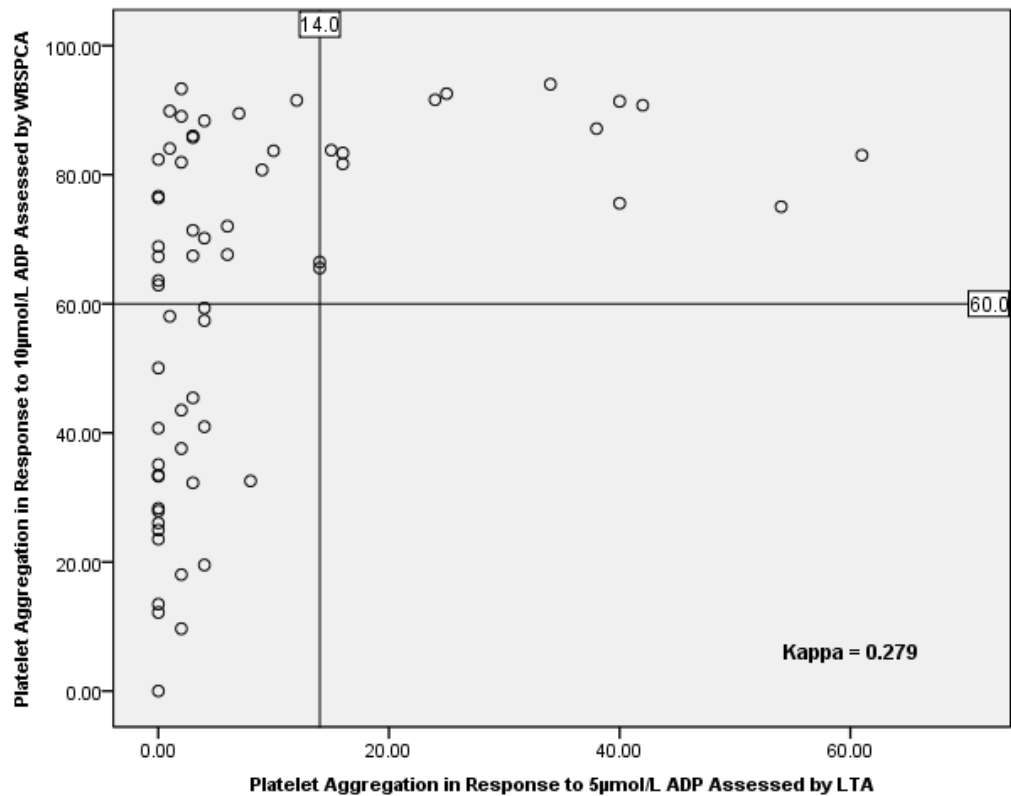


Figure 5.8:There was a fair agreement between 10µmol/L of ADP- WBSPCA and 5µmol/L –LTA,(kappa co-efficient = 0.279).

5.5.2. Agreement between 10µmol/L-ADP-WBSPCA and 20µmol/L-ADP-LTA

The incidence of responders and non-responders to clopidogrel assessed by LTA with stimulation by 20µmol/L of ADP as agonist after maximum

time of contact with the agonist was 73% (N = 45/62), 27% (N = 17/62) respectively. As with 5µmol/L of ADP in LTA, all the patients who were identified as responders by WBSPCA were also grouped as responders by LTA (N= 24). However 25 patients who were classed as responders by LTA were classed as non-responders in WBSPCA (Figure 5.10). There was a fair agreement between both the assays (kappa co-efficient 0.311) (Figure 5.9).

Figure 5.9: Agreement between 20µmol/L-ADP-LTA and 10µmol/L-ADP-WBSPCA

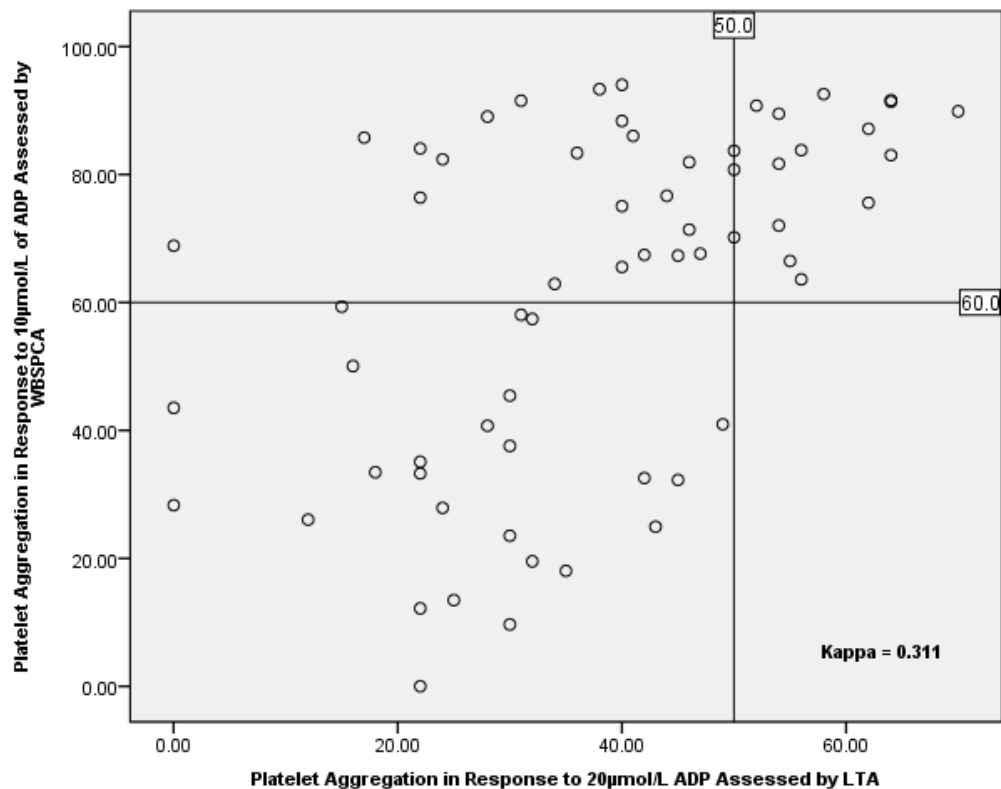


Figure 5.9: There was a fair agreement between platelet aggregation in response to 10µmol/L of ADP measured at 4 minutes assessed by WBSPCA and 20µmol/L of ADP measured at maximum time point in LTA (kappa co-efficient = 0.311).

Figure 5.10: Incidence of Responders and Non-Responders

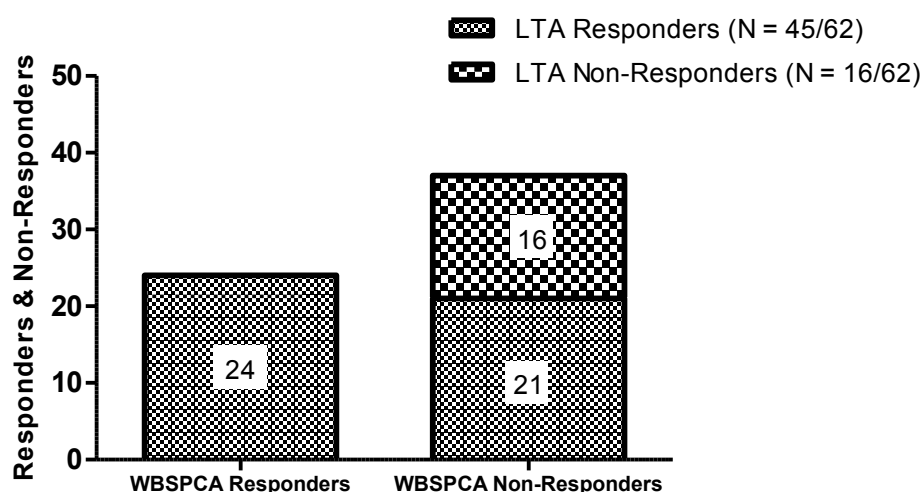


Figure 5.10: Of the 37 patients who were identified as non-responders in the WBSPCA, 21 were classed as responders by LTA.

5.6. ROC Curve Analysis

A ROC curve analysis was performed using LTA as standard and WBSPCA for comparison. The platelet aggregation in response to 20 μ mol/L and 5 μ mol/L of ADP as agonist measured after maximum point of contact with the sample assessed by LTA was compared with 10 μ mol/L of ADP measured at 4 minutes in WBSPCA. Considering 20 μ mol/L of ADP in LTA (AUC = 0.762, upper bound = 0.851 lower bound = 0.672). A cut-off of 59 % (< 60 %) in WBSPCA was 63% sensitive and 90% specific in discriminating responders from non-responders. A value of 69% (< 70%) was 48% sensitive and 98% specific in identifying responders from non-responders (Figure 5.11).

Considering 5 μ mol/L of ADP, (AUC = 0.803, upper bound = 0.884, lower bound = 0.722). A cut-off of 60% (< 60%) was 60% sensitive and 97% specific in identifying responders from non-responders. Likewise a cut-off of 70% was 49% sensitive and 100% specific in defining responders from non-responders, (figure 5.12).

Figure 5.11: ROC Analysis 20 μ mol/L of ADP measured at Maximum Time LTA as Control (N=63)

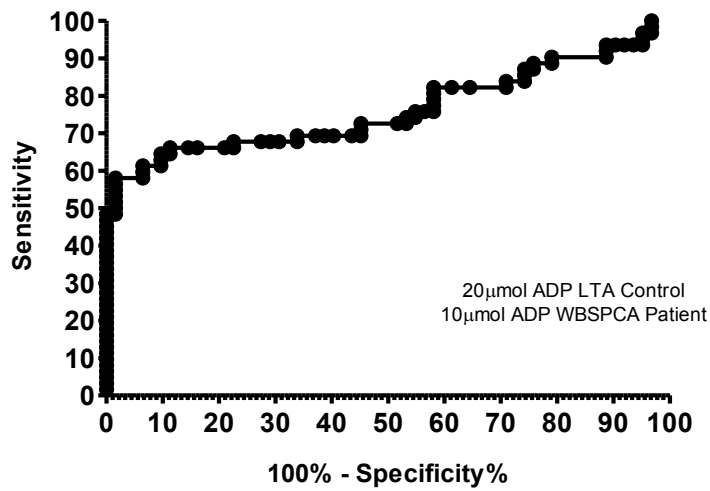


Figure 5.11: (AUC = 0.762, Lower Bound = 0.672, Upper Bound = 0.851) (N=63)

Figure 5.12: ROC Analysis 5 μ mol/L of ADP measured at Maximum Time LTA as Control (N=63)

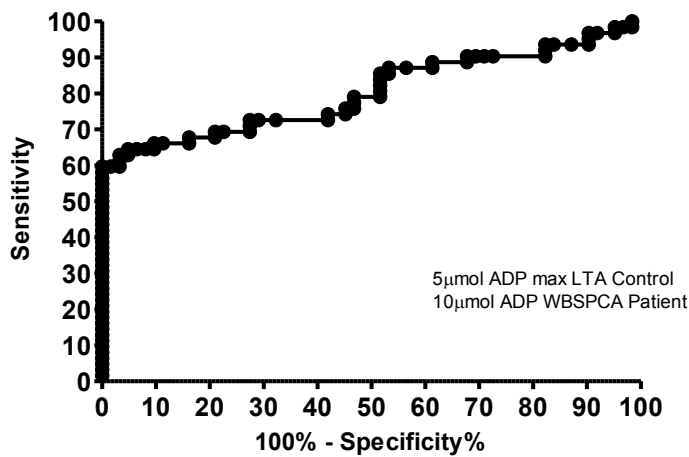


Figure 5.12: (AUC = 0.803, Lower Bound = 0.722, Upper Bound = 0.884) (N=63).

5.7. Discussion

Although numerous tests are available to determine platelet function, the search for the ideal test still continues. Different cut-off values were advocated by different tests to discriminate clopidogrel responders from non-responders. Given the same test, for example the LTA, which used to be considered as the gold standard, various structured prospective studies have used different percentages of platelet aggregation to discriminate clopidogrel responders from non-responders. In one study, Lau and colleagues categorized the patients as clopidogrel non-responders, low responders and responders depending on the relative inhibition of platelet aggregation in response to 20 μ mol/L of ADP assessed by LTA. A platelet aggregation of <10% was classed as responders, 10-29% as low responders and > 30% as non-responders (266). Other clinicians have used a cut-off value ranging between <10% to <40% to differentiate non-responders from responders (267). In the CREST study, the patients were categorized as high post treatment platelet reactivity and low post treatment reactivity. The high post-treatment platelet reactivity was defined as >75th percentile for 5 and 20 μ mol/L ADP-stimulated aggregation (268). Furthermore, a patient who has been classed as responder by one test may well be a non-responder using the criteria set for another test. There is no uniformity in the prevalence of non-responders assessed by different test (269). However, we do have some clarity on this subject now and there are accepted values for an individual test that identifies rather high on-treatment platelet reactivity to clopidogrel. They include PRI > 50% by VASP-P analysis, > 235 to 240 PRU units by VerifyNow assay, > 46% maximal 5- μ mol/l ADP-induced aggregation and > 468 arbitrary aggregation units/min in response to ADP by Multiplate analyzer.

When we explore the reasons for such ambiguity in defining clopidogrel responders and non-responders, two main reasons need consideration. One is the significant interindividual variation in the inhibition of platelet aggregation to clopidogrel and the other, the pitfalls in the *invitro* measurement of platelet aggregation per se, and the limitations of various

tests that measure the platelet aggregation. This has been discussed in the introduction chapter.

Interindividual variation to the platelet aggregation in response to ADP in clopidogrel treated patients is a well-documented phenomenon and several factors are known to cause this. Genetic reasons and drug interactions, particularly with those metabolized by CYP activity, need to be taken into consideration. Other clinical parameters like high BMI, diabetes mellitus (particularly insulin dependent DM), and HPR in the context of ACS all contribute to the variability in response to clopidogrel (101).

Similarly, measuring platelet aggregation using different PFT has its own limitations(270). It is well agreed that platelets exert their actions in a complex multifactorial fashion and it's very difficult to measure all the modifications of platelet function by a single test(271). The ideal PFT should be able to study the global stimulus by various agonists rather than individual agonist particularly if employed for comprehensive assessment of antiplatelet and anticoagulant medications. Furthermore none of the tests is able to measure the contribution of thrombin-induced platelet activation towards clot formation since the tests are performed in the presence of an anticoagulant that prevents the formation and/or action of thrombin in order to prevent fibrin formation and consequent coagulation(269). Although LTA is considered as gold standard, it does not precisely measure all aspects of platelet function (271). Strong evidence exist regarding how poorly the available PFT namely LTA, VASP and VerifyNow agree with each other despite a strong correlation(269).

The prevalence of non-responders assessed by WBSPCA in our study is 60%. By LTA it is 23% and 27% for 5 μ mol/L of ADP measured at final time point and 20 μ mol/L of ADP measured at maximum time point respectively. As with other studies there is a substantial discrepancy in the prevalence of clopidogrel non-responders assessed by LTA and WBSPCA.

The level of correlation in our study between WBSPCA and LTA is predominantly moderate, but statistically significant and there is a strong correlation for 5 μ mol/L of ADP measured at maximum time point in LTA and 10 μ mol/L of ADP in WBSPCA ($r=0.611$, $P <0.001$). A fair correlation between WBSPCA and LTA has been previously reported by Storey and colleagues who used the tool to measure the platelet inhibition achieved by GP IIb/IIIa inhibitors (244). Limited data is available regarding the correlation between WBSPCA and LTA in measuring the platelet aggregation inhibition in patients treated with clopidogrel. Never-the-less, the results of our study suggest that the WBSPCA and LTA correlate the same in comparison with other PFT.

In one study that compared LTA with VASP and VerifyNow assay a correlation coefficient of 0.55 and 0.64 were obtained for VASP and VerifyNow respectively (272). In another study that compared Multiplate impedance platelet aggregometry (IPA) with that of LTA using ADP as the agonist significant correlation was found with an r value of 0.73(273).

WBSPCA and LTA failed to agree with each other with a kappa statistic in the range of fair agreement, (0.279 for 5 μ mol/L of ADP) and (0.311 for 20 μ mol/L of ADP). Again, limited data is available comparing WBSPCA and LTA in assessing agreement. LTA measures macro aggregation and WBSPCA measures micro aggregation and this could explain the lack of agreement between both the assays. Reviewing the evidence on the concordance of other assays with LTA the results seems to be similar to our results, with a kappa value of 0.35 and 0.36 for VASP and VerifyNow respectively (272). However, a statistically significant agreement was found between LTA and Multiplate IPA with a kappa value of .74(273).

The ROC analysis suggests that a cut-off of <70% in WBSPCA with 10 μ mol/L ADP as agonist measured at 4 minutes is more specific and equally sensitive in discriminating responders from non-responders using LTA as reference. Although in our study we have adopted a cut-off value of <60%, in other studies a cut-off value of <70% has been adopted(274).

In summary, our study demonstrated that WBSPCA correlates moderately but with statistical significance with LTA. Although there was a fair agreement only, this is acceptable given the fact that LTA predominantly measures macro aggregation and WBSPCA measures micro aggregation. The flow cytometric VASP assay has now been recommended to assess the platelet function on the P2Y₁₂ receptor. The main limitations of the assay are that it is labour intensive and expensive, hence more suitable as a research tool than as a point-of-care assay. The available VerifyNow point-of-care tool also lacks concordance with LTA and further data is needed on the same before advocating it as the preferred assay for platelet function. Given this caveat for the need for an ideal test, the WBSPCA might have a role in assessing the platelet aggregation inhibition in patients treated with P2Y₁₂ inhibitor like clopidogrel and newer agents like prasugrel.

5.8. Conclusion

In conclusion, there is a highly significant and moderate correlation between LTA and WBSPCA in the assessment of ADP-induced platelet aggregation in clopidogrel-treated ACS patients.

Chapter 6: Percutaneous Coronary Intervention, Related increase in Troponin and its Relationship with Platelet Aggregation and Inflammatory Markers.

6.1. Background and Aim

Currently cardiac troponins are considered to be the gold standard biochemical markers for the diagnosis of myocardial necrosis (275). Myocardial necrosis subsequent to percutaneous intervention is a common occurrence (276). Troponin elevation occurs in about a quarter of patients who undergo PCI. Troponin elevation post PCI has been associated with increase in long-term adverse outcomes. It is recommended that post-procedure biomarker assay is performed when an intra-procedural angiographic complication is identified or a patient has signs or symptoms suggestive of MI during or after PCI. However measuring the troponin levels after all elective PCI is not recommended routinely (277). PCI-related MI (Type 4a) is diagnosed when there is troponin elevation within 48hrs post procedure of 5 x upper limit of normal (ULN) with either symptoms of myocardial ischaemia, new ischaemic ECG changes or documented complications during the procedure (278). HPR at the time of PCI is associated with an increased incidence of post PCI myonecrosis and increased incidence of recurrent CV events following PCI(279). However, no study has so far used WBSPCA to evaluate the platelet reactivity at the time of PCI in patients who are treated with aspirin and clopidogrel and its relation with post PCI

We aim to investigate the relationship between post PCI myonecrosis and non-responsiveness to antiplatelet agents namely clopidogrel and aspirin in patients with ACS assessed by WBSPCA.

6.2. Results - Demography

WBSPCA and LTA were performed in all 63 patients. Of these 55 patients had both pre and post PCI cTnI data available and were included in this analysis. For the purpose of this analysis, we used the same threshold as provided in the 3rd Universal Definition of MI to indicate significant PCI

related myonecrosis, that is an increase in cTnI of more than 5 times the ULN (>0.5ng/ml). The demographic details of this patient group are presented in table 6.1

Table 6.1

Demographic data	N = 50/55		
Age	62		
Sex	50	39(M)	16(F)
Risk Factors		Yes	No
Diabetes Mellitus	50	6/50	44/50
Hypertension	50	30/50	20/50
Smoking	50	26/50	24/50
Dyslipidaemia	50	31/50	1/50
CKD	50	4/50	46/50
Previous H/o IHD	50	15/50	35/50
NSTEMI	50	34/50	
STEMI	50	7/50	
Unstable Angina	50	9/50	

6.3. Procedure-Related Elevation in cTnI

The median (IQ range) of the cTnI values before PCI was 0.10 (0.10-0.57 ng/ml), and post PCI levels were 0.62 (0.18-2.02 ng/ml). There was a statistically significant difference in the median compared between both the values. (Mann Whitney U test $P < 0.0001$). Data were not normally distributed by Shapiro-Wilk normality test, ($w=0.5136$). The mean with SE data is presented in figure 6.1.

Figure 6.1: Troponin I Elevation Related to PCI (N=55)

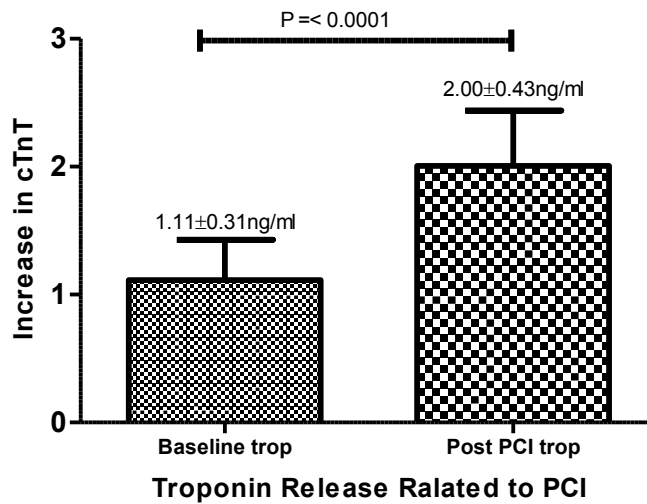


Figure 6.1: Mean (\pm SE) values of cTnI levels pre and post PCI ($P < 0.0001$) (N=55)

6.4. Incidence of PCI Related Myonecrosis

In this study, the proportion of patients who met the criterion for significant PCI related release of troponin was 38% (N = 21/55). In the remaining 62 % who did not meet the criterion, 24 % had some increase in cTnI following PCI. The mean (\pm SD) percentage of platelet aggregation in patients who did not have PCI related release of troponin was 62 ± 25 % and that of those who had PCI related release of troponin was 60 ± 27 %. There was no difference in the median compared between the groups ($P = 0.578$, figure 6.2)

6.5. Incidence of PCI Related Myonecrosis in Responders vs. Non-Responders Assessed by WBSPCA

The incidence of clopidogrel responders was 40% as assessed by WBSPCA. The incidence of PCI-related myonecrosis in responders was 41% and in non-responders was 36%. This was not statistically significant, (Fishers exact test $P = 0.782$), (figure 6.3). The mean (\pm SD) increase in cTnI in responders was $0.86 (\pm 1.36$ ng/ml) and that of non-responders was $1.08 (\pm 2.40$ ng/ml). There was no difference in the median compared

between the values, (Mann Whitney U test $P = 0.478$). The mean with SE value is presented in figure 6.4.

Figure 6.2: Platelet Aggregation as Assessed by WBSPCA in PCI Related Myonecrosis (N=55)

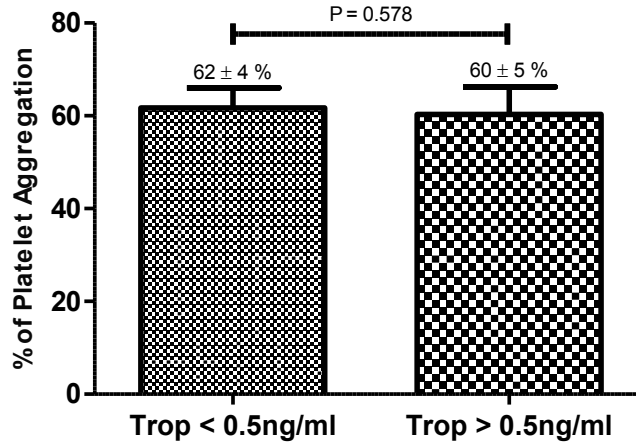


Figure 6.2: No difference in platelet reactivity in those who developed PCI related myonecrosis as assessed by WBSPCA (N= 55) ($P = 0.578$).

Figure 6.3: Incidence of post PCI Myonecrosis in Responders and Non-Responders to Clopidogrel Assessed by WBSPCA (N=55)

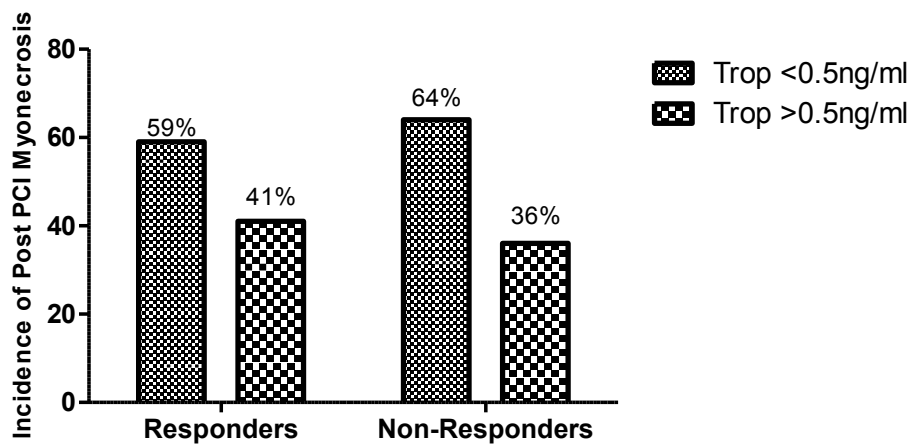


Figure 6.3: There was no significant difference in the incidence of PCI related myonecrosis between responders and non-responders ($P = 0.782$) (N=55)

Figure 6.4: The Increase in cTnI Compared between Responders and Non-Responders to Clopidogrel as Assessed by WBSPCA (N=55)

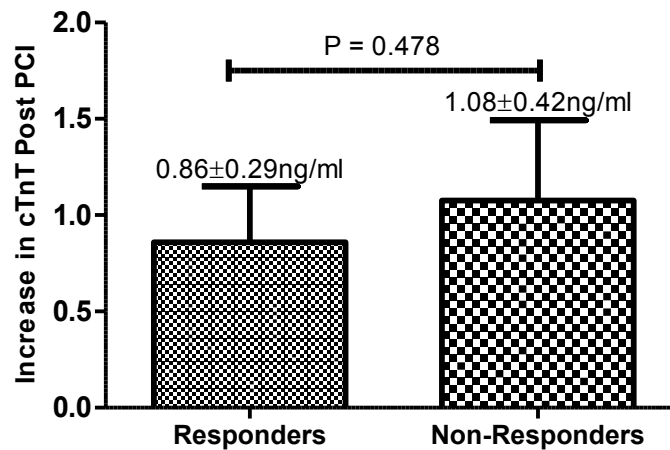


Figure 6.4: There was no difference in the mean elevation of cTnI compared between responders and non-responders as assessed by WBSPCA (P= 0.478) (N=55)

6.6. Incidence of PCI Related Myonecrosis in Responders vs. Non-Responders to Clopidogrel Assessed by LTA

When assessed by LTA with 20 μ mol/L of ADP as agonist measured as maximal response, the incidence of clopidogrel responders was 76%. PCI related myonecrosis happened in 44% of the responders and 23% of the non-responders. The difference in the incidence was not statistically significant (Fishers exact test P=0.211) (figure 6.5). The mean (\pm SD) increase in cTnI in responders was 0.96 (\pm 1.61 ng/ml) and that of non-responders were 1.10 (\pm 3.13 ng/ml). The difference in the median was not statistically significant, (Mann Whitney U test P= 0.398) (figure 6.6).

Figure 6.5: PCI Related Myonecrosis in Responders and Non-Responders as Assessed by LTA (N=55)

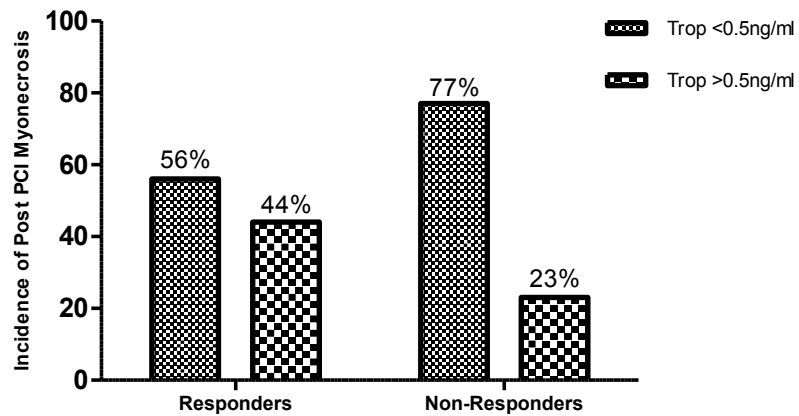


Figure 6.5: No significant difference in the incidence of PCI related myonecrosis between responders and non-responders assessed by LTA (P=0.211).

Figure 6.6: PCI Related Myonecrosis in Responders and Non-Responders as Assessed by LTA

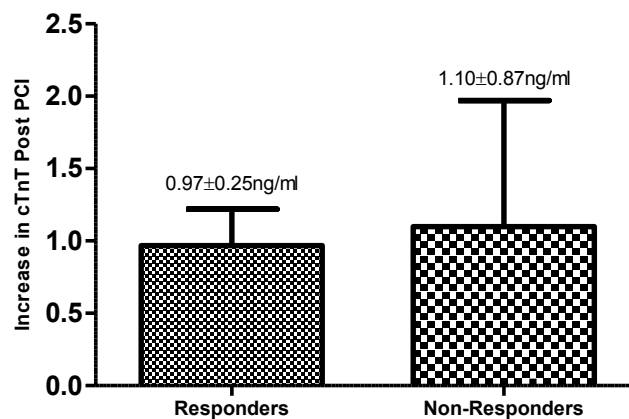


Figure 6.6: There was no significant difference in the mean increase in cTnI between responders and non-responders (P= 0.398).

6.7. Correlation between Post-PCI Myonecrosis and Platelet Aggregation in Response to 10µmol/L ADP Assessed by WBSPCA

Pearson's correlation showed no correlation between platelet aggregation measured by WBSPCA in response 10µmol ADP measured at 4 minutes and PCI-related troponin release. Pearson's correlation coefficient ($r=0.013$, $P=0.926$ and $R^2 = 1.677 \times 10^{-4}$ Figure 6.7)

Figure 6.7: Correlation between PCI-Related Myonecrosis and Platelet Aggregation Assessed by WBSPCA (N=55)

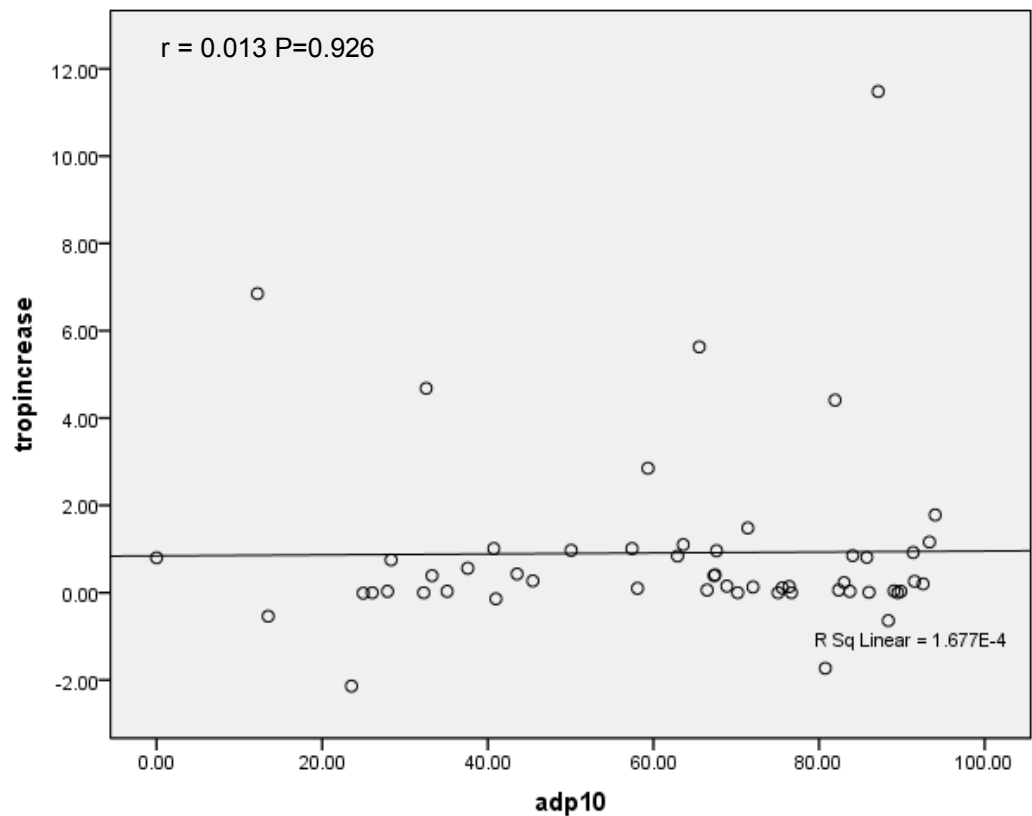


Figure 6.7: There was no correlation between the PCI related myonecrosis and platelet aggregation as measured by WBSPCA. ($r=0.013$, $P= 0.926$, $R^2 = 1.677 \times 10^{-4}$).

6.8. PCI-Related Myonecrosis and its Relation with Platelet Reactivity in Aspirin-Treated Patients Assessed by WBSPCA

55 patients had available complete data on AA-induced platelet aggregation and were included in the analysis. Using the study definition, 36/55 (65%) patients were found to be aspirin responders and the remaining 19/55 (35%) patients were low-responders to aspirin. The mean \pm SD elevation in troponin in aspirin responders was 0.82 ± 1.60 ng/ml. The corresponding value in aspirin low-responders was 1.31 ± 2.69 ng/ml. There was no difference in the median between the groups (Mann Whitney U test, $P = 0.233$, figure 6.8).

Figure 6.8: Mean Increase in Troponin between Responders and Low-Responders to Aspirin Assessed by WBSPCA (N=55)

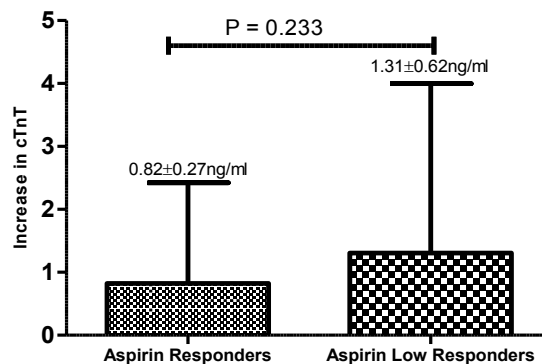


Figure 6.8: cTnI increase in aspirin responders and low-responders ($P=0.233$)

6.9. Platelet Aggregation Assessed by WBSPCA and its Relation to PCI Related Myonecrosis

Of the 55 patients, 21 patients met the criterion for significant PCI related myonecrosis (38%). The mean \pm SD percentage of platelet aggregation in patients who had PC related myonecrosis was 35 ± 25 % and for those who did not develop PCI related myonecrosis was 24 ± 11 %. There was no difference in the median compared between both the categories, Mann Whitney U test $P = 0.125$ (figure 6.9). Of the 21 patients who the criterion for significant PCI related myonecrosis, 12 were responders to aspirin ($N = 12/36$, 33%). The remaining 9 patients were low-responders to aspirin ($N = 9/19$, 47%) (Figure 6.10)

Figure 6.9: Platelet Aggregation Assessed by WBSPCA in Aspirin Treated Patients and its relation with PCI Related Myonecrosis (N=55)

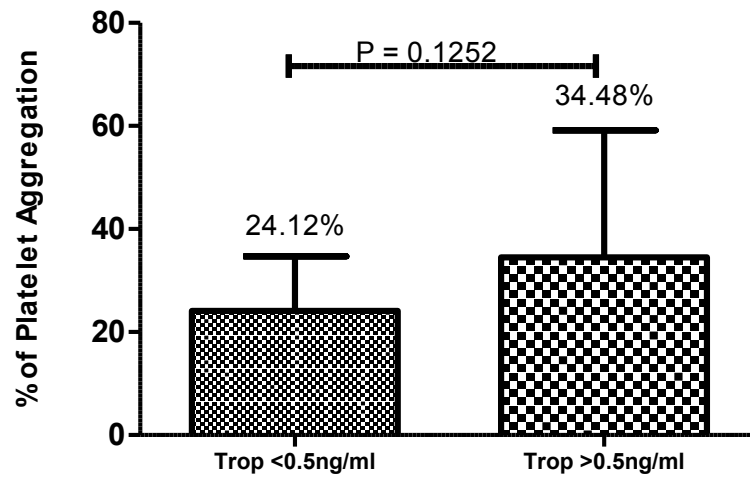


Figure 6.9: Percentage of platelet aggregation in patients who developed PCI related myonecrosis compared with those who did not develop the same (P= 0.125) (N=55)

Figure 6.10: Incidence of PCI Related Myonecrosis in Aspirin Treated Patients (N=55)

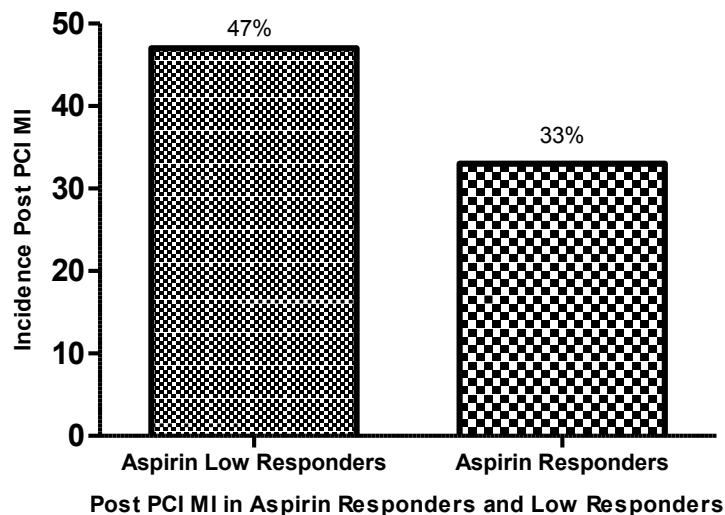


Figure 6.10: The incidence of PCI related myonecrosis was higher in aspirin low-responders compared to responders (47% vs. 33%, N=55)

6.10. Correlation of PCI Related Myonecrosis and Platelet Aggregation Assessed by WBSPCA in Aspirin Treated Patients

Spearman's nonlinear correlation showed no correlation between the PCI related myonecrosis and platelet aggregation in response to 0.8mM AA in aspirin treated patients at the time of PCI measured by WBSPCA at 4 minutes. Correlation co-efficient ($r=0.148$, $P=0.280$, figure 6.11).

Figure 6.11: PCI Related Myonecrosis and its Correlation to Platelet Aggregation at the Time of PCI Assessed by WBSPCA (N=55)

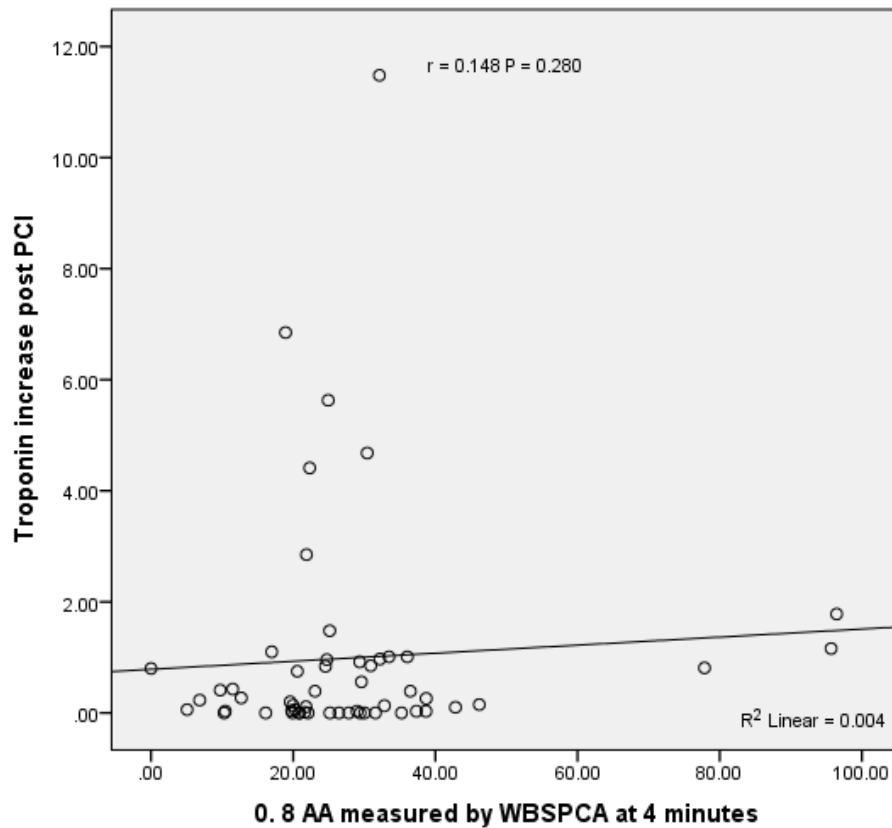


Figure 6.11: PCI related myonecrosis and its correlation with platelet aggregation assessed by WBSPCA in aspirin treated patients ($r= 0.148$, $P= 0.280$).

6.11 PCI-Related Myonecrosis Compared between Diabetics and Non-diabetics

The mean percentage of platelet aggregation in diabetics with PCI related myonecrosis was 75% assessed by WBSPCA stimulated by 10µmol/L ADP and 34% measured by LTA using 20µmol/L of ADP as agonist. The mean percentage of platelet aggregation in patients who were non-diabetic with no PCI related myonecrosis was 55% assessed by WBSPCA and 14% measured by LTA (figure 6.12). The detailed descriptive data is attached (Appendix 5).

Figure 6.12: Platelet Aggregation in Diabetics and Non-Diabetics with or Without PCI Related Myonecrosis

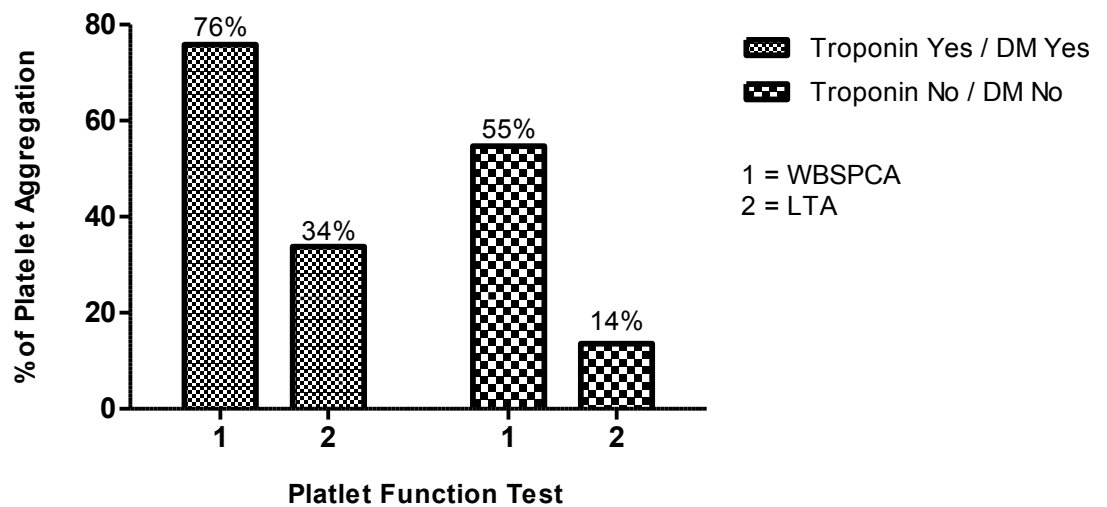


Figure 6.12: The mean percentage of platelet aggregation in diabetics with PCI related myonecrosis was 76%-WBSPCA and 34%-LTA. 55%-WBSPCA and 14% LTA were the similar value in non-diabetic without PCI related myonecrosis.

6.12. Relationship between Pre PCI-Inflammatory Markers and PCI Related Myonecrosis

There was no significant difference in the mean (\pm SD) of pre-PCI levels of inflammatory markers namely CRP, sCD40L, IL-6 and TNF- α in patients who developed PCI related myonecrosis with those who did not develop the same, (Mann Whitney U test $P > 0.05$). Data presented in table 6.2, (figure 6.16-6.19).

Table 6.2: Inflammatory Markers Pre PCI and PCI Related Myonecrosis

Inflammatory markers	N	cTnl <0.5 ng/ml	cTnl> 0.5 ng/ml	P
CRP pre-PCI (mg/dl)	54	7.150±10.8	6.94±10.06	0.097
sCD40L pre-PCI (ng/ml)	54	2.23±0.95	2.30±1.36	0.401
IL-6 pre-PCI (pg/ml)	54	26.84±20.55	19.63±14.55	0.174
TNF-α pre-PCI (pg/ml)	49	11.06±6.73	9.71±5.23	0.460

Figure 6.13: CRP pre-PCI Compared with PCI-Related Myonecrosis

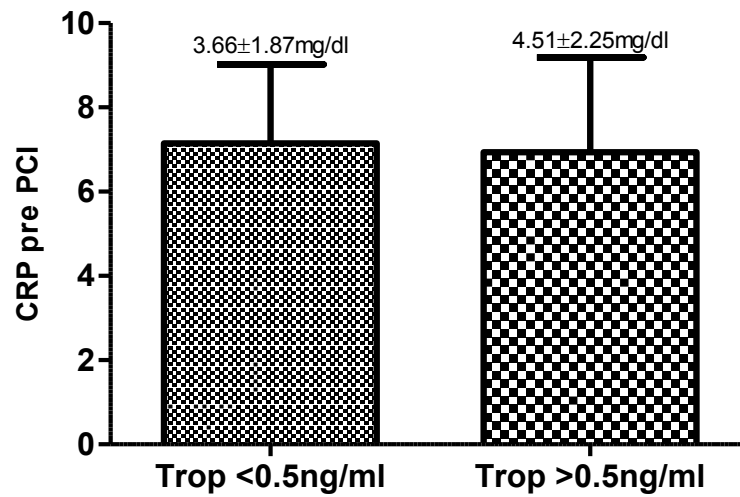


Figure 6.13: There was no significant difference in CRP levels pre-PCI in comparison with those who developed PCI related myonecrosis (P=0.097, N=54)

Figure 6.14: sCD40L pre-PCI Compared with PCI Related Myonecrosis (N= 54)

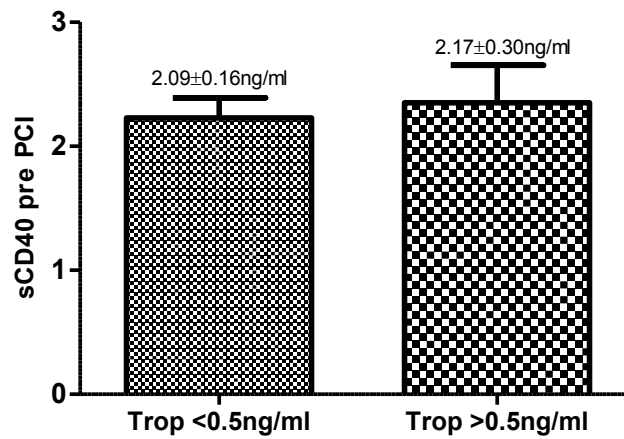


Figure 6.14: There was no difference in the levels of pre-PCI sCD40L in those who had PCI related myonecrosis (P=0.401, N=54)

Figure 6.15: IL-6 pre-PCI vs. PCI Related Myonecrosis (N=54)

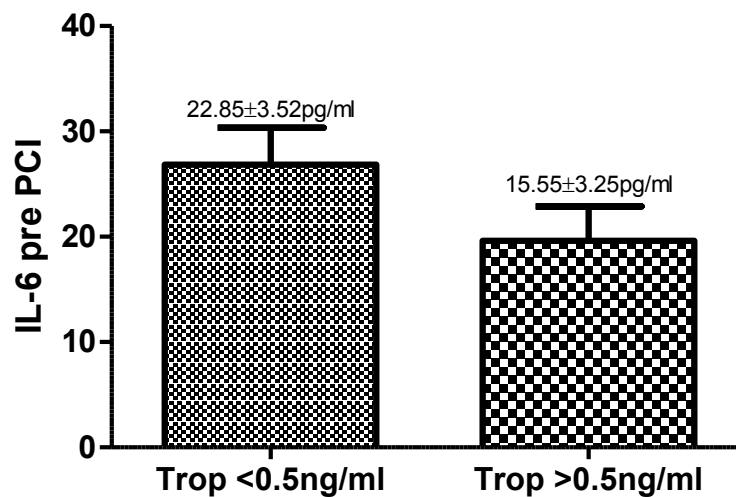


Figure 6.15: There was no difference in the levels of IL-6 pre-PCI in those who had PCI related myonecrosis (P = 0.174, N=54)

Figure 6.16: TNF- α pre-PCI vs. PCI Related Myonecrosis (N=49)

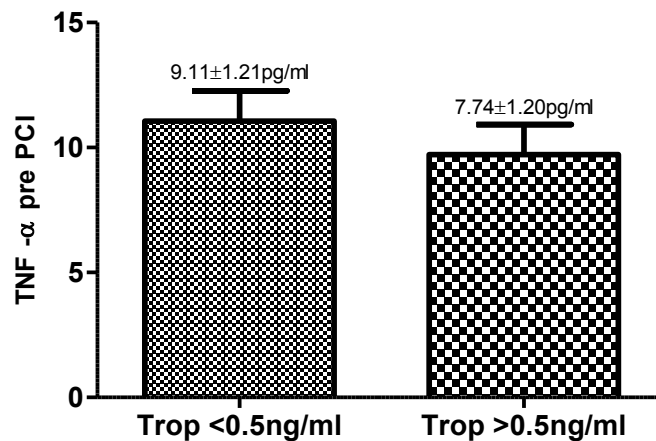


Figure 6.16: There was no difference in the levels of pre-PCI TNF- α in those who PCI related myonecrosis (P = 0.460, N=49)

6.13. Discussion

We studied the relationship between PCI related myonecrosis and its relationship with platelet reactivity at the time of PCI in patients treated with aspirin and clopidogrel. There was a statistically significant rise in PCI related release in cTnI (P=0.0014). There was non-significant difference in the incidence of PCI related myonecrosis in clopidogrel non-responders compared to responders assessed by both WBSPCA and LTA. Similarly, no significant difference was found in the mean percentage of platelet aggregation in patients who had PCI related myonecrosis compared to those who did not. The release of cTnI following PCI was numerically higher in clopidogrel non-responders than responders. There was no correlation between PCI related release in cTnI and platelet reactivity in response to 10 μ mol/L of ADP at the time of PCI in patients treated with clopidogrel assessed by WBSPCA.

The incidence of PCI related myonecrosis was numerically higher in aspirin low responders compared to responders, but not statistically significant. The mean percentage of platelet aggregation in response to 0.8mM AA at the time of PCI was higher in the patients who developed

PCI related myonecrosis. Similarly, the mean increase in cTnI post PCI was higher in aspirin low-responders compared to responders. There was no correlation between platelet aggregation in response to 0.8mM AA and post PCI release of cTnI.

No study has so far compared platelet reactivity at the time of PCI assessed by WBSPCA and occurrence of PCI related myonecrosis. However mixed results have been published with other point-of-care tools. Cuisset and colleagues have demonstrated an increased incidence of periprocedural MI (PMI) in patients who were non-responders to clopidogrel assessed by point- of-care assay(206). On the contrary, Buch and colleagues found no association between periprocedural MI and platelet aggregation inhibition assessed by Rapid Platelet Function Assay P2Y12 assay (280). In the BRIEF-PCI study, there was no increased incidence of PCI-related myonecrosis in aspirin or clopidogrel non-responders (281). In the ARMYDA-PRO study the incidence of periprocedural MI occurred more often in patients who had a higher platelet reactivity unit (PRU) assessed by Verify-Now assay (282). Patti and colleagues have shown that the incidence of PCI related myonecrosis is higher in clopidogrel non-responders undergoing elective PCI for low to moderate risk CAD (283).

The 2012 ACCF consensus document on PCI related myonecrosis has acknowledged that, troponin release in the context of PCI is related to adverse outcomes only when the release is due to ischaemic events unrelated to the procedure, or the troponin release is caused by intraprocedural complications and when the baseline troponin is high. Moreover defining the PCI related myonecrosis as increase in troponin 5 times above the ULN may be too sensitive a criterion which has increased the incidence of PCI related myonecrosis to 33% when compared to previous studies where the incidence was only 20% when older generation less sensitive assays were used (278). HPR is now being increasingly recognized as an independent risk factor for future occurrences of MACE regardless of the PCI-related increase in myonecrosis (284). Its relevance in the incidence of PCI related

myonecrosis is debatable. Our study has provided preliminary data on the relationship between clopidogrel non-responders assessed by WBSPCA and its relationship to the incidence of PCI-related myonecrosis.

6.14. Conclusion

In our study, the incidence of PCI-related myonecrosis was not significantly different in clopidogrel responders compared to responders. The incidence of post PCI myonecrosis is numerically higher in aspirin low responders compared to responders. The mean increase in cTnI was higher in clopidogrel non-responders and aspirin low responders, but not significant. A study with a large sample size is required to assess whether the results of WBSPCA-assessed platelet reactivity at the time of PCI is associated with the incidence of post-PCI myonecrosis.

Chapter 7: Relationship between Plasma Inflammatory Markers and Platelet Aggregation in Patients Undergoing Percutaneous Coronary Intervention.

7.1. Introduction

Atherosclerosis is well acknowledged as an inflammatory condition (285, 286). Platelets, when activated, not only are involved in thrombus formation but also contribute to an inflammatory process following vascular injury (287). Various inflammatory markers from diverse sources are released during the active process of plaque rupture and thrombus formation. Of note are sCD40L, predominantly released by the platelets but also from the macrophages, and interleukin-6 (IL-6) and TNF- α released by the macrophages and T cells. In addition, CRP is released from the hepatocytes and adipocytes in response to IL-6 and TNF- α (288). These inflammatory markers are increased during the acute presentation of ACS and during PCI. Aspirin and clopidogrel are recognized treatment options for a spectrum of disease conditions including UA, NSTEMI, STEMI, acute ischaemic stroke and symptomatic PVD (289). Inhibition of platelet aggregation can modulate the release of both platelet related inflammatory markers, particularly sCD40L, and non-platelet systemic inflammatory markers such as IL-6, TNF- α and CRP (290).

Aspirin and clopidogrel, in addition to inhibiting platelet aggregation, also have anti-inflammatory effects, which can be indirect (291, 292). Several studies have demonstrated a mixed relationship between HPR assessed by various platelet function tests and increased inflammatory markers in patients with CAD treated either medically or by PCI (293-295). However, little evidence exists regarding the utility of WBSPCA in evaluating the platelet reactivity in patients treated with aspirin and clopidogrel and its relationship with the inflammatory markers.

We aimed to study the relationship between aspirin low responders as assessed by WBSPCA and its relationship with inflammatory markers

namely CRP, IL-6 and TNF- α . In addition, we assessed whether variable P2Y₁₂ receptor blockade by clopidogrel assessed by WBSPCA and LTA leads to variability in inflammatory response as measured by CRP and sCD40L in patients undergoing percutaneous coronary intervention.

7.2. Results - Demography

WBSPCA and LTA were performed in all 63 patients. Although we aimed to check the inflammatory markers viz. CRP, IL-6, TNF- α and sCD40L at three time points, namely at baseline before PCI (B) 2-4 hours following PCI (4H) and 18-24 hours after PCI (24H), there are some missing values. Hence patients in whom complete set of information was available, including inflammatory markers at all three time points (B, 4H, and 24H) and their corresponding platelet aggregation data were only considered for the analysis. In that respect, we included data on CRP analysed on 52 patients, TNF- α on 49 patients, IL-6 on 52 patients and sCD40L on 52 patients. A detailed demographic data on these patients is already presented in chapter 3.

7.3. Descriptive Statistics of Inflammatory Markers

Except for the sCD40L-B levels, inflammatory marker levels were not normally distributed. There was a significant increase in the level of CRP-24H from CRP-B, 4.42 (\pm 13.57mg/dl, P=0.003). Such difference was not seen with the CRP-4H, 1.28 (\pm 8.68mg/dl, P= 0.127). Although there was a marginal increase in sCD40L-4H and sCD40L-24H, it was not statistically significant (P= 0.557, P= 0.115). The IL-6-4H was elevated from IL-6-B, 4.65 (\pm 16.41pg/ml, P=0.073). There was no increase in IL-6-24H from IL-6-B 3.28 (\pm 14.47pg/ml, P= 0.242). The TNF- α -4H and TNF- α -24H were marginally elevated compared to TNF- α -B (B= 10.39 \pm 6.07pg/ml, 4H= 10.93 \pm 6.85pg/ml, 24H=10.68 \pm 6.30pg/ml). There were some extreme outliers in the CRP levels. Particularly, one value determined the results obtained regarding all analysis pertaining to CRP. There was no clinical correlation as to why an extreme increase in CRP was seen in that particular patient. Hence, we considered this value to be anomalous and removed it in all our further analysis of CRP. Considering

this change, there was no significant increase in CRP-24H from CRP-B. The data are attached (Appendix 6). The mean with SE value of each inflammatory marker are presented in figure 7.1-7.4.

Figure 7.1: CRP (N= 52)

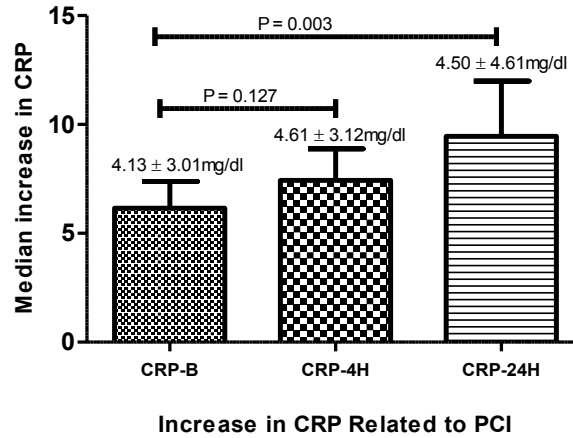


Figure 7.1: There is a statistically significant difference CRP-24H and CRP-B (N=52)

Figure 7.2: sCD40L (N=52)

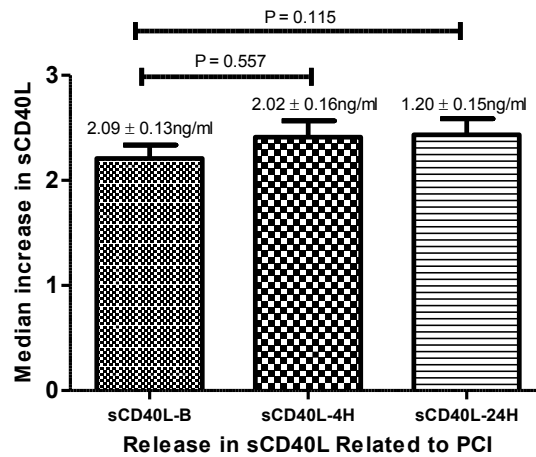


Figure 7.2: There is no significant difference between sCD40L-24H and sCD40L-B (N=52)

Figure 7.3: TNF- α (N=49)

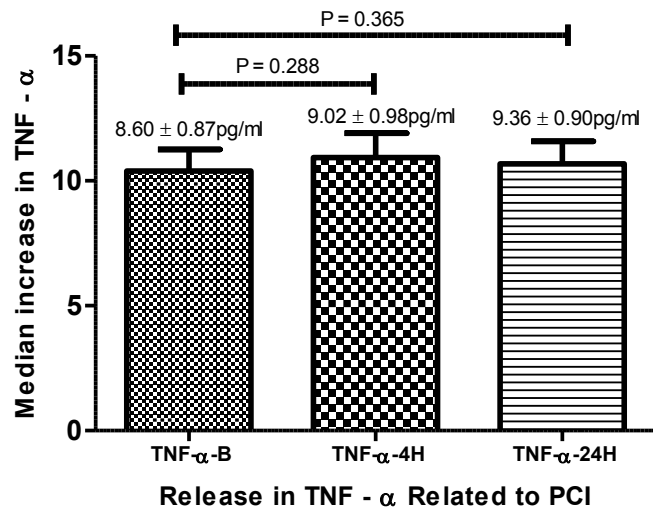


Figure 7.3: There is no significant difference between TNF- α -24H and TNF- α -B (N=49)

Figure 7.4: IL-6 (N=52)

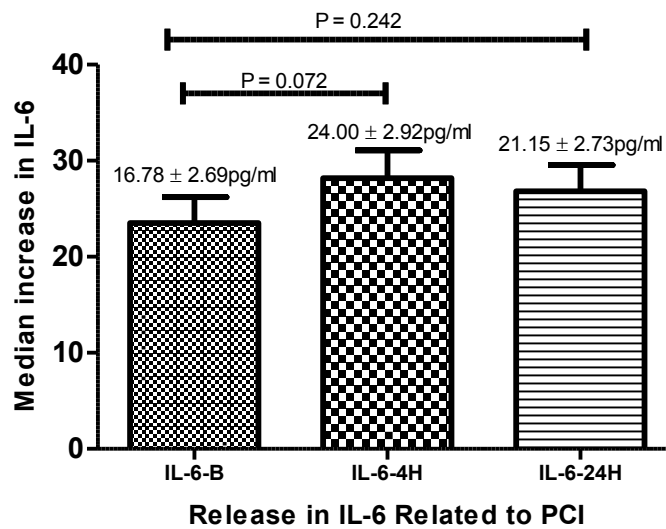


Figure 7.4: There is no significant difference between IL-6-24H and IL-6-B (N=52)

7.4. Post PCI Release in sCD40L and Clopidogrel Response

The relationship between platelet aggregation in response to 10µmol/L of ADP measured at 4 minutes assessed by WBSPCA and to three concentrations of ADP namely 2, 5, 20µmol/L assessed by LTA in patients treated with clopidogrel and sCD40L release before and after PCI was studied in 52 patients. Patients were categorized into groups based on the percentage of platelet aggregation. The mean increase in sCD40L levels in each group was compared by ANOVA.

7.4.1. SCD40L-4H and its Relation with Platelet Aggregation in Response to 10µmol/L of ADP Assessed by WBSPCA

Platelet aggregation in response to 10µmol/L of ADP in clopidogrel treated patients was categorized into four groups. Group 1= percentage of platelet aggregation 0-< 30%, group 2= 30-<60%, group 3= 60- <90%, and group 4= percentage of platelet aggregation > 90%. Their corresponding differences in the levels of sCD40L namely sCD40L-B, sCD40L-4H, sCD40L-24H were compared by one way ANOVA.

Of the 52 patients studied, 9 were classed as group 1, 11 patients belonged to group 2, 25 patients were in group 3, and 5 patients were classed as group 4. The mean (\pm SD) of the sCD40L-4H in group 1 was 1.98 (\pm 0.65ng/ml) and in group 4 was 2.69 (\pm 1.11ng/ml). The values in group 2 and 3 were not much different at 2.53 (\pm 1.43ng/ml) and 2.53 (\pm 1.14ng/ml) respectively. There was no difference in the mean compared between all the groups ($P=0.588$ $F= 0.648$) by one way ANOVA. The mean with SE for each group is presented in figure 7.5. Their ANOVA table is attached (Appendix 7).

7.4.2. SCD40L-24H and its Relation with Platelet Aggregation in Response to 10µmol/L of ADP Assessed by WBSPCA

The mean (\pm SD) increase in sCD40L-24H in group 1 was 1.74 (\pm 0.75ng/ml). Similar data for group 2, 3, 4 are 2.13 (\pm 0.86nm/ml), 2.66 (\pm 1.08ng/ml), 3.72 (\pm 0.92ng/ml) respectively. There was a statistically significant difference in the mean compared between group 1 and group 4,

as well as group 2 and group 4 ($P=0.004$, $F=5.221$). Mean with SE presented in figure 7.6. Details of statistics attached (Appendix 8)

Figure 7.5: sCD40L-4H Compared between All the Groups of Platelet Aggregation Assessed by WBSPCA (N= 52)

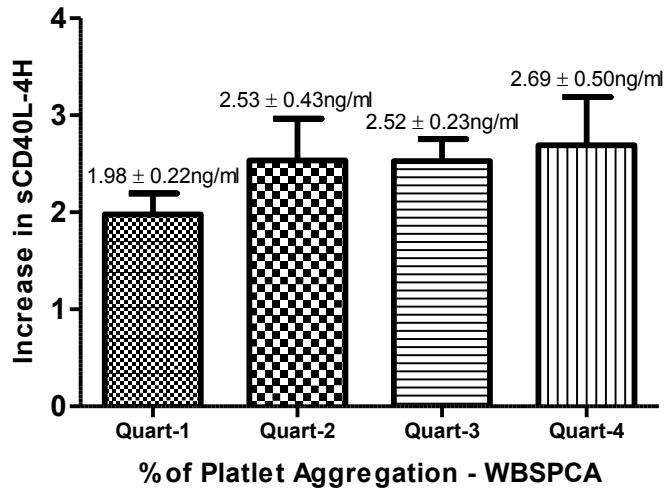


Figure 7.5: No differences in the mean compared between the groups (N=52)

Figure 7.6: sCD40L-24H Compared between all Groups of Platelet Aggregation Assessed by WBSPCA (N=52)

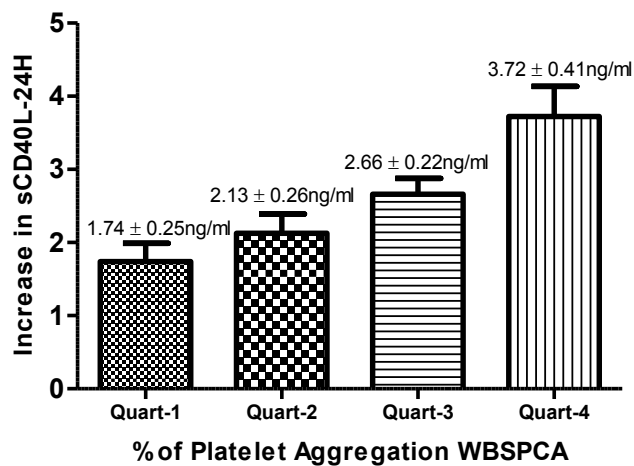


Figure 7.6: Significant difference in the mean between group 1 and group 4, group 2 and group 4 ($P= 0.004$, $F= 5.22$).

7.4.3. sCD40L-24H and its Relation with Platelet Aggregation in Response to 5µmol/L of ADP Assessed by LTA

The percentage of platelet aggregation in response to 5µmol/L of ADP in clopidogrel treated patients assessed by LTA measured at final time point was divided into three groups. Group 1 = 0%, Group 2= 0 -< 14% and group 3=> 14%. 16/52 was categorized in group 1, 22/52 in group 2, and 12/22 in group 3. The mean (\pm SD) value of sCD40L in group 1 was 2.16 (\pm 0.91ng/ml) and that of group3 was 2.86 (\pm 1.09ng/ml). There was no significant difference in the means between the groups ($P = 0.242$, $F= 1.46$). Mean with SE is presented in figure 7.7. ANOVA table attached (Appendix 9).

Figure 7.7: sCD40L-24H Compared between all Groups of Platelet Aggregation Assessed by LTA (N=52)

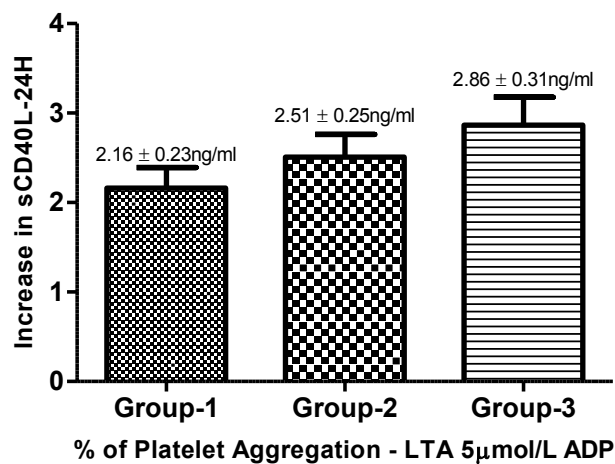


Figure7.7: There was no difference in the mean ($P= 0.242$).

7.4.4. sCD40L-24H and its Relation with Platelet Aggregation in Response to 20µmol/L of ADP Assessed by LTA

The percentage of platelet aggregation in response to 20µmol/L-ADP in clopidogrel-treated patients measured by LTA at maximum time point was categorized as three groups. Group 1 = percentage of platelet aggregation < 25%, Group 2 = 25-< 50 % and group 3 >50 %. 13/52 of patients were in

group 1, 24/52 were in group 2 and 13/52 were in group 3. The mean (\pm SD) of sCD40L in group 1 was 2.24 (\pm 0.95ng/ml) and that of group 3 was 2.47 (\pm 0.95ng/ml). There was no difference in the mean between the groups ($P = 0.570$, $F= 0.569$). The mean with SE is presented in figure 7.8. The ANOVA table is presented in Appendix 10.

Figure 7.8: sCD40L-24H Compared between all Groups of Platelet Aggregation Assessed by LTA (N=52)

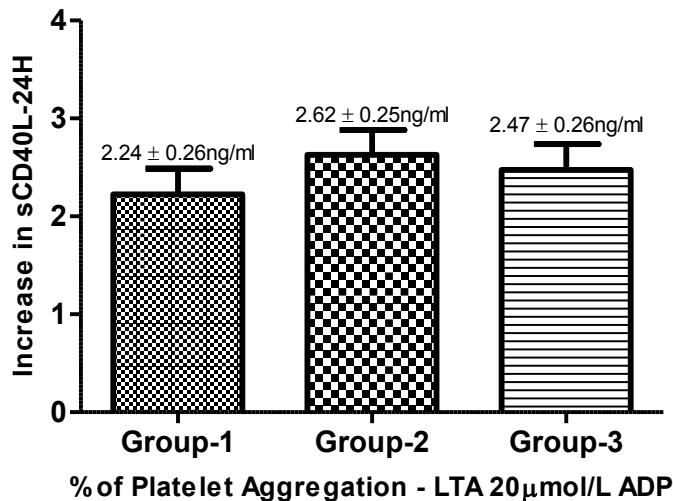


Figure7.8: No difference in the mean compared between the groups ($P= 0.570$).

7.5. Correlation between sCD40L and Platelet Aggregation in Clopidogrel Treated Patients

Spearman’s linear correlation demonstrated a moderate but significant correlation between platelet aggregation in response to 10 μ mol/L ADP measured at 4 minutes assessed by WBSPCA and sCD40L-B ($r=0.274$, $P= 0.031$). Likewise a moderate but significant positive correlation was seen between platelet aggregation in response to 10 μ mol/L ADP measured at 4 minutes assessed by WBSPCA and sCD40L-24H ($r= 0.343$, $P= 0.008$, figure 7.9). There was no correlation between the platelet aggregation assessed by LTA and the levels of sCD40L at 24H ($r= 0.154$, $P= 0.251$ for 20 μ mol/L). The detailed table with all correlation co-efficient is attached (Appendix 11).

Figure 7.9: Correlation between sCD40L-24H and Platelet Aggregation Measured by WBSPCA (N=52)

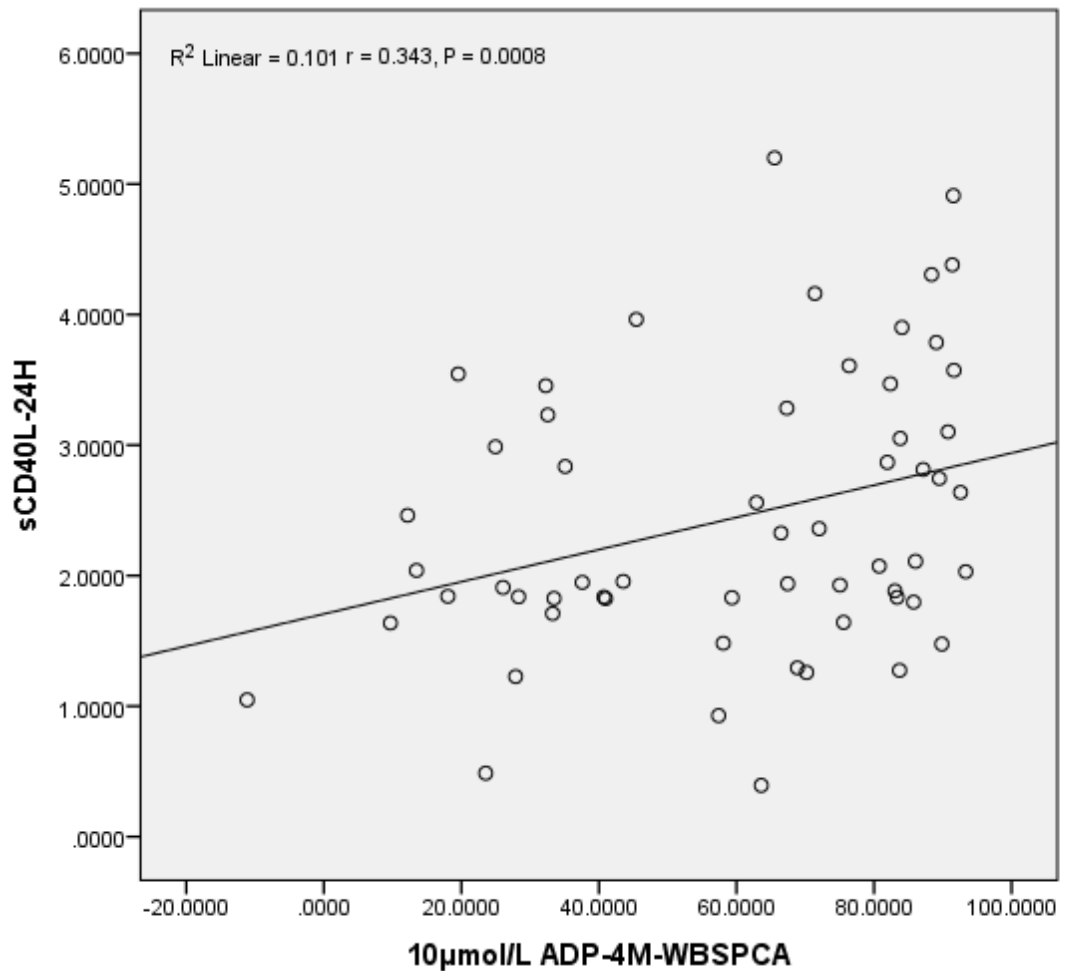


Figure 7.9: Moderate, but significant correlation between platelet aggregation and sCD40L -24H ($r = 0.343$, $P = 0.008$).

7.6. CRP and its Relationship with Platelet Aggregation in Response to Aspirin and Clopidogrel

51 patients were considered for analysis. They were grouped into four categories. Aspirin/clopidogrel responders (N = 13/51), aspirin responders only (N = 19/51), clopidogrel responders only (N = 7/51) and aspirin/clopidogrel non-responders (N = 12/51). The mean (\pm SD) level of the CRP-4H in the aspirin/clopidogrel responders was 4.55 (\pm 4.02mg/ml) and that of aspirin/clopidogrel non-responders was 5.24 (\pm 6.32mg/ml).

Although the means were numerically high in the non-responders, it was not significant ($P > 0.05$, figure 7.10). Considering CRP-24H, the mean (\pm SD) level of CRP in the aspirin/clopidogrel responders was 5.12 (\pm 4.63mg/ml) and that of the aspirin/clopidogrel non-responders were 5.71 (\pm 6.62mg/ml). There was no difference in the mean compared between the groups ($P > 0.05$, figure 7.11).

Figure 7.10: CRP-4H (N=52)

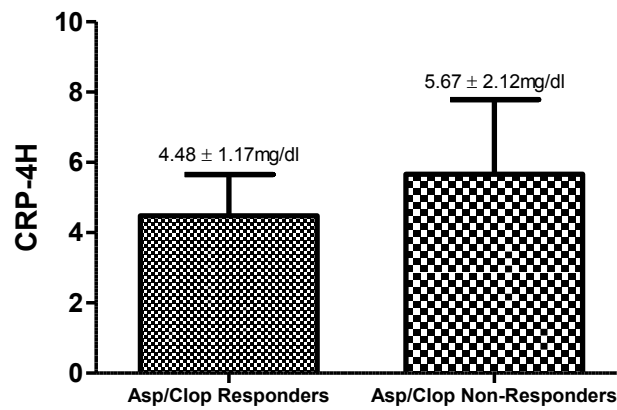


Figure 7.10: Median difference in the CRP-4H levels between aspirin/clopidogrel responders and non-responders measured ($P > 0.05$) (N=52)

Figure 7.11: CRP-24H (N=52)

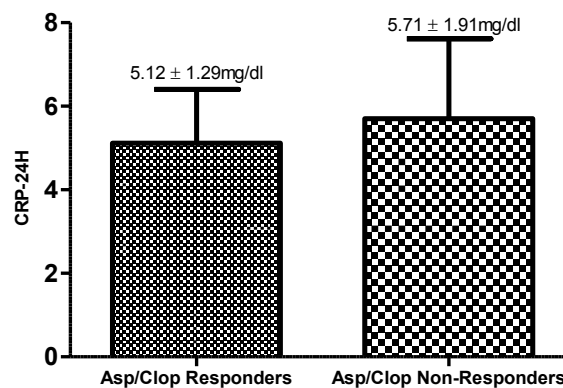


Figure 7.11: Median difference in the CRP-24H levels between aspirin/clopidogrel responders and non-responders ($P > 0.05$) (N=52)

7.6.1. Correlation between Platelet Aggregation in Response to 0.8mM AA and CRP

Spearman's correlation showed fair but significant correlation between platelet aggregation in response to 0.8mM AA measured at 4 minutes assessed by WBSPCA and CRP-4H ($r= 0.274$, $P=0.039$, figure 7.12). There was no correlation with CRP measured at other time points. Detailed r and p value of all the correlations between aspirin and CRP is attached (Appendix 12).

Figure 7.12: Correlation between CRP-4H and Platelet Aggregation in Response to 0.8mM AA (N=52)

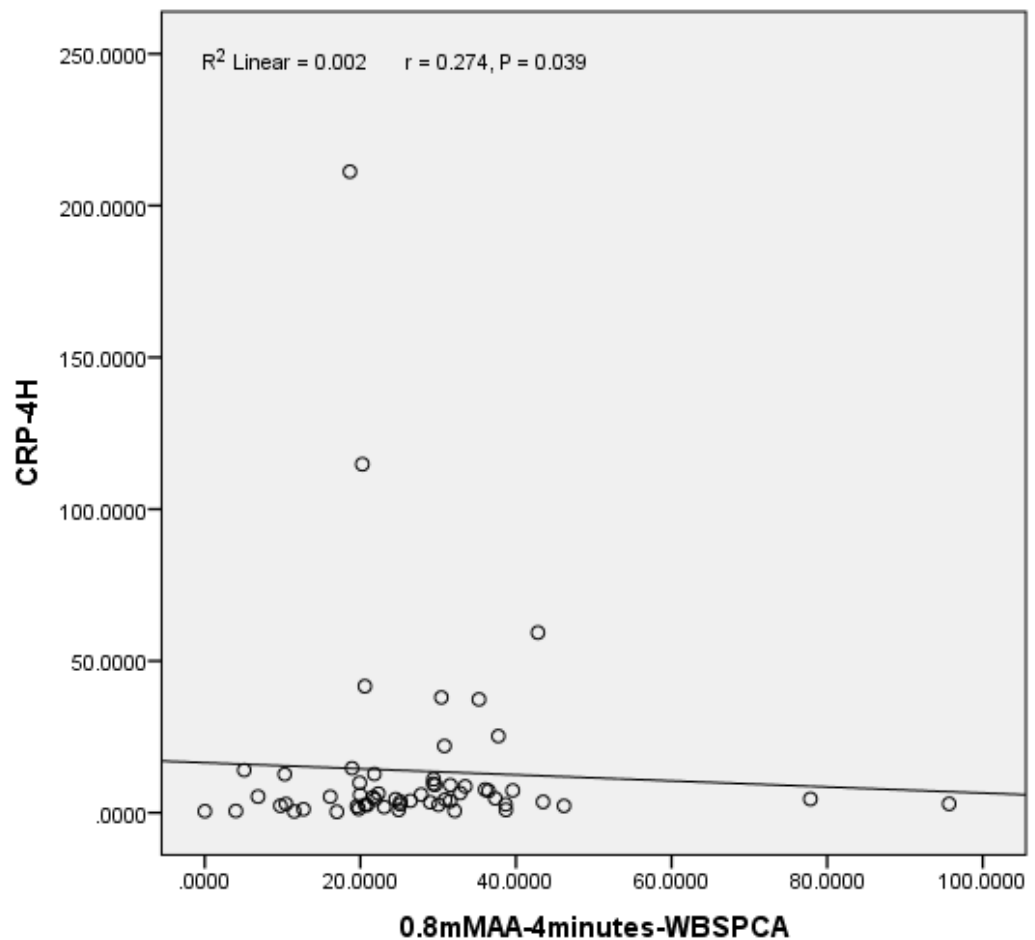


Figure 7.12: A fair but significant correlation was seen between CRP-4H and platelet aggregation in response to 0.8mM AA ($r= 0.274$, $P= 0.039$) (N=52)

7.6.2. Correlation between Platelet Aggregation in Response to 10µmol/L ADP and CRP

There was no correlation between platelet aggregation in response to 10µmol/L ADP measured at 4 minutes assessed by WBSPCA and CRP-24H in clopidogrel-treated patients, Spearman's correlation coefficient ($r = -0.158$, $P = 0.235$, figure 12). No correlation was seen with CRP measured at other time points, data attached (Appendix 12).

Figure 7.13: Correlation between Platelet Aggregation in Response to 10µmol/L ADP and CRP-24H (N=52)

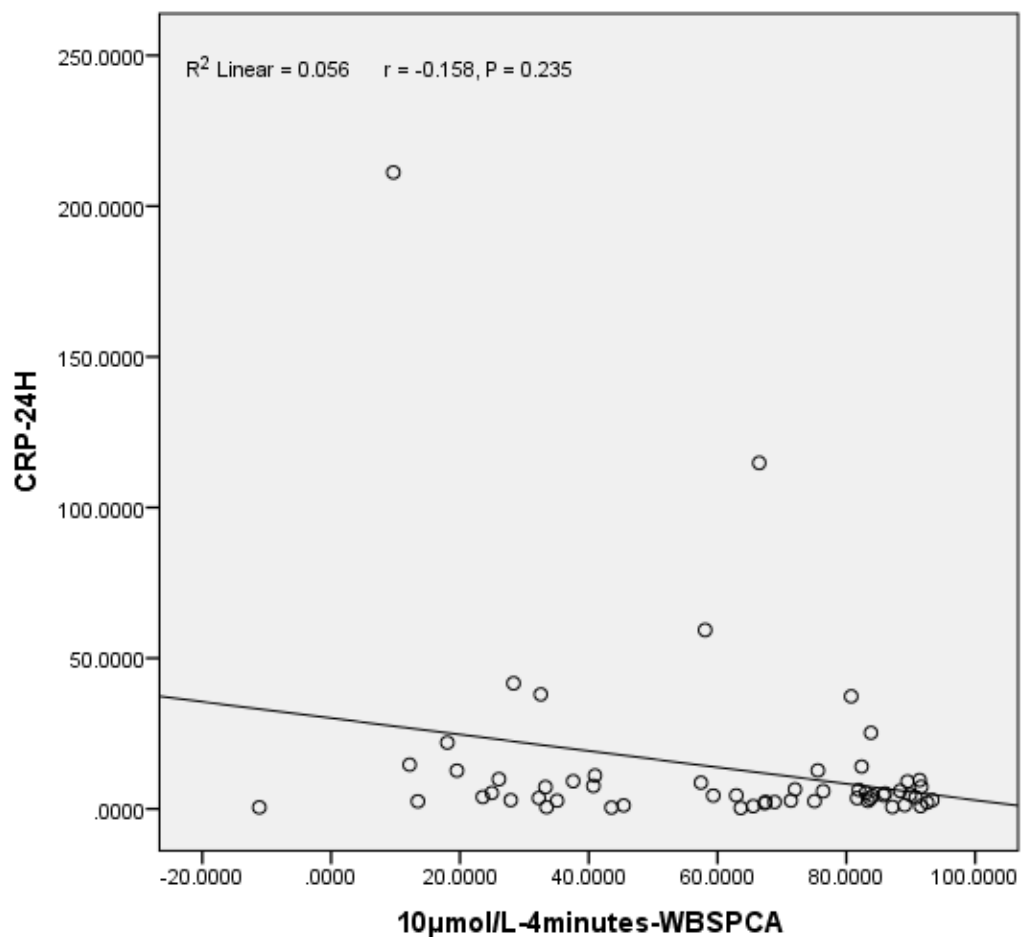


Figure 7.13: Correlation between CRP-24H and platelet aggregation in response to 10µmol/L ADP ($r = -0.158$, $P = 0.235$) (N=52).

7.7. TNF- α and its Relationship with Platelet Aggregation in Response to 0.8mM AA in Aspirin Treated Patients

49 patients were considered for analysis (N = 39/49, 80%) of patients were aspirin responders, remaining (N = 10/49, 20%) were aspirin low-responders. The mean (\pm SD) levels of TNF- α -4H in aspirin responders was 10.40 (\pm 7.46pg/ml) and that of the low-responders was 11.75 (\pm 5.84pg/ml). There was no significant difference in the mean (Wilcoxon signed rank test P= 0.324, figure 7.14). Similar values for TNF- α -24H was 10.30 (\pm 7.12pg/ml) and 11.27 (\pm 4.87, P= 0.456, figure 7.15).

Figure 7.14: TNF- α 4H

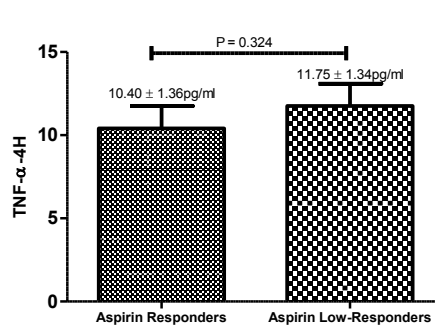


Figure 7.15: TNF- α 24H

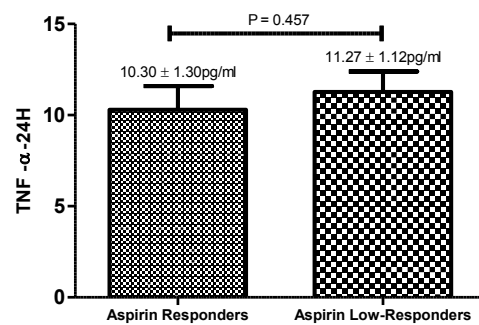


Figure 7.14: No difference in the mean between responders and low-responders (P= 0.324) (N=49)

Figure 7.15: No difference in the mean between responders and low-responders (P= 0.456) (N=49)

7.7.1. Correlation between TNF- α and Platelet Aggregation in Response to 0.8mM AA in Aspirin Treated Patients

Spearman's correlation showed a moderate but significant correlation between TNF- α -4H and platelet aggregation in response to 0.8mM AA measured at 4 minutes assessed by WBSPCA in aspirin-treated patients, (r = 0.307, P= 0.025, figure 7.16). Fair and significant correlation was seen with TNF- α measured at other time points (Appendix 13).

Figure 7.16: Correlation between TNF- α -4H and Platelet Aggregation in Response to 0.8mM AA in Aspirin Treated Patients (N=49)

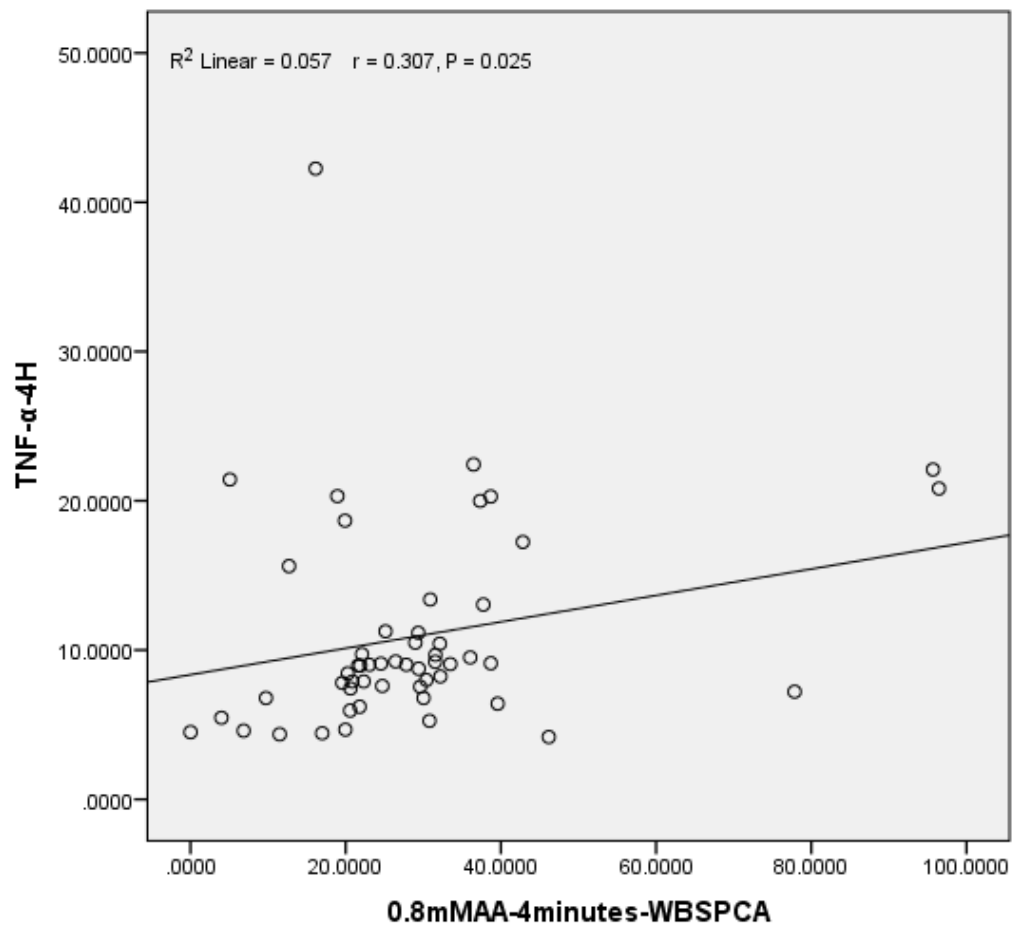


Figure 7.16: Correlation between TNF- α -4H and platelet aggregation in response to 0.8mM AA ($r = 0.307$, $P = 0.025$, $R^2 = 0.057$) (N=49)

7.8. IL-6 and its Relationship with Platelet Aggregation in Response to Aspirin

52 patients were considered for analysis. 33/52 (64%) were aspirin responders and the remaining (N=19/52, 36%) were aspirin low-responders. The mean (\pm SD) value of IL-6-4H in aspirin responders was 28.29 (\pm 23.00pg/ml) and in low-responders was 22.72 (\pm 4.84pg/ml). There was no significant difference in the mean (Wilcoxon signed rank test $P = 0.794$, figure 7.17). Likewise the mean (\pm SD) value of IL-6-24H in aspirin responders was 29.16 (\pm 21.89pg/ml) and that of non-responders

was 22.72 (\pm 14.84pg/ml). There was no significant difference in their mean, (Wilcoxon signed rank test $P= 0.481$, figure 7.18).

Figure 7.17: IL-6-4H

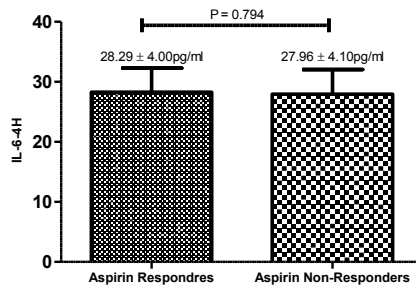


Figure 7.17: No difference in the mean ($P=0.794$) ($N=49$)

Figure 7.18: IL -6-24H

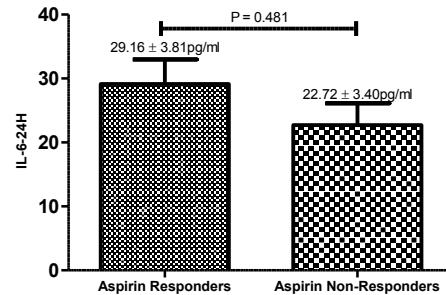


Figure 7.18: No difference in the mean ($P=0.481$) ($N=49$)

7.8.1. Correlation between IL-6 and Platelet Aggregation in Response to 0.8mM AA in Aspirin Treated Patients

There was no correlation between IL-6 levels at any point in time (B, 4H, 24H) and platelet aggregation in response to 0.8mM AA assessed by LTA. Data are included in Appendix 14.

7.9. Incidence of Ischaemic Events

61 patients out of 63 patients completed 12 months follow up. Ischaemic events occurred in 8 patients. Of these, four patients developed in stent re-stenosis. One had an acute presentation two days following the procedure with stent thrombosis. One patient died. This was an elderly patient who had a multivessel PCI and ventricular fibrillation (VF) arrest during the procedure and was successfully resuscitated. He died at one month following the procedure. One patient presented with NSTEMI and was referred for CABG surgery. One patient presented with ACS and was found to have a new lesion that needed to be stented. There was no difference in the levels of inflammatory markers in this group compared with those who did not have any ischaemic events.

7.10. Discussion

To summarize the findings of our study, there was no significant increase in the levels of inflammatory markers related to PCI at B, 4H and 24H. This is seen in all four markers studied. sCD40L levels were significantly higher in group 4 of the patients who had a platelet aggregation >90 % compared with those in group 1, platelet aggregation <30%. This was however seen only in the levels measured at 24H, not with 4H and in particular only when assessed by WBSPCA and not by LTA. There was a moderate, but significant positive correlation between platelet aggregation in response to 10µmol/L of ADP assessed by WBSPCA and sCD40L levels at 24H. TNF-α levels correlated well with platelet aggregation in response to aspirin. CRP was no different in clopidogrel responders and non- responders. CRP was numerically higher in aspirin non-responders compared to responders. No difference was seen in the levels of TNF-α and IL-6 in aspirin responders and non-responders. Neither the inflammatory markers nor post-PCI myonecrosis were any different in patients who developed MACE compared with those who did not.

Atherosclerosis, thrombus formation and inflammation are interlinked. Hence it's logical to accept that effective suppression of platelet aggregation may also have an anti-inflammatory response (296). Prevailing evidence suggests that aspirin has an effect on levels of CRP, IL-6 and TNF-α (288). Similarly, the levels of sCD40L were reduced in patients who received clopidogrel compared with placebo who were already on aspirin. However there was no significant reduction in the level of CRP in this study (297).

Mixed evidence prevails over the role of aspirin and clopidogrel in reducing the levels of inflammatory markers. On balance, aspirin seems to have a consistent effect in reducing the levels of CRP and clopidogrel in reducing the levels of sCD40L as found in our study also (288, 297). Again the increase in sCD40L was significant only 18-24 hours post PCI, but not immediately after the PCI as found by Quinn and colleagues (298). In this study they did not find any relation between clopidogrel pretreatment and

the rise of CRP post PCI. In another study by Azar and colleagues, there was no difference in the levels of CRP in patients who received clopidogrel compared with placebo, but a reduction in the levels of sCD40L was observed (297).

In our study, there was a strong positive correlation between platelet aggregation and plasma sCD40L levels. This correlation was not found with other inflammatory markers like CRP, IL-6 and TNF- α in patients treated with clopidogrel. Similar findings have been reported by Qing Yang and colleagues (299).

CRP has been positively linked with the incidence of post-PCI myonecrosis. Our study failed to demonstrate this in a significant manner although numerically the mean increase in CRP levels was higher in patients who developed post-PCI myonecrosis compared with those who did not (276).

The limitations of our study are small sample size and, although prospective, it was not a randomized study. The loading dose of clopidogrel varied between 300 and 600 mg. When comprehensively assessing all data together, the sample size was further reduced due to some missing values. There were some extreme outliers at least with the results of CRP who influenced the results to a greater extent. These abnormal levels could not be explained by clinical events. There was no accepted cut-off for inflammatory markers discriminating elevated levels from non-elevated levels. The presentations of the patients were not uniform. Although they were acute presentations, they varied in their clinical diagnosis from UA to STEMI and the time of interventions varied from 2 days from their presentation to more than 7 days. The outcomes were too small in numbers to make any conclusion about the role of inflammatory markers in predicting the outcomes.

7.11. Conclusion

sCD40L levels were significantly higher in patients with percentage of platelet aggregation > 90% in response to 10µmol/L ADP treated with clopidogrel and low in patients with a percentage of platelet aggregation < 30% in response to 10µmol/L ADP assessed by WBSPCA. There was a positive correlation between platelet aggregation in response to 10µmol/L of ADP assessed by WBSPCA and sCD40L levels 18-24 hours post PCI.

Chapter 8: Discussion and Conclusions

To summarize the findings of our study

- The incidence of ischaemic events was numerically higher in clopidogrel non-responders compared to responders but this difference was not statistically significant. The incidence of bleeding events was numerically higher in clopidogrel responders compared to non-responders although not significant. The mean percentage of platelet aggregation was higher in patients who developed ischaemic complications compared to those who developed bleeding complications.
- The incidence of ischaemic events was higher in aspirin low-responders compared to aspirin responders. Likewise the incidence of bleeding complications was higher in aspirin responders compared to low-responders, but not statistically significant. The mean percentage of platelet aggregation in response to 0.8mM AA was higher in patients who developed ischaemic complications and lower in patients who developed bleeding complications. There was moderate but significant correlation between platelet aggregations in response to 0.8mM AA measured at 4 minutes by WBSPCA and serum TXB₂ levels.
- There was moderate and significant correlation between WBSPCA and LTA
- The incidence of post PCI myonecrosis was no different in clopidogrel non-responders compared to responders. The incidence of post PCI myonecrosis was higher in aspirin low-responders compared to responders, but not significant. There was no association between the levels of pre PCI inflammatory markers and the extent of post PCI related myonecrosis.
- The levels of sCD40L 18-24 hours post PCI was significantly higher in patients whose percentage of platelet aggregation was more than 90% compared to patients whose percentage of platelet

aggregation was either less than 30% or between 30-60%. There was a moderate, but significant correlation between sCD40L levels 18-24 hours post PCI and percentage of platelet aggregation in response to 10 μ mol/L of ADP assessed by WBSPCA in clopidogrel-treated patients. There was moderate and significant correlation between TNF- α levels and percentage of platelet aggregation in response to 0.8mM AA in aspirin-treated patients.

Limited data are available about WBSPCA in monitoring the efficacy of clopidogrel and aspirin. However, previous studies have shown that WBSPCA could be a useful tool in monitoring the antiplatelet effect of GP IIb/IIIa inhibitors (244). There is compelling evidence to suggest a strong association between HPR and future periprocedural and long term clinical outcomes notwithstanding the lack of an optimal method to define HPR and risk stratify the patients (300). In the majority of the prospective studies, clopidogrel has been widely studied: the higher the residual platelet reactivity, the higher the risk of cardiovascular adverse events (301).

HPR on clopidogrel treatment using LTA has been linked with recurrent ischaemic events although this test is not specific for the P2Y₁₂ pathway. Platelet reactivity index, determined by VASP phosphorylation assay, has also been associated with recurrent ischaemic events after PCI and most importantly has a strong negative predictive power below a certain cutoff. The VASP assay however does not include the contribution of the P2Y₁ receptor to the platelet response. The VerifyNow assay is a user friendly bedside tool. A higher PRU value assessed by this assay is associated with adverse cardiovascular events. Most clinical trials linking low response to clopidogrel with clinical outcomes have employed LTA and VerifyNow assay. Although we now have cut-off values that define HPR for each PFT, these values may have different weights in different settings, like urgent versus elective PCI, and periprocedural setting versus maintenance treatment phase. Moreover, these cutoff values had significant negative predictive values for recurrence of ischaemic events

compared to positive predictive value. It is important to acknowledge that although HPR is a major risk factor for future thrombotic events, many other factors contribute to these events(241).

In our study we have shown that clopidogrel non-responders, as defined using WBSPCA, had a numerically higher incidence of ischaemic events conversely the mean percentage of platelet aggregation in clopidogrel treated patients was higher in those who developed ischaemic events.

Gaglia et al attempted to evaluate the degree of agreement and correlation among VASP, LTA and VerifyNow assay in patients on clopidogrel therapy undergoing PCI. HPR was defined according to the latest consensus recommendation. It was found that there was only a fair degree of agreement between those tests. Nevertheless, although the agreement among tests may be modest, it still remains that the individual tests have all demonstrated significant correlation with occurrence of adverse CV outcomes beyond a certain cut-off value adopted by consensus (302). In our study we found that there was a moderate and significant correlation between WBSPCA and LTA.

Guidelines have rather mixed recommendations regarding the applicability of PFT in patients treated with antiplatelets. The 2012 update of the Society of Thoracic Surgeons guidelines stated that, in patients treated with antiplatelets, a point-of-care test to assess platelet function may be useful in identifying patients with high residual platelet reactivity and hence with less risk for bleeding during CABG (303). The 2014 ACC/AHA guideline for the management of patients with NSTEMI-ACS does not recommend PFT to determine platelet inhibitory response (304). The 2015 ESC guidelines for the management of ACS in patients with NSTEMI do not recommend the routine performance of PFT. In addition, the document notes that, "PFT may be considered in selected patients treated with clopidogrel including those with a history of stent thrombosis, suspected non-compliance, as well as persistent high on-treatment platelet reactivity or high bleeding risk in the presence of stents in critical coronary segments" (62).

Lately PFT have gained an important role in the monitoring of modern antiplatelet therapy but still uncertainties remain about its clinical utility. The indications for PFT in the field of CV medicine is not just restricted to monitoring the effects of antiplatelets, but also have an extended role in acute care settings like CABG and PCI. These interventions are prone to thrombotic and bleeding complications regardless of the use of antiplatelets and antithrombotics. Hence obtaining baseline information pertaining to platelet function is suggested to improve the outcomes of the procedure. Moreover the need to use antiplatelets and antithrombotics in this clinical situation is likely; hence it is even more compelling to screen patients for their platelet function. The platelet count, its response and the distribution of platelet receptors on the platelets is varied. Therefore the way in which each individual responds to antiplatelet therapy is unique and forms a strong basis for individualizing their treatment based on their platelet function (305).

Growing evidence suggests that platelet hyper-reactivity by itself is an independent risk factor for future MACE and platelet hyper-reactivity is present in all CV risk conditions like smoking, hypertension, obesity and diabetes. Hence PFT as a routine screening test to predict future adverse CV events is a remote possibility (306). Equally PFT might become a valid tool to predict bleeding complications, at least in high risk patients, such as those taking anticoagulants along with antiplatelets and in the elderly population (265).

Until the introduction of the newer P2Y₁₂ inhibitors like prasugrel and ticagrelor, clopidogrel, and its less safe predecessor ticlopidine, remained the only other antiplatelet agent to be used in conjunction with aspirin for the treatment of ACS in both medically managed patients and patients treated by PCI. Although prasugrel and ticagrelor are now recommended for the treatment of ACS in preference to clopidogrel, except in those with contraindications or with an indication for oral anticoagulant therapy, clopidogrel still has a class I indication in the treatment of ACS. This is because the newer agents are not widely available globally or may not be affordable in some healthcare settings (307). This makes clopidogrel the

most widely used antiplatelet agent globally. Interindividual variation in the platelet aggregation response in clopidogrel treated patients led to the concept of individualizing the loading dose of clopidogrel (300mg, 600mg and 900mg) dependent on the platelet reactivity. Currently that model is not recommended as dose adjustment failed to establish clinical benefits (227, 233).

Plateletworks is a point-of-care tool that works on the principle of single platelet counting techniques. It uses ADP as agonist and hence is used to measure the effect of P2Y₁₂ inhibitor particularly clopidogrel. It has good results in monitoring the effectiveness of GP IIb/IIIa inhibitor (308). It measures platelet micro aggregation as opposed to LTA that measures platelet macro aggregation. Plateletworks has demonstrated good agreement with LTA assay in measuring the effectiveness of clopidogrel in patients undergoing PCI (309), although the assay is more time dependent. WBSPCA works on the same principle as PlateletWorks. It uses ADP as agonist and has shown promising results in monitoring GP IIb/IIIa inhibitors (244, 310). In our study we have shown that that mean platelet aggregation assessed by WBSPCA in patients presenting with ACS and treated with antiplatelets was higher in patients who developed ischaemic complications and was low in patients who developed bleeding complications. WBSPCA is a simple test that is cost effective and user friendly. This could be considered in the context of assessing the baseline platelet reactivity of an individual before commencing antiplatelet medications.

Our study has several limitations. Patients belonged to all spectrum of ACS including UA/NSTEMI and STEMI. In addition, patients were treated both medically without any interventions and with interventions. Patients received varying dose of clopidogrel ranging from 75 mg of maintenance dose alone to 300 and 600 mg of loading dose followed by 75 mg of maintenance dose. The definitions for responders and non-responders to clopidogrel and aspirin were not standardized. The sample size is small and the original design of the project is to obtain sample data on the utility of this test in assessing the efficacy of clopidogrel. Hence our study was

not adequately powered to detect ischaemic and bleeding events. When the study was conducted between 2005 and 2009, limited information was available about PFT in general. However with the evidence from more large scale studies that was published subsequently, we now have better clarity on the role of PFT in clinical scenarios particularly its role in high risk and low risk ACS. Moreover we currently have precise cut-off and a consensus about the definition of HPR based on different PFT. This information was not available to us during the conduct of this study. At the same time, the work has suffered some delay and the data that we now have might be slightly outdated. However, as WBSPCA is not widely studied the information generated from this study surely adds to the wealth of knowledge that we have about the subject and will certainly form a platform for future work.

Available evidence does not support PFT-guided antiplatelet therapy for the treatment of ACS. However, this conclusion is drawn from studies related to CAD. More evidence is warranted in other disease conditions where aspirin and clopidogrel are equally used like stroke, TIA, neuro-interventions, PAD and left ventricular assist device. A number of small studies have shown some initial promising results in these clinical settings and in the paediatric age group. More large scale studies are needed to confirm the findings of such small studies. Until future studies exclude the usefulness of PFT in the above mentioned clinical scenarios, we cannot dismiss the usefulness of PFT prematurely. As long as there is an utility for PFT, WBSPCA can always be a potential tool as our study although small in size has produced results comparable with other large scale studies with other PFT. Other potential use of PFT is to monitor compliance to antiplatelet therapy. PFT can also help us to determine timing of cardiac surgery following withdrawal of clopidogrel particularly in whom an early surgery is indicated who otherwise has to wait for the guidelines stipulated time before surgery could be done. Assessing the usefulness of WBSPCA assay to particularly predict bleeding outcomes offers scope for future research (249).

It has been suggested that the ability of the PFT to assess the hazard ratio for a given complication should be taken into account in assessing its clinical utility since HPR to ADP is a risk factor and not a diagnosis (311). P2Y₁₂ based PFT assess the overall effectiveness of the receptor and not necessarily just the effect of the P2Y₁₂ inhibitors on the receptor, this concept needs consideration while interpreting the results of the assay (312). HPR to ADP does not appear to predict future events in medically managed ACS patients and in low risk ACS patients (313). Hence platelet function testing in this group is not recommended. Low platelet reactivity (LPR) to ADP is associated with increased bleeding complications in patients treated with P2Y₁₂ receptor inhibitors. LPR to ADP is an independent risk factor for bleeding in patients undergoing PCI (314). In the future, we might aim to establish a therapeutic window for each P2Y₁₂ inhibitor where the combined risk of bleeding and thrombotic events is minimized.

Both LTA and VASP are not recommended as routine tests to assess the HPR to ADP as they are very cumbersome and methodological errors are possible (265). Simple POCT are preferred over these assays in clinical settings. WBSPCA has the potential to meet the above said criteria. VASP and LTA play a crucial role as research tools to assess HPR to ADP. WBSPCA is a simple, cost effective and user friendly assay that has the ability to monitor the efficacy of aspirin, GP IIb/IIIa inhibitors and P2Y₁₂ inhibitors. We have shown in our study that the assay correlates moderately with LTA, and serum TXB₂. Globally, clopidogrel still remains the widely used P2Y₁₂ inhibitor in addition to aspirin in the management of ACS both medically and by intervention. Availability and cost are the advantages of clopidogrel over the newer agents like prasugrel and ticagrelor in countries with limited healthcare expenditure. In such setting, use of PFT-guided therapy may be an option in high risk individuals undergoing PCI. Given this scenario, there is a huge potential for a point-of-care instrument that can give reliable and quick results based on which therapeutic decisions can be made. This gives a promising hope for tools like WBSPCA.

Conclusion

The overall consensus about the ideal test is one which accurately predicts both ischaemic and bleeding risks. A simple but sophisticated tool that gives reliable and reproducible information and that meets quality control standards based on which clinical judgments could be safely taken is highly needed. Future work on whole blood single platelet counting assay can determine its usefulness as an effective tool to monitor the effects of P2Y₁₂ inhibitors.

9. Reference

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10. Appendix

Appendix 1-Consent Form



The University of Sheffield

CONSENT FORM

Title of Project: Pilot study of the relationship between inhibitory effects of antiplatelet agents and safety and efficacy in patients with acute coronary syndromes: towards designing the optimal monitored antiplatelet strategy

(A study of the effects of routine anti-clotting drugs in patients with unstable angina and heart attacks)

Name of Researcher: DR. ROBERT F. STOREY

Please initial box

1. I confirm that I have read and understand the information sheet dated 30th September 2004 Version 3 for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study.

Name of Patient

Date

Signature

Name of Person taking consent

Date

Signature

1 for patient; 1 for researcher

Appendix 2- Amendment



NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at <http://eudract.emea.eu.int/document.html#guidance>.

To be completed in typescript by the Chief Investigator and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available in section 5 of our Standard Operating Procedures available at www.corec.org.uk/applicants/help/docs/SOPs.doc.

Details of Chief Investigator:	
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Full title of study:	: Pilot study of the relationship between inhibitory effects of antiplatelet agents and safety and efficacy in patients with acute coronary syndromes: towards designing the optimal monitored antiplatelet strategy
Name of main REC:	North Sheffield
REC reference number:	NS2003 11 1800
Date study commenced:	6 October 2004
Protocol reference (if applicable), current version and date:	Version 4, 8 November 2005
Amendment number and date:	Amendment no. 3, 8 November 2005

Appendix 3

Pilot study of the relationship between the inhibitory effects of antiplatelet agents and safety and efficacy in patients with acute coronary syndromes: towards designing the optimal monitored antiplatelet strategy

Schedule of questions for outpatient telephone interview

Before contacting patients by telephone, the hospital information system will be interrogated to ensure there is no record of death since the last contact and the General Practice surgery will be contacted to ensure the same.

The following questions will be asked during the telephone interview at 30 days:

1. Have you had any further admissions to hospital since you were discharged from the Northern General Hospital on ...(date)...?
2. Have you had any new problems since that date?
3. Have you had any problems with bruising or bleeding since that date?
4. Have you required any further treatment for your heart condition since that date?
5. Have any changes been made to your medication since that date, such as stopping any medication, or are any changes planned?
6. Have you required any blood tests since that date and, if so, have you been informed of any abnormal results?

The following questions will be asked during the telephone interview at 3 months and at 12 months:

7. Have you had any further admissions to hospital since on our last telephone discussion on ...(date)...?
8. Have you had any new problems since that date?
9. Have you had any problems with bruising or bleeding since that date?
10. Have you required any further treatment for your heart condition since that date?
11. Have any changes been made to your medication since that date, such as stopping any medication, or are any changes planned? [If appropriate] What was the reason, if any, for the change in medication?
12. Have you required any blood tests since that date and, if so, have you been informed of any abnormal results?

Appendix 4 - Descriptive Statistics of Platelet Aggregation Assessed by LTA and WBSPCA

ADP Concentrations	N	Minimum	Maximum	Mean	SD
ADP 2max♣	63	.01	55.000	22.79	12.78
ADP 2final♣	63	.01	42.000	2.56	7.34
ADP 5max♣	62	.01	62.000	30.89	15.11
ADP 5final♣	62	.01	61.0000	8.87	14.22
ADP 20max♣	62	.01	70.0000	37.35	16.59
ADP 20final♣	62	.01	66.00000	19.13	18.814
ADP 10min♣	63	.01	94.0200	61.21	26.69

♣ Light transmission aggregometry ♣ Whole blood single platelet counting assay

Appendix 5

Smoking / Troponin													
Group	10 ADP 4MIN %	2 ADP MAX %	2 ADP FINAL %	5 ADP MAX %	5 ADP FINAL %	20 ADP MAX %	20 ADP FINAL %	cTn ng/ml	N	%	P Value		
Smoking Yes	60.31	22.00	1.33	30.88	6.31	40.23	20.35	1.28	27/50	54%	0.066		
Smoking No	63.12	20.52	3.00	31.00	10.52	34.26	17.39	0.52	23/50	46%			
Smoking Yes / cTn Yes	62.48	22.81	1.94	30.73	8.93	38.67	23.60	2.16	16/50	32%			
Smoking No / cTn No	55.91	18.67	0.00	33.00	11.00	34.83	12.83	0.01	6/50	12%	0.2769		
Smoking Yes / cTn No	57.17	20.82	0.45	31.09	2.73	42.36	15.91	0.01	11/50	22%			
Smoking No / cTn Yes	65.66	21.18	4.06	30.29	10.35	34.06	19.00	0.71	17/50	34%	0.4639		
Troponin / CAD													
CAD Yes	58.10	24.93	5.00	31.53	8.07	34.13	15.20	2.11	15/50	30%	0.070		
CAD No	63.10	19.77	0.86	30.68	8.38	38.88	20.62	0.43	35/50	70%			
cTn Yes / CAD Yes	61.56	22.75	5.42	29.67	6.67	31.67	13.17	1.69	12/50	24%			
cTn No / CAD No	61.24	19.60	0.33	31.87	6.20	40.33	16.13	0.01	15/50	30%	0.3879		
cTn No / CAD Yes	22.87	23.50	0.00	31.00	1.50	35.00	5.00	0.00	2/50	4%			
cTn Yes / CAD No	64.50	19.90	1.25	29.74	10.11	37.74	24.16	0.74	20/50	40%	0.0653		
Troponin / DM													
DM Yes	78.10	29.83	10.67	42.17	19.00	47.33	34.00	0.33	6/50	12%	0.3565		
DM No	59.35	20.16	0.93	29.37	6.79	36.05	16.86	1.02	44/50	88%			
cTn Yes / DM Yes	75.82	30.60	12.80	42.80	21.40	46.00	33.80	0.39	5/50	10%			
cTn No / DM No	54.67	19.69	0.31	31.31	5.56	38.81	13.56	0.01	28/50	56%	0.0893		
cTn No / DM Yes	89.49	26.00	0.00	39.00	7.00	54.00	35.00	0.00	1/50	2%			
cTn Yes / DM No	62.03	20.43	1.29	28.22	7.52	34.41	18.81	1.59	16/50	32%	0.2901		

Appendix 6

Table 1: Descriptive statistics of inflammatory markers					
Inflammatory markers	N	KS normality test	Mean \pm SD	Median with IQR	P value
CRP base	52	no	8.92 \pm 21.72	4.13 \pm (1.72-7.82)	
CRP 2-4hr	52	no	10.20 \pm 22.51	4.61 \pm (1.97-8.62)	
CRP 18-24hr	52	no	13.34 \pm 33.23	4.50 \pm (2.36-9.17)	
CRP 2-4-base	52	no	1.28 \pm 8.68	0.20 \pm (-0.30-0.82)	0.1272
CRP 18-24-base	52	no	4.42 \pm 13.57	0.48 \pm (-0.04-2.20)	0.0031
sCD40L-B	52	yes	2.21 \pm 0.93	2.09 \pm (1.48-2.78)	
sCD40L-4H	52	no	2.41 \pm 1.12	2.02 \pm (1.68-2.78)	
sCD40L-24H	52	no	2.43 \pm 1.11	2.00 \pm (1.73-3.20)	
sCD40L-4H-B	52	no	0.20 \pm 0.98	0.05 \pm (-0.38-0.42)	0.5569
sCD40L-24H-B	52	no	0.22 \pm 0.76	0.04 \pm (-0.14-0.42)	0.1151
TNF- α -B	49	no	10.39 \pm 6.07	8.60 \pm (6.36-11.62)	
TNF- α -4H	49	no	10.93 \pm 6.85	9.02 \pm (7.00-12.15)	
TNF- α -24H	49	no	10.68 \pm 6.30	9.36 \pm (6.31-12.24)	
TNF- α -4-B	49	no	0.54 \pm 2.49	0.12 \pm (-0.76-1.36)	0.2884
TNF- α -24H-B	49	no	0.29 \pm 2.99	0.25 \pm (-0.94-1.40)	0.3654
IL-6-B	52	no	23.52 \pm 19.39	16.78 \pm (6.40-38.30)	
IL-6-4H	52	no	28.17 \pm 21.09	24.00 \pm (9.02-44.33)	
IL-6-24H	52	no	26.80 \pm 19.70	21.15 \pm (8.65-44.95)	
IL-6-4H-B	52	no	4.65 \pm 16.41	3.13 \pm (-5.05-11.71)	0.0728
IL-6-24H-B	52	no	3.28 \pm 14.47	2.45 \pm (-5.80-7.58)	0.2419
* KS = Kolmogorov-Smirnov normality test: only sCD40LB is normally distributed					
*There is a significant difference in the elevation of CRP-24H from CRP-B P=0.0031					

Appendix 7 - ANOVA Analysis of the sCD40L-4H Compared with WBSPCA

ANOVA	Mean Diff	q	95% CI of Diff	P<0.05
0-<30% vs. 30-<60%	-0.56	1.53	-1.93 to 0.81	No
0-<30% vs. 60-<90%	-0.55	1.76	-1.74 to 0.63	No
0-<30% vs. >90%	-0.71	1.59	-2.42 to 0.99	No
30-<60% vs. 60-<90%	0.01	0.02	-1.10 to 1.11	No
30-<60% vs. >90%	-0.16	0.36	-1.80 to 1.49	No
60-<90% vs. >90%	-0.16	0.41	-1.66 to 1.33	No

Appendix 8 - ANOVA Analysis of the sCD40L-24H and its Relationship with 10 ADP – WBSPCA

ANOVA	Mean Diff	q	95% CI of Diff	P<0.05
0-<30% vs. 30-<60%	-0.39	1.26	-1.56 to 0.78	No
0-<30% vs. 60-<90%	-0.92	3.45	-1.93 to 0.09	No
0-<30% vs. >90%	-1.98	5.17	-3.43 to -0.54	Yes
30-<60% vs. 60-<90%	-0.53	2.14	-1.47 to 0.41	No
30-<60% vs. >90%	-1.59	4.29	-2.99 to -0.19	Yes
60-<90% vs. >90%	-1.06	3.15	-2.33 to 0.21	No

Appendix 9 - ANOVA analysis of the sCD40L-24H and its Relationship with 5 ADP final –LTA

ANOVA	Mean Diff	q	95% CI of Diff	P<0.05
0% vs. 0-<14%	-0.348	1.39	-1.21 to 0.51	No
0% vs.<14%	-0.70	2.41	-1.70 to 0.30	No
0-<14%	-0.36	1.29	-1.30 to 0.59	No

Appendix 10 - ANOVA analysis of the sCD40L-24H and its relationship with 20 ADP max – LTA

ANOVA	Mean Diff	q	95% CI of diff	P<0.05
>25% vs. 25-49%	-0.40	1.51	-1.32 to 0.52	No
>25% vs. ≥50%	-0.25	0.82	-1.30 to 0.80	No
25-49% vs. ≥50%	0.16	0.58	-0.76 to 1.07	No

Appendix 11 - Correlation co-efficient and P values of inflammatory markers in relation with 0.8mM AA measured by WBSPCA

	CRP-B	CRP-4H	CRP-24H	TNF-B	TNF-α 4H	TNF-α 24H	IL-6B	IL-6-4H	IL-6-24H
r	0.182	0.274	0.160	0.291	0.307	0.270	0.062	0.132	0.061
P	0.156	0.039	0.229	0.028	0.025	0.051	0.630	0.329	0.652

Appendix 12 - Spearman's correlation co-efficient and p values of inflammatory markers in relation with 10 ADP measured by WBSPCA

	CRP B	CRP-4H	CRP-24H	sCD40L-B	sCD40L-4H	sCD40L -24H
R	-0.015	-0.125	-0.158	0.274	0.205	0.343
P	0.907	0.354	0.235	0.031	0.129	0.0085

Appendix 13 - Spearman's correlation co-efficient and P values of inflammatory markers in relation with 20 ADP max measured by LTA

	CRP B	CRP 2-4	CRP 18-24	sCD40LB	sCD40L-4H	sCD40L-24H
R	0.1737	0.03298	0.06119	0.2031	0.07146	0.1545
P	0.1806	0.8093	0.6512	0.1164	0.6041	0.2511