Platelet activation status, on-treatment platelet reactivity and reticulated platelets in the early and late phases after TIA or ischaemic stroke

A thesis submitted to the University of Dublin, Trinity College, 2020 for examination for a PhD in Clinical Medicine / Clinical Neurosciences

by

Soon Tjin Lim MB, BCh, BAO, LRCP & SI, DTM, DGM, DSCM, MRCPI, MRCP UK, MRCP SCE (Neurology)

Department of Neurology,
The Adelaide and Meath Hospital, Dublin, incorporating the National Children's Hospital / Tallaght University Hospital
Trinity College Dublin

Declaration

I designed all of the studies described in this thesis with the advice and supervision of my colleagues listed in the acknowledgements section. I recruited and clinically assessed all of the patients studied in this thesis. I performed venepuncture on all of the study subjects, prepared the samples for analysis, carried out the flow cytometry experiments, PFA-100® assays, VerifyNow® assays, Multiplate® assays and FBC measurements and prepared the platelet poor plasma for further analysis. I entered all of the data onto a database and performed the statistical analysis of the data with the help and supervision of Prof. Dominick J.H. McCabe and Prof. Vincent Thijs.

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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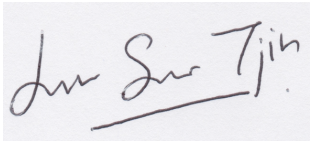

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Dr. Soon Tjin Lim

Summary

Aims: This thesis aimed to assess platelet activation status, on-treatment platelet reactivity and the profile of reticulated platelets in patients with ischaemic cerebrovascular disease (CVD) who were commencing or changing antiplatelet therapy.

Methods: We conducted a collaborative systematic review and meta-analysis of relevant published data on Antiplatelet-high on treatment platelet reactivity (Antiplatelet-HTPR) following TIA/ischaemic stroke. We performed independent, observational analytical studies with comprehensive, longitudinal follow-up in eligible adult patients within 4 weeks of TIA/ischaemic stroke onset who were commencing or changing antiplatelet therapy. Patients underwent detailed clinical and laboratory assessment with venepuncture before (**baseline**), 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after starting aspirin, clopidogrel or after adding dipyridamole to aspirin, with additional clinical follow-up at ≥ 1 year after symptom onset. Platelet activation status was assessed with whole blood flow cytometry to quantify the expression of platelet surface activation markers (CD62P and CD63), circulating leucocyte-platelet complexes, and the percentage of circulating reticulated platelets (%RP). The prevalence of *ex vivo* high on-treatment platelet reactivity (HTPR) in whole blood was assessed with the PFA-100®, VerifyNow® and Multiplate® platforms.

Results: Our meta-analysis suggests that identification of Antiplatelet-HTPR predicts the risk of recurrent vascular events and outcomes in CVD patients, especially on aspirin. 124 patients were assessed at baseline in the longitudinal studies assessing platelet activation and HTPR status in patients commencing aspirin, clopidogrel or dipyridamole. 211 patients were assessed at baseline in the collaborative case-control studies assessing the %RP in patients with recent TIA/stroke. There were no changes in platelet activation status over time in the cohort of patients who commenced aspirin or clopidogrel. The % monocyte-platelet complexes initially increased at 14d, but this increase was not sustained at 90d in

the overall cohort of patients who commenced dipyridamole treatment; we confirmed this rise was driven by data from the subgroup of patients with dipyridamole-HTPR. The PFA-100®, VerifyNow® and Multiplate® assays were found to be effective at detecting the *ex vivo* antiplatelet effects of aspirin, clopidogrel and dipyridamole. The prevalence of **Aspirin-HTPR** by a 'cross-sectional/case-control definition' across all devices was 9.5 - 23.8% at 14 days and 7.7 - 30% at 90d. The prevalence of aspirin-HTPR by our 'novel longitudinal definition' was 4.8% at 14 days and 7.7% at 90d. The cross-sectional prevalence of **Clopidogrel-HTPR** was 31.6 - 60.5% overall in the early phase and 20.8 - 56% in the late phase after TIA/ischaemic stroke. The prevalence of Clopidogrel-HTPR with our novel longitudinal definition was lower at 14d (13.9 - 21%) and 90d (4 - 4.5%). The prevalence of **Dipyridamole-HTPR** with our novel longitudinal definition was 75-83.3% during the early phase and 71.4 - 81.5% during the late phase after TIA/stroke. The %RP was increased in the late phase (> 90 days), but was not significantly increased in the early phase (≤4 weeks) after TIA or ischaemic stroke overall compared with healthy controls. The %RP was significantly elevated only in the subgroup of patients with TIA/stroke due to small vessel disease at baseline and at 90d, but not at 14d *vs*. controls, with the findings in this small vessel disease subgroup at 14d possibly reflective of a type II error.

Conclusions: An important proportion of CVD patients have Antiplatelet-HTPR with 'user-friendly' assays. The prevalence of Aspirin-HTPR and Clopidogrel-HTPR was numerically lower with novel longitudinal *vs.* cross-sectional definitions. Simultaneous assessment of platelet activation status and reticulated platelets may improve our understanding of the pathogenesis of Antiplatelet-HTPR in CVD. Large, multi-centre studies have been designed to definitively assess whether testing HTPR status can facilitate personalised antiplatelet therapy in CVD patients to optimise secondary prevention following TIA/ischaemic stroke.

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Copyright Statement

Publications and Presentations Completed During The Course of this thesis

Publications in Journals

1) Stephen J. X. Murphy, **Lim ST**, Justin A. Kinsella Sean Tierney, Bridget Egan T. M. Feeley Sinead M. Murphy Richard A. Walsh D. R. Collins Tara Coughlan Desmond O'Neill, McCabe DJH**.** Increased Leucocyte–Platelet Complex Formation in Recently Symptomatic versus Asymptomatic Carotid Stenosis Patients and in Micro-emboli Negative Subgroups. *Thrombosis and Haemostasis 2019*
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2) Murphy SJX, **Lim ST**, Coughlan CA, Kinsella JA, Tierney S, Egan B, Feeley TM, Murphy SM, Walsh RA, Collins DR, Coughlan T, O'Neill D, Harbison J, Madhavan P, O'Neill SM, Colgan MP, Cox D, Moran N, Hamilton G, McCabe DJH. Increased platelet count and lymphocyte-platelet complex formation in patients with recently symptomatic versus asymptomatic carotid stenosis: Results from the HaEmostasis In carotid STenosis (HEIST) study. *Eur Stroke Journal* 2016; I (IS): 405.

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Coughlan, Desmond O'Neill, Joseph Harbison, Prakash Madhavan, Sean O'Neill, Mary Paula Colgan, Dermot Cox, Niamh Moran, George Hamilton, Dominick McCabe. Evidence of ongoing platelet activation in micro-emboli negative recently symptomatic versus asymptomatic carotid stenosis. *Stroke* 2018; 49: AWP113.

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1) Poster presentation at the Irish Neurological Association Meeting; 6th – 7th June 2019, Cork, Ireland: **Simultaneous Assessment of Plaque Morphology, Micro-embolic Signal Status and Platelet Biomarkers Enhances Understanding of The Pathogenesis Of Symptoms In Symptomatic And Asymptomatic Carotid Stenosis Patients.**

2) Poster presentation at the American Academy of Neurology Meeting; 4th - 11th May 2019, Philadelphia, United States of America. **On-treatment platelet reactivity' under both high and low shear stress conditions and relationship with cerebral micro-embolic signals in asymptomatic and symptomatic carotid stenosis: Results from the Haemostasis In Carotid Stenosis (HEIST) study.**

3) Poster presentation at the International Society on Thrombosis and Haemostasis Meeting; 18th-21st July 2018, Dublin, Ireland: **Emerging Value of Platelet Function Testing at Predicting the Risk of Recurrent Vascular Events and Outcomes after TIA / Ischaemic Stroke: A Systematic Review of the Literature.**

4) Poster presentation at the Irish Neurological Association Meeting; 10th November 2017, Dublin, Ireland: **Evidence of Ongoing Platelet Activation in Micro-Emboli Negative Recently Symptomatic Versus Asymptomatic**

Carotid Stenosis: Results from The Haemostasis In Carotid Stenosis (HEIST) Study.

5) Poster presentation at the Irish Heart Foundation Meeting; 8th April 2016, Dublin, Ireland: **Optimal Antiplatelet Therapy in Stroke as Guided by Platelet Function Testing**

6) Poster presentation at the European Stroke Organisation (ESO) Meeting; 10-12th May 2016, Barcelona, Spain: **Utility of Platelet Function Testing in TIA and Stroke.**

7) Platform and Poster presentation at the Irish Neurological Association Meeting; 28th-29th May 2015, Galway, Ireland**: Outcomes in TIA or Stroke Patients with Patent Foramen Ovale, Inter-Atrial Septal Aneurysm or Both.**

8) Poster presentation at the European Stroke Organisation Conference (ESO) Meeting; 17th-19th April 2015, Glasgow, United Kingdom: **Prevalence of Arterial and Venous Thrombophilia in TIA or Ischaemic Stroke Patients With A Patent Foramen Ovale, With or Without an Inter-Atrial Septal Aneurysm.**

9) Poster presentation at the Irish Neurological Association Meeting; 8th -9th November 2013, Dublin, Ireland: **To TPA or Not To TPA That is the Question.**

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Abbreviations

AA	Arachadonic acid
ADAMTS-13	A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif number 13
AMP	Adenosine Monophosphate
APTT	Activated Partial Thromboplastin time
ARU	Aspirin Reaction Unit
ATP	Adenosine Triphosphate
C-ADP	Collagen-ADP
cAMP	Adenosine 3′, 5′-cyclic monophosphate
CAST	Chinese Acute Stroke Trial
CD	Cluster Differentiation
CDUS	Carotid Duplex Ultrasound
CE	Conformité Européene
CEA	Carotid Endarterectomy
C-EPI	Collagen-Epinephrine
$\chi 2$	Chi-Squared statistic
CI	Confidence Interval
cGMP	Guanosine 3′, 5′-cyclic monophosphate
COX	Cyclo-oxygenase
CT	Computerised Tomography
CTA	Computerised Tomography Angiography
CVD	Cerebrovascular Disease
Cy5	Cyanine 5
DNA	Deoxyribonucleic Acid
DP	Dipyridamole
DVT	Deep Vein Thrombosis
DWI	Diffusion Weighted Imaging
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ESO	European stroke organisation
ESPS-2	Second iteration of the European Stroke Prevention Study
FBC	Full Blood Count

FITC	Fluorescein Isothiocyanate
FLAIR	Fluid Attenuation Inversion Recovery
FS	Forward Scatter
G Proteins	Guanine Nucleotide Binding Regulatory Proteins
Gp	Glycoprotein
GTP	Guanosine Triphosphate
HBS	HEPES buffered saline
HBSS	Hanks Balanced Saline Solution
HTPR	High On-Treatment Platelet Reactivity
H2O	Water
ICA	Internal Carotid Artery
Iso-TRAP	iso- thrombin receptor activating peptide
IST	International Stroke Trial
LAMP	Lysosomal-Associated Membrane Proteins
LPCs	Leukocyte-platelet Complexes
MAC-1	Macrophage 1 antigen
MCA	Middle Cerebral Artery
MCL	Multi-tube Carousel Loader
MCP-1	Monocyte Chemotactic Protein-1
MFI	Mean Fluorescence Intensity
MMP-9	Matrix Metalloproteinase-9
MPV	Mean Platelet Volume
MR	Modified Release
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRS	modified Rankin Scale
NSAID	Non-steroidal Anti-inflammatory Drug
OCS	Open Canalicular System
OR	Odds Ratio
PAD	Peripheral Arterial Disease
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor-1
PARs	Protease Activated Receptors
PAU	Platelet Aggregation Unit

PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PDW	Platelet Distribution Width
PE	Phycoerythrin
PECAM	Platelet Endothelial Cell Adhesion Molecule Platelet Endothelial Cell Adhesion Molecule
PF-4	Platelet Factor 4
PFA-100®	Platelet Function Analyser-100, Dade-Behring, Germany
PG	Prostaglandin
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-biphosphate
PMT	Photomultiplier Tube
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PRU	P2Y12 Reaction Unit
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PT	Prothrombin Time
PTE	Phosphatidylethanolamine
RCT	Randomised controlled trial
RNA	Ribonucleic Acid
RP	Reticulated platelets
RPE	R-phycoerythrin
SD	Standard deviation
SS	Side Scatter
TF	Tissue factor
TIA	Transient Ischaemic Attack
TO	Thiazole Orange
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
tPA	tissue-type Plasminogen Activator
TxA_2	Thromboxane A_2
VWF:Ag	von Willebrand factor

1. General Introduction

1.1. Overview of Transient Ischaemic Attack (TIA) and ischaemic stroke

1.1.1. Definition of Transient Ischaemic Attack

Until 10 years ago, a transient ischaemic attack (TIA) was **clinically defined** as a transient episode of focal brain, retinal or spinal cord neurologic dysfunction that lasted for less than 24 hours in duration, and which, after adequate investigations, was presumed to be of a non-traumatic vascular origin (Bamford 1992; Easton, Saver et al. 2009). This clinical 'time-based definition' had some limitations because some patients still had a risk of permanent tissue damage and infarction, even though their presenting symptoms lasted for less 24 hours.

The advent of newer imaging modalities, in particular MR-DWI, but also CT and MR perfusion imaging, has revealed that 30-50% of patients with 'clinically-defined TIAs' have evidence of brain ischaemia or infarction on neuroimaging investigations (Kidwell, Alger et al. 1999; Rovira, Rovira-Gols et al. 2002; Ay, Koroshetz et al. 2005; Boulanger, Coutts et al. 2005; Coutts, Simon et al. 2005). These pathological radiological lesions, combined with the patient's clinical profile, have been shown to be associated with an increased risk of subsequent ischaemic stroke (Redgrave, Coutts et al. 2007; Merwick, Albers et al. 2010). This has prompted some to develop a new definition of TIA which incorporates both clinical findings and 'tissue-based abnormalities' on neuro-imaging (Albers, Caplan et al. 2002). A 'tissue-based definition of TIA is dependent on the 'absence of permanent end-organ damage' on the basis of clinical, neuro-imaging or neuropathological findings rather than using an arbitrary 'time-based cut point'. However, it is worth noting that incidental asymptomatic DWI lesions on their own have limited ability to predict the risk of subsequent stroke in some studies (Purroy, Montaner et al. 2004; Prabhakaran, Chong et al. 2007). The American Stroke Association opted to

adopt the tissue-based definition of TIA in 2009 to include any 'transient episode of neurological dysfunction caused by focal brain, retinal or spinal cord ischaemia, without acute infarction on neuro-imaging within 24 hours', with MRI being the most sensitive imaging modality. This revised tissue-based definition of TIA essentially redefines a TIA as a 'stroke' if there is evidence of ischaemia or infarction in patients with symptoms lasting for < 24 hours.

However, the European Stroke Organisation has not adopted the tissue-based definition of TIA because there are several limitations inherent in such an approach when one is trying to compare and combine data from different centres worldwide. The diagnostic classification of a TIA will obviously be heavily influenced by the extent and type of neuro-imaging evaluation performed in each individual patient, which will in turn impact on clinical and epidemiological data on this topic. For example, infarction has been noted in 4-34% of patients on CT *vs*. 21% - 67% on diffusion-weighted MRI in patients with a clinical 'time-based' definition of TIA (Sorensen and Ay 2011), but many centres worldwide, especially in lower income countries, do not have easy access to MRI. In addition, conventional neuroimaging, especially CT and to a lesser degree 'non-DW-MRI', has limited sensitivity in detecting changes of early acute ischaemia or very small infarctions (e.g. < 1ml in volume) (Ay, Koroshetz et al. 2005), and some other patients with acute ischaemic stroke do not have initial neuroimaging changes at the time of presentation but these may develop later. On the contrary, diffusion-weighted MRI, with its very high sensitivity for detecting acute ischaemia could 'inadvertently categorise' patients as having had an acute ischaemic stroke if they undergo neuro-imaging very early after symptom onset before their ischaemic DWI changes had time to resolve (Forster, Gass et al. 2012). Furthermore, the prognostic value of a tissue-based *vs*. a time-based definition in terms of predicting the risk of early recurrent stroke following an initial TIA has not been confirmed. A meta-analysis of 4574 patients showed that the seven day risk of a subsequent ischaemic stroke was 0.4% after a tissue-based definition of a TIA compared with 3.2% if one used a time-based definition of TIA in the same patient population (Giles, Albers et al. 2011).

1.1.2 Definition of Stroke

Stroke has been **clinically defined** as ‘a neurological deficit of sudden onset, with focal rather than global neurological dysfunction, with symptoms lasting for more than 24 hours or resulting in death before 24 hours, and in which, after adequate investigations, is presumed to be of a non-traumatic vascular origin’. This traditional clinical definition of stroke, although practical and reproducible in clinical and research settings, also has some limitations. For instance, trauma following head and neck injury or neck manipulation may lead to cervical artery dissection (Biller, Sacco et al. 2014) which is responsible for up to 25% of cases of stroke in patients below 45 years of age, and is the cause of 2% of all strokes (Biller, Sacco et al. 2014). Subarachnoid haemorrhage commonly presents with headache and meningism, often without focal or global neurological dysfunction (van Gijn and Rinkel 2001). In addition, severe subarachnoid haemorrhage, bilateral hemispheric or brainstem ischaemic stroke may present with global rather than focal neurological deficits. Subdural, extradural and traumatic intracranial haemorrhages are not classified as strokes (Whisnant, Basford et al. 1990), and cerebral infarction due to infectious processes such as infective endocarditis, or due to underlying neoplasia are also excluded by some authors (Warlow 2001).

As alluded to above, following their proposals in 2009, the American Heart Association / American Stroke Association also updated the definition of stroke in 2013 to include patients with features of infarction on neuroimaging (Sacco, Kasner et al. 2013). There is considerable heterogeneity in the response of brain tissue to acute ischaemia. In some patients, brain tissue may be robust enough to withstand an acute ischaemic injury for hours to days, whereas in others, severe irreversible damage occurs in a very short space of time (Sacco, Kasner et al. 2013). As previously discussed under the TIA section, with the advent of modern neuroimaging techniques including DW-MRI, certain patients presenting with seemingly very brief clinical events have evidence of permanent infarction on brain imaging. On the contrary, some patients with prolonged symptoms for over 24 hours may not have any persistent changes on neuroimaging. Therefore, the updated tissue-based definition defines stroke as ‘an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction’. Infarction in this context was

defined as (a) ‘brain, spinal cord, or retinal cell death attributable to ischaemia, based on imaging, pathological or other objective evidence of cerebral, spinal cord, or focal retinal ischaemic injury in a defined vascular distribution; or (b) clinical evidence of cerebral, spinal cord, or retinal focal ischaemic injury based on symptoms persisting ≥ 24 hours or until death, when other aetiologies have been excluded’.

However, as stated above in the TIA section (section 1.1.1), due to difficulties obtaining rapid diagnostic MRI in various parts of the world, establishing a tissue-based diagnosis of TIA *vs*. stroke is not always practical. Therefore, for the purpose of this thesis, clinical time-based definitions of TIA and stroke were used, unless otherwise specified.

1.1.3 Epidemiology of Stroke

Approximately 80% of strokes are due to ischaemic cerebral infarction and 20% are attributed to haemorrhage (Krishnamurthi, Moran et al. 2014). The overall incidence of ischaemic and haemorrhagic stroke has risen over the past decade to between 85-94 per 100,000, and is as high as 1151-1216 per 100,000 in patients older than 75 years old, with a higher incidence of haemorrhagic stroke in lower and middle-income countries (Feigin, Lawes et al. 2009; Krishnamurthi, Moran et al. 2014). Stroke is the now the commonest cause of acquired disability in adults worldwide and is the second most common cause of mortality in middle to high income countries (Lozano, Naghavi et al. 2012). The combined incidence of TIA and stroke exceeds the incidence of acute cardiovascular events in Europe (Rothwell, Coull et al. 2005). The lifetime risk for adult men and women, aged 25 years and older, is around 25% (Collaborators, Feigin et al. 2018). Countries which have the highest risk of stroke are in Eastern Asia, Central Europe and Eastern Europe (Lozano, Naghavi et al. 2012) and China has the greatest burden of stroke in the world with one of the highest mortality rates (Wang, Jiang et al. 2017). However, most patients survive their initial ischaemic stroke with 95% still alive at seven days, 80-90% alive at 30 days, and 77% alive at one year (Feigin, Lawes et al. 2009). Therefore, survivors are at risk of recurrent vascular events with a pooled

cumulative risk of stroke recurrence of 3.1% at 30 days, 11.1% at 1 year, 26.4% at 5 years and 39.2% at 10 years after initial symptom onset (Mohan, Wolfe et al. 2011).

Because this thesis focused specifically on patients with TIA and ischaemic stroke, I will not deal with haemorrhagic stroke in any further detail.

1.1.4. Classification of Ischaemic Stroke and TIA according to Aetiology

The TOAST criteria have been one of the most widely used aetiological classification system for patients with ischaemic cerebrovascular disease over the past three decades and have been shown to have good inter-observer agreement (Adams, Bendixen et al. 1993). These criteria are based on clinical features and the results of additional studies, including brain and neurovascular imaging, cardiac investigations and laboratory tests for pro-thrombotic conditions etc. Patients are categorised into five subtypes of TIA or ischaemic stroke: 1) large artery atherosclerotic, 2) cardio-embolic 3) small vessel occlusion 4) stroke of other determined aetiology and 5) stroke of undetermined aetiology. The last subtype of stroke of undetermined aetiology includes cases in which the cause of the stroke cannot be determined conclusively, there are two or more potential competing causes, or insufficient investigations have been performed.

Since the original TOAST classification system was developed, advances in available laboratory, neuro-imaging and cardiac investigations have enabled more frequent identification of potential cardiac and other causes of TIA or stroke (Ay, Furie et al. 2005). These developments could paradoxically result in an increasing number of patients being classified as having had a TIA or stroke of undetermined aetiology, particularly where two or more potential causes are identified. This led to the development of an update of the TOAST criteria, known as the SSS-TOAST classification system (Ay, Furie et al. 2005). The SSS-TOAST classification system subdivides each of the original TOAST categories into three subcategories: ‘evident’, ‘probable’, or ‘possible’ based on all available diagnostic information according to pre-defined clinical and imaging criteria. This system was further

refined to develop an automated version of the SSS-TOAST criteria, known as the Causative Classification System (CCS) which employs a computerised algorithm with a standardised questionnaire with the aim of improving the accuracy of TIA/stroke subtyping (Ay, Benner et al. 2007). The CCS system has been found to have good inter-rater reliability between various centres (Arsava, Ballabio et al. 2010), but the overall agreement between the original TOAST and CSS classification systems was only moderate (McArdle, Kittner et al. 2014).

In 2009, a new approach to TIA and stroke subtyping was developed based on aetiological 'phenotyping'. This classification system differed from prior classification systems such as the TOAST criteria which subtyped patients according to the single most likely aetiology of their TIA or stroke. With this system, each patient is categorised under 4 subheadings of 'A-S-C-O': 'A' for atherosclerosis, 'S' for small vessel disease, 'C' for a cardiac source and 'O' for other causes (Amarenco, Bogousslavsky et al. 2009). Each of the four phenotypic categories is graded as 1, 2 or 3: 1 for 'definitely a potential cause of the index TIA or stroke', 2 for 'causality uncertain', 3 for 'unlikely a direct cause of the index TIA or stroke but the disease is present'. If the disease is 'absent', a grading of 0 is allocated for that category. When grading cannot be assigned because of 'inadequate work-up', a grade of 9 is given. For instance, a patient with a large MCA infarct due to probable symptomatic ipsilateral 70% carotid stenosis, who also has leukoaraiosis, paroxysmal atrial fibrillation and platelet count of 600 x 10^9/L would be classified as 'A1-S3-C1-O3'. A similar patient with a 70% ipsilateral carotid stenosis, who had no brain imaging, a normal ECG, normal cardiac imaging, but an elevated platelet count would be classified as 'A1-S9-C0-O3'. Ancillary diagnostic tests are graded as 'A', 'B', or 'C': 'A' indicates direct demonstration by gold-standard diagnostic tests or criteria; 'B' indicates indirect evidence or use of less sensitive or specific tests or criteria; and 'C' indicates weak evidence in the absence of specific tests or criteria. This classification system has been used in some studies in patients with stroke with good - excellent agreement between both the CCS and ASCO classification systems with the TOAST criteria (Marnane, Duggan et al. 2010), but application of the ASCO criteria is a more time-consuming process that application of the TOAST criteria.

In 2013, there was a minor modification of the ASCO criteria to incorporate ‘dissection (D)’ into the possible aetiological categories, and hence their renaming as the ‘ASCOD’ criteria (Amarenco, Bogousslavsky et al. 2013).

For the purpose of this thesis, we utilised both the original TOAST and the ASCOD subtyping systems for patients with TIA or stroke.

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1.2. Thrombus Formation in Transient Ischaemic Attack (TIA) and ischaemic stroke

The process of thrombosis, thrombus expansion, migration and dissolution are closely linked. Thrombus generation involves a number of steps, including platelet activation, activation of the coagulation system with fibrin formation and subsequent dissolution (Furie and Furie 2008). A key aspect of each of these processes is the formation of thrombin from pro-thrombin. Thrombin then promotes the generation of the fibrin network within a thrombus via the cleavage of soluble circulating fibrinogen (Lane, Philippou et al. 2005) to form insoluble stands of fibrin. Excess vascular fibrin deposition may lead to thrombus growth, whereas excess fibrin breakdown may lead to haemorrhage. Plasminogen is converted to plasmin by various plasminogen activators. Plasmin acts to degrade fibrin and fibrinogen. Understanding of this pathway has led to the development of plasminogen activators to assist with thrombolysis of acute vascular thrombi in ischaemic stroke. For example, tissue plasminogen activator has been proven to improve clinical outcomes typically when it is administered within 4.5 hours of acute stroke onset in patients screened with CT brain imaging to rule out intracranial haemorrhage or very large infarcts (del Zoppo, Ferbert et al. 1988; Mori, Tabuchi et al. 1988; del Zoppo, Poeck et al. 1992; Mori, Yoneda et al. 1992; Hacke, Kaste et al. 1995; National Institute of Neurological and Stroke rt 1995; Hacke, Kaste et al. 1998; Hacke, Kaste et al. 2008) .

1.2.1. The role of platelets in artherothrombosis

The process by which a blood clot is formed at the site of vessel injury is known as haemostasis. Haemostasis can be divided into phases, although the process is dynamic with a complex interplay between various pathways *in vivo* (Furie and Furie 2008). Under physiological conditions, the initial step in haemostasis involves activation of platelets at the site of endothelial injury within the vessel wall to form of a platelet plug to control any bleeding. Injury to the endothelium also results in exposure of the circulating blood to subendothelial components, including adhesive macromolecules like collagen and von Willebrand factor (Ruggeri 2002). In addition, endothelial cell activation occurs, which in turn enhances recruitment of platelets to the site of endothelial injury, and activates other cell types and procoagulant factors. Ultimately, a platelet-rich thrombus forms that helps to stabilise the site of endothelial injury, but in the setting of a pathological thrombo-embolic state, this also leads to a risk of distal platelet thrombo-embolism.

Platelet activation is mediated via a number of physiological stimuli, including adenosine diphosphate (ADP), epinephrine (adrenaline), thrombin and collagen, but ADP and epinephrine are believed to be less potent platelet agonists *in vivo* than collagen or thrombin. In addition to platelet activation by various stimuli, there are 2 important physiological pathways implicated in the process of *in vivo* platelet activation: (1) the 'collagen pathway' which is triggered by exposure to sub-endothelial collagen and (2) the 'tissue factor pathway' which is independent of sub-endothelial collagen (Fig. 1.1)(Furie and Furie 2008). The relative role which these two pathways play in the process of *in vivo* platelet activation in individuals is not known. However, their respective roles are dependent on the underlying disease process (Furie and Furie 2008) and vary according to the degree of shear stress to which platelets are exposed *in vivo* (Zhang, Bergeron et al. 2002).

The Collagen Pathway

The collagen pathway is activated when sub-endothelial collagen and tissue factor are exposed following traumatic injury to a blood vessel wall, thus promoting physiological haemostasis, or following rupture of an atherosclerotic plaque, thus promoting pathological thrombus formation. In the resting, unstimulated state, platelets normally roll along the intact endothelium without adhering to same (Ruggeri 2000). When endothelial damage or injury occurs, platelets adhere to exposed sub-endothelial collagen directly via the platelet glycoprotein (Gp) VI receptor, and via interaction of the platelet GpIb-V-IX complex receptor with von Willebrand factor bound to sub-endothelial collagen (Ruggeri 2002). These processes lead to the conversion of the plasma protein Factor XII to XIIa, triggering the generation of thrombin. However, platelet activation in this collagen-initiated pathway is initially independent of thrombin.

The Tissue Factor Pathway

The tissue factor pathway does not require exposure to collagen, and is independent of both von Willebrand factor and glycoprotein VI (Furie and Furie 2008). Protein disulphide isomerase (PDI) is a thiol isomerase which is secreted by activated platelets and endothelium (Cho, Furie et al. 2008; Reinhardt, von Bruhl et al. 2008). PDI stimulates tissue factor which then activates Factor X. Factor X, in turn, promotes the conversion of prothrombin to thrombin, and finally the conversion of fibrinogen to fibrin. Thrombin is a potent platelet agonist and cleaves protease-activated receptor 1 (PAR1) on the platelet surface, leading to platelet activation, enhancing platelet degranulation with secretion of adenosine diphosphate (ADP), serotonin, and thromboxane $A_{2.}$ Thrombin and these other agonists subsequently activate platelets further in a positive feedback loop (Furie and Furie 2008).

Overall, the functional response of activated platelets involves the following four processes described below:

Platelet adhesion to the subendothelial matrix and shape change

Following platelet activation, platelet adhesion mainly occurs via binding of the platelet surface GPIb/IX/V complex receptor to von Willebrand factor (VWF) in the subendothelial matrix in the collagen pathway described above (Ruggeri 2002). Other processes which contribute to platelet adhesion include the binding of the platelet collagen receptor GPIa/IIa to collagen fibrils in the matrix (Sixma, van Zanten et al. 1997). Upon activation, platelets also undergo a conformational change from a resting discoid shape to a more spherical shape and they spread out with extension of elongated spiny pseudopodia which confer additional adhesive capacity (Blockmans, Deckmyn et al. 1995).

Platelet aggregation

Following platelet activation, a conformational change occurs in the GPIIb/IIIa complex (integrin alpha IIb beta 3) receptor on the platelet surface which enables receptor binding to fibrinogen and immobilized VWF (Bennett and Vilaire 1979; Savage, Shattil et al. 1992; Savage, Saldivar et al. 1996). In addition, the cytosolic part of the activated GPIIb/IIIa complex binds to the platelet cytoskeleton, leading to further platelet spreading and enhanced clot retention (Shattil, Kashiwagi et al. 1998).

Platelet secretion of granule contents

There are four distinct populations of granules within the organelle zone of platelets: alpha granules, dense granules, lysosomes and peroxisomes (Blockmans, Deckmyn et al. 1995). The two populations which are most relevant to this thesis are the alpha and dense granules which will be described in more detail below.

Alpha-granules are the most abundant of platelet granules (Blair and Flaumenhaft 2009). Alpha granules contain many proteins, including e.g. P-selectin (CD62P), von Willebrand factor, fibrinogen, platelet factor 4, thrombospondin and platelet-derived growth factor (PDGF).

Dense granules are the second most common sub-population of granules and contain ADP, ATP, ionized calcium, histamine and serotonin.

Many of these substances are secreted from these granules upon platelet activation.

ADP and serotonin act to stimulate and recruit more platelets to the haemostatic/thrombotic zone (Kroll and Schafer 1989). ADP-activated platelets enhance the surface expression of intercellular adhesion molecules (ICAM)-1 on endothelial cells (Gawaz, Neumann et al. 1998). When fibrinogen is secreted from platelet alpha granules, this provides an additional source of fibrinogen at the site of endothelial damage to supplement that already contained within the plasma. Fibronectin and thrombospondin are adhesive macromolecules that also act to strengthen and preserve platelet aggregates. Platelet derived growth factor (PDGF) is believed to play a role in tissue repair. The prostaglandin metabolite, Thromboxane A2, mediates vasoconstriction and further platelet aggregation. Protein disulphide isomerase (PDI) stimulates tissue factor and increases the synthesis of fibrin to enhance platelet thrombus formation (Cho, Furie et al. 2008; Reinhardt, von Bruhl et al. 2008).

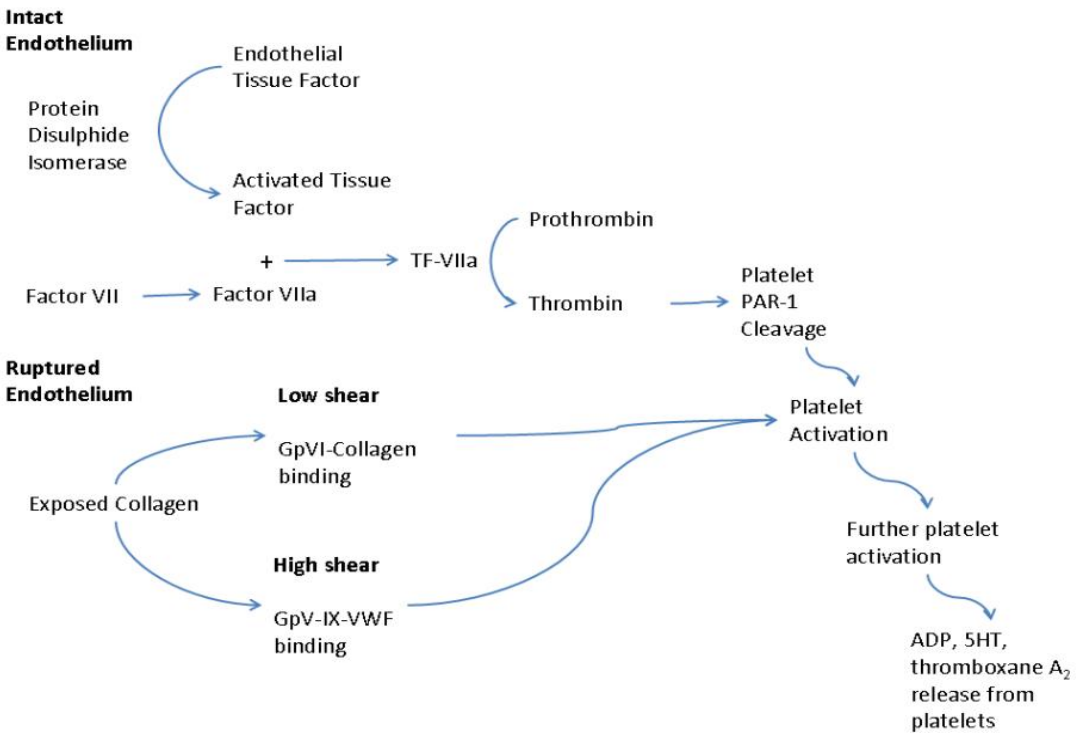
Procoagulant process mediated via the boost in thrombin generation

A procoagulant process arises following platelet activation, and includes the exposure of procoagulant phospholipids, especially phosphatidylserine on the platelet surface, with the ensuing formation of enzyme complexes in the clotting cascade on the platelet surface (Kojima, Newton-Nash et al. 1994).

Figure 1. 1 Platelet Activation Pathways

(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

Two distinct pathways may act in tandem or independently to activate platelets. In the presence of intact endothelium, PDI 'decrypts' circulating Tissue Factor which then activates Factor VII to form activated Factor VIIa. The activated Tissue Factor-Factor VIIa complex ultimately catalyses the conversion of Prothrombin to Thrombin. Thrombin subsequently cleaves and activates PAR-1 on the platelet surface to promote platelet activation. In some disease states, PDI may not be required, because Tissue Factor may already be present in a decrypted form. In the presence of a damaged endothelium, for example due to trauma or atherosclerotic plaque rupture, shear-dependent platelet surface receptors bind collagen under low shear stress conditions and von Willebrand Factor (vWF) under high shear stress conditions leading to 'outside in' platelet activation.



2. Platelet Biology

2.1. Historical background

Platelets were first identified by the 19th century anatomist, Max Shultze, who noted the frequent presence of what he described as 'clumps of colourless spherules' while studying the constituents of human blood, and recognized that they were related to coagulation (Brewer 2006). The earliest detailed analysis of platelet anatomy and its role in thrombosis and haemostasis subsequently followed, elucidated by Giulio Bizzozero, who observed the processes of platelet adherence, aggregation, and platelet-leucocytic interactions via *in vitro* and *in vivo* experiments (Brewer 2006). Soon thereafter, William Osler identified the presence of platelets in atheromatous plaques in humans, thereby alluding to role of platelets in thrombotic disease. In the early 20th century, the pathologist James H. Wright recognized megakaryocytes as precursors to platelets (Coller and Michelson 2019). Around this time, the association between platelet count and bleeding time was identified, lending further support to the capacity of platelets in haemostasis. Over the following decades, the role of platelets in haemostasis and thrombo-embolism became clearer with the detection of a number of key components, including fibrinogen (de Gaetano 2001), collagen (de Gaetano 2001), von Willebrand factor (Coller and Michelson 2019), and crucially, adenosine-5'-diphosphate (ADP), a potent inducer of platelet aggregation (Gaarder, Jonsen et al. 1961). The latter discovery helped pave the way for the development of platelet aggregometry to detect defects in platelet function (Born 1962; O'Brien J 1962), an approach which

has now expanded into a wide range of commercialized techniques using different aggregating agents.

2.2. Platelet structure

Platelets are formed from megakaryocytes which are derived from multipotent bone marrow stem cells. From their early form, megakaryocyte-erythroid progenitor cells undergo a protracted differentiation process of around 5 – 7 days duration aided by various transcription factors. Megakaryocyte-erythroid progenitor cells are initially transformed into megakaryocyte colony-forming cells (Meg-CFC), then into polyploid precursor cells through endomitosis, before eventually entering a maturation phase characterized by organelle formation and cytoplasmic membrane invagination, following which the mature megakaryocytes enter the bone marrow endothelial cells and shed platelets and proplatelets into the circulation (Machlus, Italiano et al. 2019). The average lifespan of a platelet is between 8 and 10 days (Thomas and Michelson 2019).

Platelets in the non-activated or resting state are relatively small, anucleate, discoid-shaped structures measuring 2.0 – 3.0 μm in diameter (Paulus 1975), with a mean corpuscular volume (MCV) of 6 - 10 $x10^{-15}$ L (Thomas and Michelson 2019).

The platelet structure comprises of four zones: (a) the peripheral zone, (b) the sol-gel zone, (c) the organelle zone, and (d) the membrane zone (Figure 1.1) (White and Gunay-Aygun 2011).

(a) The peripheral zone

The exterior of the peripheral zone is formed by the glycocalyx layer, comprised of glycoproteins, glycolipids and mucopolysaccarides. The glycocalyx absorbs albumin and other plasma proteins via endocytosis (Ware 1995). The glycocalyx contains a net negative charge at rest which repels other platelets, blood cells, and the vessel endothelium.

Beneath the glycocalyx is the outer platelet membrane, comprised of a phospholipid bilayer which contains around half of the total platelet phospholipid content (Blockmans 1995). The outer membrane of the lipid bilayer is mainly composed of phosphatidylcholine (PC) and sphingolipid sphingomyelin (SM) (Sims & Wiedmer 2001). The inner cytoplasmic layer of the lipid bilayer contains the lipids phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PTE), which are the source of prostaglandins (PG) and other signaling molecules synthesized via the action of phospholipases (Blockmans 1995; Shapiro 2000). This phospholipid layer facilitates a number of processes in the coagulation cascade, including the formation of factor Xa complex and the generation of thrombin from prothrombin, when exposed during the process of platelet activation (Colman 1994; Monroe 2002). Glycoproteins embedded in the lipid bilayer contain surface-facing glycosylated receptors which trigger platelet activation in response to cellular and humoral stimuli.

The plasma membrane invaginates into the interior of the platelet, forming the surface-connected, open canalicular system (OCS). The OCS serves to increase the membrane surface area, thus increasing the spreading capacity of platelets with

extension of filopodia during activation, as well as facilitating the entry of extracellular components and exocytosis of the contents of the alpha granules and dense bodies located within the cytoplasm (Thomas 2019).

(b) The sol-gel zone

The sol-gel zone represents the matrix of the platelet cytoplasm and contains three main components (the microtubular coil, the membrane skeleton, and actin microfilaments) which maintain the shape of the platelet and allow for morphological changes to occur. The circumferential microtubule coil, composed of tubulin, provides support to the cytoskeleton and helps maintain the discoid shape of the resting platelet (Blockmans 1995). The membrane skeleton is comprised of a network of short actin filaments, cross-linked by actin-binding proteins, spectrin and dystrophin-related protein (Fox 2001); it maintains the structure of the surface membrane, stabilizes the lipid bilayer, and is linked to the plasma membrane via glycoprotein receptors, primarily GP Ib/IX and GP Ia/IIa (Fox 2001). Long actin microfilaments comprise the bulk of the platelet cytoskeleton. Proteins such as tropomyosin, a-actinin and actin-binding protein help maintain the organization of actin filaments (Blockmans 1995). During platelet activation, myosin may bind to the actin filaments, initiating processes which lead to platelet shape change, pseudopod extension, internal contraction and release of granular constituents (Shapiro 2000).

(c) The organelle zone

This zone contains secretory granules and cellular components such as mitochondria and lysosomes required for platelet haemostatic function. In addition, young (i.e.

reticulated) platelets recently derived from megakaryocytes contain megakaryocyte-derived RNA and ribosomes, essential for protein translation (Schubert 2014).

There are four distinct populations of granules within the organelle zone: α-granules, dense granules, lysosomes, and peroxisomes (Blockmans 1995).

α-granules are the most numerous of the organelles (Blair & Flaumenhaft 2009). They contain integrins (e.g. αIIb, α6, β3), immunoglobulin family receptors (e.g. GPVI, Fc receptors, PECAM), leucine-rich repeat family receptors (e.g. GPIb-IX-V complex), tetraspanins (e.g. CD9) and other receptors (CD36, Glut-3), most of which are also present on the resting platelet membrane. Other proteins, such as the integral membrane proteins fibrocystin L, CD109, and CD62P, are expressed upon platelet activation (Blair & Flaumenhaft, 2009). α-granules also contain von Willebrand Factor (VWF), which can contribute to, but is not necessary for, normal haemostasis and thrombus formation in its 'platelet-derived form' (Blair & Flaumenhaft, 2009). A number of other coagulation factors and co-factors involved in haemostasis are also found in α-granules, including Factors V, IX and XIII, which are secreted upon platelet activation. Factors V and XI are involved in activation of Factor X which takes place on the platelet surface membrane. Other contents of α-granules include inhibitory proteases such as plasminogen activator inhibitor-1 (PAI-1) and α2-antiplasmin, which limit plasmin-mediated fibrinolysis, indicating a role of α-granules in perpetuating and stabilising the fibrin thrombus; antithrombin which cleaves activated clotting factors in both the intrinsic and extrinsic pathways; C1-inhibitor, which degrades plasma kallikrein, Factor XIa, and

Factor XIIa; and platelet-derived growth factor (PDGF) which stimulates formation of connective tissue cells (Kaplan et al, 1979).

Although many α-granule proteins are synthesized within the megakaryocyte, some plasma proteins, such as immunoglobulins and albumin are also taken up by the α-granule via pinocytosis (Blair & Flaumenhaft, 2009). Subpopulations of α-granules which vary in morphology have been demonstrated, with functions not limited to haemostasis and thrombosis, but also involved in other processes such as inflammation, antimicrobial host defence, atherosclerosis, wound repair, angiogenesis, and malignancy (Blair & Flaumenhaft, 2009), revealing a wide spread of physiological and pathological functions of these structures.

The second most common sub-population of granules are the ***dense granules*** which contain CD62P, CD63 (Isreals et al 1992), ATP, ADP, serotonin, calcium and a cytoplasmic pool of adenine nucleotide reserves (Shapiro 2000).

Lysosomes are tiny vesicles containing β-glucuronidase, cathepsins, collagenase and elastase which are secreted when platelets are activated (Ciferri et al. 2000). They also contain lysosomal-associated membrane proteins 1 and 2 (LAMP 1 and LAMP 2) and CD63 (Grau et al. 1998) which are expressed on the plasma membrane after platelet activation. The exact function of CD63 remains unclear, however it is thought to form ‘lateral associations’ with integrins that may serve as 'organisers' of multi-molecular networks modulating integrin-mediated signalling, cell morphology, motility and migration (Israels and McMillan-Ward 2005).

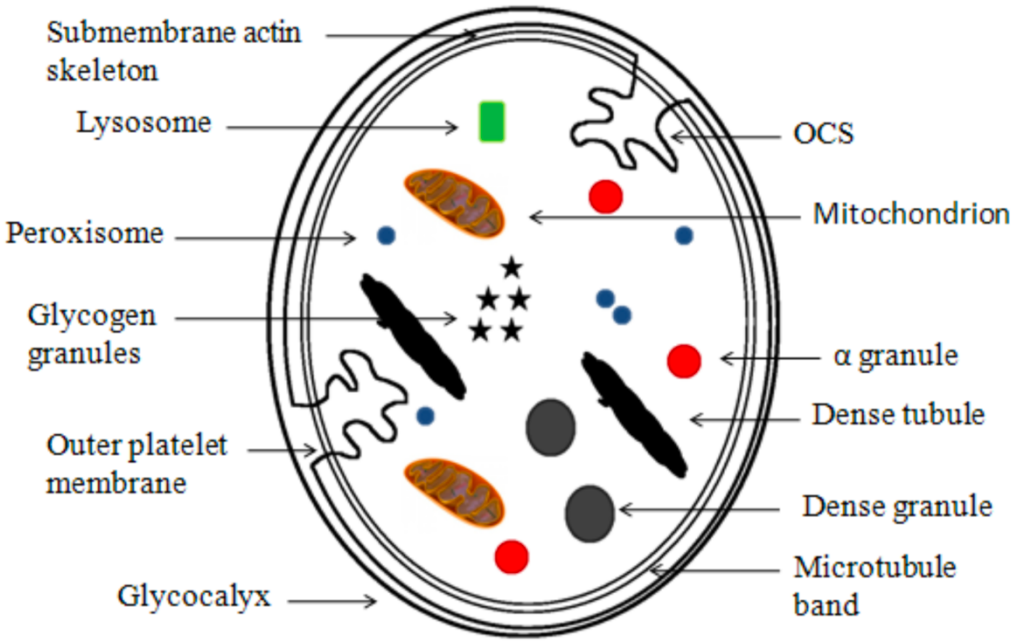
Peroxisomes are very small organelles, few in number, that play a role in lipid metabolism in platelets.

(d) **The membrane zone:**

The membrane zone is composed of the OCS and the dense tubule system (Shapiro 2000). The dense tubule system is a branching network of membrane-bound tubules, isolated from the remainder of the cell's contents. It serves an equivalent function to the smooth endoplasmic reticulum (SER) in other cells within which proteins, calcium and protein disulphide isomerase are sequestered, in addition to the enzymes involved in prostaglandin synthesis (Blockmans, Deckmyn et al. 1995; Shapiro 2000; van Nispen Tot Pannerden, van Dijk et al. 2009). Upon activation, platelets may secrete thromboxane A2 and platelet activating factor from the dense tubular system (Ebbeling, Robertson et al. 1992).

Figure 2. 1 Diagrammatic representation of the ultrastructure of a platelet in cross section

(adapted from Figure 1 in monograph by Shapiro, 1999 and Murphy SJX, PhD Thesis, TCD 2017)



2.3. Platelet agonists and their receptors

The most common platelet agonists include thrombin, ADP, epinephrine and thromboxane A2. These agonists act to activate cell surface receptors which span the platelet membrane. The cytoplasmic surface of these receptors interacts with heterotrimeric G-proteins which consist of a GTP-binding α-subunit and β-γ heterodimers (Offermanns 2000). The G-proteins are inactive when GDP is bound to the α subunit. When receptors are stimulated by agonists, GDP is converted to GTP, which in turn activates G-proteins. These G-protein coupled receptors are

subsequently turned off by phosphorylation after a certain period of time and are occasionally excreted from the cell surface by endocytosis.

Platelet agonists are occasionally, classified as ‘strong’ or ‘weak’ which is dependent on the intracellular signals generated. Strong agonists, such as thrombin and collagen, potently stimulate phosphoinositide hydrolysis and are not affected by inhibitors of cyclooxygenase (COX), such as aspirin. Weaker agonists, such as ADP and epinephrine are unable to cause phosphoinositide hydrolysis. These receptors are reliant on intermediate pathways to mediate their effects e.g. by stimulating thromboxane A2 formation.

Collagen Receptors (GPIa/IIa and GPVI): Subendothelial collagen plays a vital role in platelet adhesion and is a potent platelet agonist. The interaction between platelets and collagen is complex. The collagen receptor glycoprotein Ia/IIa is part of the integrin family of adhesion receptors and mainly functions as an anchor for platelets to adhere to exposed collagen following damage to the vascular endothelium (Offermanns 2000). GPVI acts as collagen agonist receptor and plays an important role in collagen-induced platelet aggregation and secretion. It also acts as a collagen-adhesion receptor under low shear conditions (Poole, Gibbins et al. 1997; Holtkotter, Nieswandt et al. 2002). The collagen-mediated processes include phosphoinositide hydrolysis, thromboxane A2 formation, protein phosphorylation and an increase in cytosolic Ca^{++}. Cyclooxygenase inhibitors dampen but do not completely inhibit the platelet response to collagen.

ADP Receptors ($P2Y_1$ and $P2Y_{12}$): ADP is contained within platelet dense granules and is secreted when platelets are activated. The G-protein coupled receptors, $P2Y_1$ and $P2Y_{12}$ interact with ADP (Kunapuli, Dorsam et al. 2003; Cattaneo 2010; Cunningham, Nisar et al. 2013). Stimulation of the platelet G α-subclass q-coupled $P2Y_1$ receptor by ADP causes phosphoinositide hydrolysis, thromboxane A_2 formation, protein phosphorylation and an increase in cytosolic Ca^{++}. Furthermore, ADP also stimulates the G α-subclass i-coupled $P2Y_1$ receptor that inhibits cAMP formation. Previous experiments in knockout mice have shown that stimulation of both $P2Y_1$ and $P2Y_2$ are required for the full ADP-response in platelets (Kunapuli, Dorsam et al. 2003).

Adrenergic Receptors: The effects of epinephrine on platelets are mediated via G α-subclass i-coupled α(2) adrenergic receptors (Yang, Wu et al. 2000). High doses of epinephrine have been shown to cause platelet activation in *in vitro* studies; however, normal physiologic doses of epinephrine do not cause platelet aggregation. Stress-induced epinephrine is thought to 'sensitise' platelets to the effects of low doses of other platelet agonists *in vivo*. Epinephrine is also a potent inhibitor of cyclic AMP formation, and high doses have been shown to have an effect on phospholipase C activation *ex vivo*.

Thromboxane receptor: Thromboxane A_2 is a product of the cyclooxygenase pathway where it is formed from arachidonic acid. Thromboxane A_2 is able to diffuse across the plasma membrane to activate other platelets via the thromboxane receptor. It functions to amplify the initial platelet activation response and mediates further activation of other platelets.

3. Platelet function testing in transient ischaemic attack and ischaemic stroke: A comprehensive overview of the literature

3.1. Background

Stroke is the commonest cause of acquired disability in adults in higher-income countries (Strong, Mathers et al. 2007) and the second commonest cause of death worldwide (Lozano, Naghavi et al. 2012). Although the incidence of stroke has fallen over the last decade in higher-income countries (Koton, Schneider et al. 2014), the global burden of stroke-related disability is rising (Di Carlo 2009; Feigin, Forouzanfar et al. 2014)

Cerebral or ocular ischaemia/infarction are the underlying pathogenic mechanisms responsible for all transient ischaemic attacks (TIAs) and 80-90% of first strokes{Warlow C.P, Dennis, et al. 1996 233 /id} (Lovelock, Molyneux et al. 2007; Marnane, Duggan et al. 2010; O'Donnell, Xavier et al. 2010). Several studies have shown that platelets are excessively activated or hyper-reactive in the early (Mulley, Heptinstall et al. 1983; Grau, Ruf et al. 1998; Meiklejohn, Vickers et al. 2001; Marquardt, Ruf et al. 2002; Garlichs, Kozina et al. 2003; McCabe, Harrison et al. 2004), subacute (Marquardt, Ruf et al. 2002), or late phases (Grau, Ruf et al. 1998; Meiklejohn, Vickers et al. 2001; Garlichs, Kozina et al. 2003; McCabe, Harrison et al. 2004) after TIA or ischaemic stroke. Because the majority of TIAs/ischaemic

strokes are ‘non-cardioembolic’ in origin, antiplatelet agents play a key role in secondary prevention in patients with ischaemic cerebrovascular disease (CVD) (Antithrombotic Trialists 2002). Aspirin has traditionally been the most commonly-prescribed antiplatelet drug for secondary prevention following TIA/ischaemic stroke, but the majority (82-87%) of patients are not protected from further vascular events with aspirin alone (Chinese Acute Stroke Trial (CAST) Collaborative Group 1997; International Stroke Trial Collaborative Group 1997). Clinical trials in CVD have shown that aspirin-dipyridamole combination therapy is superior to aspirin alone at preventing recurrent stroke (Diener, Cunha et al. 1996) or recurrent vascular events overall (Halkes, van Gijn et al. 2006). There may also be a benefit of clopidogrel over aspirin in subgroups of ischaemic CVD patients, especially those with co-existing ischaemic heart disease (IHD) (CAPRIE Steering Committee 1996). However, the PRoFESS trial did not subsequently show any difference in the incidence of recurrent stroke or vascular events between CVD patients on aspirin-dipyridamole combination therapy and those on clopidogrel (Sacco, Diener et al. 2008).

The CHANCE trial (Wang et al. 2013) showed that aspirin- clopidogrel combination therapy started within 24 hours of onset of a high-risk TIA ($ABCD^2 \geq 4$) or minor ischaemic stroke (NIHSS ≤ 3) and continued for 21 days, followed by clopidogrel monotherapy from day 22-90 significantly reduced the risk of ischaemic stroke (7.9 *vs*. 11.4%, $P < 0.01$) and stroke or TIA (8.2 *vs*. 11.7%, $P < 0.01$), without increasing the risk of moderate or severe bleeding (0.3 *vs*. 0.3%, P = NS) compared with aspirin monotherapy. A 300 mg loading dose of clopidogrel was used in CHANCE. However, the study was only conducted in a Chinese patient population,

44% had intracranial large artery disease (Liu, Wong et al. 2015) and the proportion with extracranial carotid stenosis was not specified.

The more recently published POINT trial showed that that aspirin-clopidogrel combination therapy, started within 12 hours of onset of a high-risk TIA or minor ischaemic stroke and continued for 90 days also significantly reduced the risk of ischaemic stroke, MI or vascular death (5 *vs*. 6.5%; hazard ratio (HR) 0.75, 95% confidence interval [CI]: 0.59 - 0.95, P = 0.02) compared with aspirin monotherapy (Johnston, Easton et al. 2018). Aspirin-clopidogrel combination therapy also reduced the risk of ischaemic stroke (4.6 *vs*. 6.3%; HR 0.72, 95% CI: 0.56 - 0.92, P = 0.01), but there was a significant increase in the risk of major haemorrhage (0.9 *vs*. 0.4%; HR 2.32, 95% CI: 1.10 - 4.87, P = 0.02) and minor haemorrhage (1.6 *vs*. 0.5%; HR 3.12, 95% CI: 1.67 - 5.83, P = 0.002) compared with aspirin monotherapy (Johnston, Easton et al. 2018). A 600 mg loading dose of clopidogrel was used in this study. A secondary, exploratory analysis of the POINT data also showed that 21 days of aspirin- clopidogrel combination therapy also significantly reduced the risk of ischaemic stroke, MI or vascular death (3.6 *vs*. 5.6%; HR 0.65, 95% CI: 0.50 – 0.85, P = 0.0015) without a significant increase in the risk of major haemorrhage (0.4 *vs*. 0.2%, P = NS) compared with aspirin monotherapy (Johnston, Elm et al. 2019). The authors concluded that for every 1000 patients treated for 21 days with aspirin-clopidogrel, 20 major ischaemic events would be prevented and 2 major haemorrhages would be expected (Johnston, Elm et al. 2019).

Subsequent to the publication of the POINT trial, meta-analyses of the data from the CHANCE and POINT trials (Pan, Elm et al. 2019), and from the FASTER

(Kennedy, Hill et al. 2007), CHANCE and POINT trials have been published to add to the evidence base (Hao, Tampi et al. 2018), (Prasad, Siemieniuk et al. 2018). The FASTER trial was a randomised trial which was prematurely terminated due to slow recruitment in which patients received aspirin-clopidogrel combination therapy or aspirin antiplatelet monotherapy, either alone or in combination with simvastatin, within 24 hours of onset of a TIA or minor stroke (Kennedy, Hill et al. 2007). These meta-analyses revealed that short-term aspirin-clopidogrel combination therapy significantly reduced the absolute risk of recurrent ischaemic vascular events by 2.6% (Pan, Elm et al. 2019) or recurrent non-fatal ischaemic or haemorrhagic stroke by 1.9 - 2.6% (Hao, Tampi et al. 2018) (Prasad, Siemieniuk et al. 2018) (Pan, Elm et al. 2019) compared with aspirin monotherapy. The benefit of combination treatment in preventing recurrent ischaemic events also outweighed the risks of major haemorrhage, the absolute risk of which was increased by about 0.2% in those on combination therapy *vs*. aspirin monotherapy (Hao, Tampi et al. 2018) (Prasad, Siemieniuk et al. 2018) (Pan, Elm et al. 2019). The absolute risk of minor extracranial bleeding was also increased by 0.7% (RR 2.22; 95%CI 1.6-3.08) (Hao, Tampi et al. 2018) (Prasad, Siemieniuk et al. 2018) to 1% (adjusted HR 2.01, 95% CI: 1.41 -2.86) (Pan, Elm et al. 2019) on combination therapy *vs*. aspirin monotherapy. However, the beneficial effect of combination antiplatelet therapy over aspirin monotherapy was confined to the first 10 - 21 days after high-risk TIA or minor ischaemic stroke (Hao, Tampi et al. 2018)(Pan, Elm et al. 2019), whereas the increased risk of haemorrhage remained constant after symptom onset (Hao, Tampi et al. 2018). Therefore, limiting the duration of combination treatment to ≤ 21days, followed by antiplatelet monotherapy thereafter, is likely to maximise the benefits and minimise harms in TIA and stroke patients overall (Hao, Tampi et al.

2018)(Pan, Elm et al. 2019). It was of interest that 3 stroke survivors and one carer who were involved in the BMJ guidelines document considered a non-fatal stroke to be 2-3 times worse than a serious GI bleed (Prasad, Siemieniuk et al. 2018).

A number of other recent guideline documents refer to these data from CHANCE and POINT to provide advice re short-term aspirin-clopidogrel combination therapy for 21 days in patients with minor non-cardioembolic ischaemic stroke who have not received IV TPA (Powers, Rabinstein et al. 2019) or for 21 days (Stroke Foundation (2019). Clinical Guidelines for Stroke Management. Melbourne Australia) or 21-30 days in those with either a high-risk TIA or minor ischaemic stroke (Boulanger, Lindsay et al. 2018).

The TARDIS trial (Bath, Woodhouse et al. 2018) which employed intensive triple antiplatelet combination therapy with aspirin + clopidogrel + dipyridamole *vs*. either aspirin + dipyridamole combination therapy or clopidogrel monotherapy did not reduce the incidence or severity or stroke or TIA, but did significantly increase the risk of major haemorrhage. It was concluded that triple antiplatelet combination therapy should not be recommended following TIA or stroke. Therefore, the optimal secondary preventive antiplatelet regimen for individuals following TIA or ischaemic stroke is unclear, and many CVD patients are not protected from further vascular events with currently available, 'non-monitored' antiplatelet treatment regimens.

Several groups have employed established laboratory tests to assess *ex-vivo* 'non-responsiveness/resistance' (Aradi, Komocsi et al. 2013) or 'high on-treatment

platelet reactivity' (HTPR) to antiplatelet therapy in the physiological milieu of whole blood in IHD patients (Bonello, Tantry et al. 2010; Breet, van Werkum et al. 2010; Price, Angiolillo et al. 2011). Most definitions of HTPR are 'cross-sectional, case-control' definitions whereby patients' results at a single time point are compared with those obtained from a group of healthy controls or the manufacturer's normal range. Very few robust, prospective, 'longitudinal studies' in CVD, in which the same patients are tested before and after starting or changing antiplatelet therapy have been performed to date (see below) (Grau, Reiners et al. 2003; Raman and Jilma 2004; Serebruany, Malinin et al. 2004; Serebruany, Malinin et al. 2005; Serebruany, Steinhubl et al. 2005; Serebruany, Malinin et al. 2008; Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013). Longitudinal definitions of HTPR in CVD patients commencing or changing antiplatelet therapy have the potential to provide more clinically meaningful information than traditional cross-sectional definitions of HTPR (Tobin, Kinsella et al. 2013). Nevertheless, cross-sectional data may be clinically informative when longitudinal data are unavailable (Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013).

One recent meta-analysis reported a higher incidence of poor outcomes (stent thrombosis, myocardial infarction or death) in patients with higher on-treatment platelet reactivity compared with those with lower on-treatment platelet reactivity on the VerifyNow platelet function analyser after percutaneous coronary intervention (Brar, ten Berg et al. 2011). However, altering antiplatelet therapy based on *ex vivo* platelet function testing with the VerifyNow has not been shown to improve 'vascular outcomes' in two large recent trials in IHD (Price, Berger et al. 2011; Collet, Cuisset et al. 2012). Despite extensive interest in platelet

function/reactivity testing in IHD, relatively little attention has been paid to the phenomenon of '*ex-vivo* HTPR' in CVD. None of the aforementioned landmark clinical trials in CVD (Diener, Cunha et al. 1996; Chinese Acute Stroke Trial (CAST) Collaborative Group 1997; International Stroke Trial Collaborative Group 1997; Sacco, Diener et al. 2008) routinely incorporated platelet function testing into the study paradigm (Kinsella, Tobin et al. 2013). Because of the heterogenous aetiology of TIA or stroke that may be caused by several different embolic, thrombotic or other mechanisms (Adams, Bendixen et al. 1993), one cannot assume that one may extrapolate data on *ex vivo* HTPR from IHD patients to those with TIA or stroke.

To investigate the potential importance of *ex vivo* platelet function/reactivity testing, and the potential role such testing could play in optimising secondary preventive therapy in CVD, I performed a comprehensive collaborative review of the literature to collate available data on *ex-vivo* platelet function/reactivity in blood in CVD patients.

3.2. Methods

This chapter was prepared in 2015 following an invitation to our research group to perform a detailed review of the literature on this topic for a special edition of the journal 'Platelets' to mark the retirement of the Editor, Prof Stan Heptinstall (Lim et al 2015). A comprehensive review of the literature was performed by searching PubMed, Medline, Ovid, Embase, and Web of Science/Web of Knowledge for human studies published in English between 1993 and February 2015 on *ex vivo*

platelet function/reactivity in CVD patients who were treated with aspirin, dipyridamole or clopidogrel. We included all available data at that time and did not focus solely on data from studies with prospective follow-up **after** assessment of antiplatelet-HTPR status which is the focus of chapter 4 in this thesis. However, some studies were inevitably reviewed for and included in both chapters. The following search terms were used in different combinations: transient ischaemic attack, TIA, stroke, platelet function, platelet reactivity, platelet aggregation, platelet aggregometry, flow cytometry, antiplatelet resistance, high on-treatment platelet reactivity, aspirin, clopidogrel, dipyridamole, Platelet Function Analyser-100 (PFA-100®), VerifyNow®, Multiplate®, aspirin TIA polymorphisms, aspirin stroke polymorphisms, acetylsalicylic acid TIA polymorphisms, acetylsalicylic acid stroke polymorphisms, clopidogrel TIA polymorphisms, clopidogrel stroke polymorphisms, antiplatelet TIA polymorphisms, antiplatelet stroke polymorphisms. We excluded review articles, studies assessing platelet function *in vitro* or *ex vivo* platelet activation, reports in which it was unclear whether haemorrhagic stroke patients were included, and pharmacogenetic studies including < 200 patients. Data on *ex vivo* platelet reactivity/function testing in the subgroup with moderate-severe symptomatic or asymptomatic carotid atherosclerotic stenosis were excluded because this is the subject of a separate systematic review in preparation.

Two authors (STL and IF-C) personally read all abstracts and/or relevant articles, hand-searched reference lists of published articles, and identified papers suitable for inclusion in this review. 1162 manuscripts were initially retrieved, including 246 articles on platelet function in CVD, and 84 on the impact of pharmacogenetics on

HTPR in CVD; 96 were deemed suitable for final review and inclusion. Although multiple technology platforms are available to assess *ex vivo* HTPR (Table 3.1), we focused on data obtained from some of the most commonly available platelet function assays, namely platelet aggregometry, the PFA-100, VerifyNow and Multiplate analyser. For consistency, the terms 'non-responsiveness' or 'resistance' used in prior studies were replaced by HTPR in this review, unless specified. Data on HTPR on aspirin, dipyridamole or clopidogrel on each of these devices are reviewed, in turn, followed by a brief overview of available data on the influence of pharmacogenetic factors on HTPR in CVD.

3.3 Prevalence of ex vivo HTPR/'non-responsiveness' in TIA or ischaemic stroke patients on antiplatelet therapy

3.3.1. Platelet aggregometry

Thirteen studies focused on aggregometry in platelet rich plasma (PRP) or whole blood in CVD.

Helgason *et al*. investigated *ex vivo* 'aspirin responsiveness' in ischaemic stroke (Helgason, Tortorice et al. 1993; Helgason, Bolin et al. 1994). The first study included 113 outpatients with prior ischaemic stroke, and 24 inpatients with acute ischaemic stroke on aspirin 325-1300 mg, titrated as per protocol (Helgason, Tortorice et al. 1993). 'Aspirin-HTPR', defined as incomplete inhibition of platelet aggregation in PRP in response to arachidonic acid (AA; 500 μM), ADP (5 μM),

epinephrine (5 μM), and collagen (0.8 μg/ml) on up to 1300 mg of aspirin daily, was seen in 3-4% of CVD patients. Of interest, the majority of inpatients (79%) who had a stroke on aspirin had complete inhibition of platelet aggregation *ex vivo*, suggesting that thromboxane-independent mechanisms were involved in the pathogenesis of these strokes. Therefore, this study did not enforce the argument that platelet aggregometry in PRP might help identifying patients at high risk of stroke recurrence on aspirin. The authors subsequently measured *ex vivo* inhibition of platelet aggregation in PRP in 306 ischaemic stroke patients on ≥325 mg of aspirin daily (Helgason, Bolin et al. 1994). 26% had HTPR on the initially-prescribed aspirin dose. Of 171 patients who underwent repeat testing, 8% had aspirin-HTPR despite dose-escalation to 1300 mg daily. 31% of patients initially on a dose of aspirin sufficient to completely inhibit platelet aggregation had HTPR on the same dose at some stage during follow-up. The mechanisms responsible for fluctuation in the *ex vivo* response to aspirin during follow-up were not determined.

Ex vivo 'aspirin responsiveness' was also measured in 14 ischaemic stroke patients with stroke recurrence on aspirin ('clinical aspirin failures') and 25 patients without stroke recurrence on aspirin ('clinical aspirin responders') after 7 days on 300 mg and 600 mg of aspirin daily, respectively. (Chamorro, Escolar et al. 1999) A daily dose of 300 mg of aspirin inhibited 1.4μM AA- and 10μM epinephrine-induced platelet aggregation less effectively in 'clinical aspirin failures' than in 'clinical aspirin responders' ($p<0.01$). More complete inhibition of platelet aggregation was seen in clinical aspirin failures when the dose was increased from 300 to 600 mg daily ($p<0.01$). Because of the limited number of subjects and the lack of clinical follow-up after dose escalation, these results cannot be generalised to recommend higher aspirin doses in patients with recurrent stroke on 300 mg daily.

Sztriha *et al.* performed a cross-sectional optical platelet aggregation study in PRP on 241 patients on aspirin 100 mg-250 mg daily who had at least one TIA or stroke in the preceding 5 years, 78 of whom had >1 preceding recurrent vascular event (stroke, MI or angina) (Sztriha, Sas et al. 2008). The degree of aggregation did not differ between patients with and without a history of recurrent events on collagen- or epinephrine-induced aggregation. The retrospective clinical analysis in this study does not allow one to conclude whether HTPR status predicts risk of recurrent vascular events over time.

Gengo *et al*. performed a cross-sectional study on 653 patients with prior TIA/stroke on aspirin (<81mg to >325mg daily) (Gengo, Rainka et al. 2008). Aspirin-HTPR was seen in 14% of patients with 0.5 mM AA, and 17% in response to 1 μg/mL collagen on whole blood impedance aggregometry; 98% retained the same aspirin-HTPR status at 7 months follow-up. Patients were not prospectively followed to assess the risk of recurrent vascular events, so this study did not inform us whether one should alter antiplatelet therapy based on baseline aspirin-HTPR status (Gengo, Rainka et al. 2008).

Fong *et al* performed a retrospective analysis of optical platelet aggregometry data in PRP in 465 CVD patients (Fong, Cheng-Ching et al. 2011). Twenty-eight per cent of patients on 81-325 mg of aspirin daily had aspirin-HTPR in response to 0.5 mg/mL AA or 10 mM ADP. Twenty-eight per cent of patients on clopidogrel 75 mg daily had clopidogrel-HTPR in response to 10 mM ADP. Amongst those on dual antiplatelet therapy, 9.3% had both aspirin and clopidogrel HTPR.

Schwammenthal *et al*. performed an observational study on 105 patients within 36 hours of acute stroke; 40% were on aspirin for > 1 week prior to presentation, and all were treated with 100-325 mg of aspirin daily for at least 6 hours before blood sampling (Schwammenthal, Tsabari et al. 2008). 'Aspirin HTPR', assessed with 1.6 mmol/L AA-induced optical platelet aggregometry in PRP, was observed in 31% within 36 hours and in 45% of the 87 patients retested at days 4-5 after stroke onset. Fifty-three per cent had consistent HTPR results over time. Patients with aspirin-HTPR at baseline had more severe strokes at baseline, a more unfavourable clinical course, and worse functional outcome during follow-up than those without HTPR after adjusting for age ($p \leq 0.02$). However, one could not reliably comment on whether aspirin-HTPR had any impact on the risk of recurrent vascular events (n=7) during a median follow-up of 11.5 months.

HTPR was subsequently assessed with whole blood impedance aggregometry in 416 acute stroke patients on 100 mg or 200 mg of oral aspirin, 500 mg of IV aspirin, or clopidogrel 75mg daily (Meves, Overbeck et al. 2012). Based on response to stimulation with AA or ADP, aspirin-HTPR was identified in 36% of patients on 100 mg of aspirin, 33% on 200 mg of aspirin, 18% on 500 mg of intravenous aspirin, and clopidogrel-HTPR was seen in 46% of patients on clopidogrel. However, because this study was not designed to assess clinical outcomes in patients with HTPR, one cannot conclude that one should use higher dose IV rather than oral aspirin in acute stroke. The same authors reported the results of another cross-sectional whole blood impedance aggregometry study in 737 patients with CVD, IHD or peripheral vascular disease (PVD) on 100-200 mg of oral aspirin or 500 mg of IV aspirin daily (Meves, Hummel et al. 2014). Aspirin doses ranged between 100-500 mg daily in the CVD subgroup (Meves, Hummel et al. 2014). The

prevalence of aspirin-HTPR was 28% in patients with CVD, 18% in those with IHD, and 22% in those with PAD. However, different groups of patients were prescribed different treatment regimens and doses of aspirin, so it was unclear whether aspirin-HTPR was more common in CVD than IHD patients.

Depta *et al*. **analysed retrospective data** on 324 patients with TIA (n=74) or ischaemic stroke (n=250) to assess clinical outcomes associated with 'optical platelet aggregometry-guided modifications in antiplatelet therapy' (Depta, Fowler et al. 2012). 'Antiplatelet therapy modification' was defined as any increase in the dose of existing antiplatelet therapy, addition of another agent, or switching antiplatelet therapy (e.g. aspirin to clopidogrel) within 24 hours of platelet function testing. In this CVD population, 43% had aspirin-HTPR in response to stimulation with AA or ADP, and 35% had clopidogrel-HTPR in response to stimulation with ADP. After platelet function testing, antiplatelet therapy was altered/increased in 23%, but this decision was **not necessarily based on the results of platelet function testing**, and only 24 patients had repeat platelet function testing after modifying antiplatelet therapy. Patients who underwent modifications in therapy had higher rates of ischaemic events, bleeding or death than those who had no modifications of antiplatelet therapy (hazard ratio: 2.24; P=0.02). However, one cannot conclude that treatment changes 'based on platelet function testing' influenced outcomes in CVD patients because the study was retrospective and observational, and most importantly, the results of platelet function testing were **not** the only criteria used to alter treatment. Furthermore, diverse modifications in

antiplatelet therapy were left to the discretion of treating physicians, and only 7% had follow-up platelet function testing.

Ex-vivo HTPR was evaluated with optical aggregometry in PRP in 72 patients within 7-62 days of non-cardioembolic TIA/ischaemic stroke who were on 75 mg of clopidogrel daily for ≥1 week (Fukuoka, Furuya et al. 2011). Clopidogrel-HTPR was seen in 8.3% with 1 μmol/L ADP and 18.1% with 4 μmol/L ADP. Patients were studied on one occasion and the relationship between aggregometry data and recurrent events was not assessed.

Lago *et al*. performed a cross-sectional, observational study on 56 patients within 72 hours of ischemic stroke who were either on aspirin (100–300 mg daily in 87%, or 450 mg of intravenous acetylsalicylate of lysine daily in 13%; n=30), 75 mg of clopidogrel daily (n=16), or aspirin and clopidogrel (n=10) (Lago, Parkhutik et al. 2014). Patients on clopidogrel exhibited a small (13%) reduction in 3μm ADP-induced optical platelet aggregation in PRP, and as expected, had less pronounced inhibition of 1mM arachidonic acid-induced aggregation, and shorter C-EPI closure times on the PFA-100 than patients on aspirin or aspirin-clopidogrel combination therapy ($P<0.05$). However, the proportion of patients with HTPR on each regimen was not reported, and the C-EPI data are not informative because the C-EPI cartridge is not sensitive at detecting the antiplatelet effects of clopidogrel. Only one patient had a recurrent event on aspirin, and outcomes after discharge were not reported.

A recent cross-sectional study in CVD patients with prospective follow-up assessed *ex vivo* 'aspirin resistance' in 634 Chinese stroke patients on 200 mg of aspirin daily with optical aggregometry using PRP (Yi, Zhou et al. 2013). Aspirin resistance (AR) was defined as a mean aggregation of ≥20% with 0·5 mg/ml AA **and** ≥70% with 10 uM ADP. 'Aspirin semi-resistance (ASR)' was defined as a mean aggregation of ≥20% with 0·5 mg/ml AA **or** ≥70% with 10 uM ADP. AR was detected in 20% and ASR in 4% of patients. During a median follow-up of 19·4 months (n=600), in which all patients received 100 mg of aspirin daily, recurrent stroke, myocardial infarction, death, or vascular events occurred more frequently in patients with AR or ASR than patients with 'aspirin responsiveness' (31% *vs*. 12%, p<0.001). AR/ASR (pooled data) was an independent risk factor for ischaemic vascular events during follow-up (observed ratio 3·2, p<0·001). Larger studies in non-Chinese populations, ideally with more 'user-friendly' tests of platelet function in whole blood, and retested on their actual maintenance dose of aspirin are needed to determine whether HTPR data predict outcome in CVD patients overall.

3.3.2. Data from commonly used whole blood platelet function analysers in CVD

PFA-100®

The PFA-100 activates platelets by exposure to moderately-high shear stress (5000-6000s^{-1}) and biochemical stimulation, traditionally with collagen and epinephrine (C-EPI) or ADP (C-ADP). Previous studies have shown that aspirin prolongs C-EPI closure times in 83-100% of healthy controls (Kundu, Heilmann et al. 1995; Mammen, Alshameeri et al. 1995; Mammen, Comp et al. 1998; Harrison, Segal et al. 2005). The C-ADP cartridge is not sensitive at detecting platelet function

inhibition with Clopidogrel (Grau, Reiners et al. 2003; Kinsella, Tobin et al. 2013), but the INNOVANCE® PFA P2Y cartridge is reported to have overcome this issue (Edwards, Jakubowski et al. 2012; Tsantes, Ikonomidis et al. 2012). Fifteen studies assessing HTPR with the PFA-100 in CVD met criteria for inclusion in this review.

Alberts *et al*. reported that 37% of patients with 'acute stroke', TIA or asymptomatic extracranial or intracranial arterial stenosis had aspirin-HTPR using the C-EPI cartridge on aspirin doses between 81 mg alternate days/daily and 325 mg daily/twice daily (Alberts, Bergman et al. 2004). Aspirin-HTPR was more common amongst patients receiving 81 mg than 325 mg daily (56% *vs*. 28%; p=0.001), and 7% still had aspirin-HTPR after empirically increasing the dose to 650 mg BD. However, the precise interval between symptom onset and study inclusion in symptomatic patients was not reported, an unspecified number of patients were also taking non-steroidal anti-inflammatory drugs or cyclooxygenase-2 inhibitors in combination with aspirin, and the exact proportion of non-responders in the patient subgroup on aspirin monotherapy was not specified (Alberts, Bergman et al. 2004; McCabe, Harrison et al. 2004).

Harrison *et al.* performed a cross-sectional study to simultaneously assess aspirin-HTPR with the PFA-100, the Ultegra® RPFA (RPFA) and optical aggregometry in 100 patients with recent TIA or minor ischaemic stroke on 75-150 mg of aspirin daily for ≥4 weeks (Harrison, Segal et al. 2005). Six patients were also taking 75 mg of clopidogrel daily, and two were on 600 mg of dipyridamole daily. The prevalence of Aspirin-HTPR was 22% on the PFA-100, 17% on the RPFA, 12% with AA-induced aggregation, and 14% with ADP-induced aggregation. There was a higher prevalence of aspirin-HTPR with both point-of-care devices than with

aggregometry, but only 2% had HTPR on all 3 tests. The co-prescription of aspirin or dipyridamole in a minority of patients may have influenced the overall results, and this initial study could not assess the value of these tests at predicting risk of recurrent events during follow-up. These assays were repeated by the same authors a year later in 72 patients from the original study cohort who were still on aspirin 75mg – 150mg od (Harrison, Segal et al. 2008). The prevalence of Aspirin-HTPR during follow-up, compared with baseline prevalence data from the initial study, was reported to be 25% *vs*. 19.4% on the PFA-100, 10% *vs*. 17% on the VerifyNow, and 1% *vs*. 7% on optical aggregometry in response to AA stimulation. Only one patient was identified as having HTPR by all three tests. Levels of agreement in test results between the 2 time points were 'moderate' for the PFA-100 (kappa = 0.44, 95% CI: 0.19 - 0.68), 'fair' for the VerifyNow (kappa = 0.34, 95% CI: 0.04 - 0.64) and 'poor' for optical aggregometry (kappa = 0.14, 95% CI: -0.11 - 0.39 for ADP; kappa = 0.09, 95% CI: -0.21 - 0.39 for arachidonic acid). (Harrison, Segal et al. 2008) This follow-up study was not designed or powered to assess the predictive ability of these tests during clinical follow-up.

Prior to the introduction of the INNOVANCE® PFA P2Y cartridge, a pilot case-crossover study assessed 31 patients in the late phase after lacunar or 'atherothrombotic' ischaemic stroke on 100-300 mg of aspirin daily (Grau, Reiners et al. 2003). Patients were treated with 75 mg of clopidogrel daily, or a combination of clopidogrel and 300 mg of aspirin daily for four weeks at a time. 16% had aspirin-HTPR on the C-EPI cartridge; clopidogrel-HTPR on the C-ADP cartridge was observed in 94% of patients on clopidogrel monotherapy, and 72% on aspirin-clopidogrel combination therapy. Therefore, the C-ADP cartridge was not sensitive

at detecting the antiplatelet effects of clopidogrel in CVD (Grau, Reiners et al. 2003).

A subsequent randomised trial involving 70 patients within 3 months of ischaemic stroke showed that combination therapy with aspirin (81 mg daily) and clopidogrel (75 mg daily; n=35) led to greater inhibition of platelet function on the C-ADP cartridge than aspirin alone (n=35; p=0.01) (Serebruany, Malinin et al. 2005). The addition of clopidogrel to aspirin also resulted in greater inhibition of platelet aggregation on optical aggregometry with 5µmol/L ADP (P=0.00001) or 5µmol/L collagen (P= 0.021), but the percentage of patients with HTPR on each treatment was not specified.

Grundmann *et al.* studied patients with the C-EPI cartridge who were on 100 mg of aspirin daily for secondary prevention of vascular events (Grundmann, Jaschonek et al. 2003). Using a cross-sectional definition at one time-point, aspirin-HTPR was noted in 34% of patients within 3 days of TIA or ischaemic stroke (N=35) but in none of the patients who were free of cerebrovascular events for >2 years (N=18).

McCabe *et al*. subsequently performed a prospective, observational case-control study in patients in the early (≤4 weeks, n=57) and late phases (≥3 months, n=46) after TIA or ischaemic stroke. Sixty per cent of patients in the early phase and 43% in the late phase had aspirin-HTPR on the C-EPI cartridge on 75-300 mg of aspirin daily. None of the CVD patients studied in the late phase were defined as having aspirin-HTPR on sodium arachidonate- or ADP-induced platelet aggregometry in PRP (n=10). Overall, a high proportion of CVD patients had aspirin-HTPR, and

cyclooxygenase-independent mechanisms, including TIA/stroke subtype, appeared to play an important role in mediating aspirin-HTPR on the PFA-100. However, the study was not designed to assess the ability of the PFA-100 to predict risk of recurrent vascular events during follow-up.

Godeneche *et al*. identified aspirin-HTPR in 15% of acute ischaemic stroke patients on treatment with 160 mg of aspirin daily for ≥3 days in a cross-sectional study with the C-EPI cartridge in 3.8% citrate-anticoagulated blood (n=100) (Godeneche, Sorel et al. 2009). As noted previously, C-ADP closure times were significantly shorter (McCabe, Harrison et al. 2005), and hypertension was more common in patients with than in those without aspirin-HTPR ($p \leq 0.018$). However, medium-long term clinical or laboratory follow-up were not performed.

Boncoraglio *et al*. assessed 129 stable CVD patients with either vascular cognitive impairment, or TIA or stroke within the preceding 1-12 months who were on 75-300 mg of aspirin daily (Boncoraglio, Bodini et al. 2009). The composite outcome of recurrent TIA, stroke, myocardial infarction or cardiovascular death occurred in 15.4% (N=4) of patients with aspirin-HTPR and 14.6% (N=15) without aspirin-HTPR (p=1.0) on the C-EPI cartridge during a mean follow-up of 56 months (Boncoraglio, Bodini et al. 2009).

A further retrospective study assessed 142 CVD patients with the C-EPI cartridge who received 100 mg or 300 mg of aspirin daily, clopidogrel 75 mg daily, or both (100 mg aspirin + 75 mg clopidogrel daily) (von Lewinski, Riggert et al. 2009). Platelet aggregation in PRP was measured by optical aggregometry using 5mg/mL

collagen or 10 mmol/L ADP. 58-62% of patients had aspirin-HTPR on the C-EPI cartridge *vs*. 27-33% with collagen-induced aggregation. Clopidogrel-HTPR was seen in 44% with ADP-induced aggregation. Agreement between the PFA-100 and collagen-induced aggregometry was poor, and aggregometry could not reliably detect the individual antiplatelet effects of aspirin or clopidogrel in patients on combination therapy.

Lai *et al.* prospectively recruited 269 Taiwanese patients within 7 days of ischaemic stroke onset who were on 100 mg aspirin daily for >5 days before assessment (Lai, Chen et al. 2012). Thirty-one per cent of patients had aspirin-HTPR on the C-EPI cartridge using a cross-sectional definition of HTPR in 3.8% citrate anticoagulated blood. Patients with aspirin-HTPR were less likely to have a favourable outcome [modified Rankin Scale (MRS) score ≤2] at 30 days (47% *vs*. 60%, p=0.047) or 90 days (58 *vs*. 71%, p=0.037) than those without aspirin-HTPR. However, after controlling for differences in CRP levels between groups, aspirin-HTPR did not predict 90 day outcomes. Recurrent ischaemic stroke rates at 90 days were similar in patients with and without HTPR (3.6% *vs*. 3.8%). A lower prevalence of aspirin-HTPR was observed in this compared with previous studies (Alberts, Bergman et al. 2004; McCabe, Harrison et al. 2005); this may reflect ethnic differences between studies, and the higher concentration of sodium citrate used in this study that may prolong closure times and reduce the prevalence of 'cross-sectional HTPR' on the PFA-100 (Heilmann, Kundu et al. 1997).

The TRinity AntiPlatelet responsiveness (TRAP) study was designed to assess HTPR at baseline within 4 weeks of TIA or ischaemic stroke, and then at ≥14 days

and ≥90 days after starting/changing antiplatelet therapy. One arm of this study prospectively assessed patients changing from no medication to aspirin (75-300 mg daily; n=26), or from aspirin to clopidogrel monotherapy (75 mg daily; n=22) (Tobin, Kinsella et al. 2013). A novel 'longitudinal definition of HTPR' was defined as failure to prolong relevant closure times compared with the patient's 'baseline value' before undergoing an antiplatelet change by more than twice the coefficient of variation of the assay. Twenty-four per cent of patients at 14 days and 18% at 90 days demonstrated aspirin-HTPR with the C-EPI cartridge; 41% at 14 days and 35% at 90 days demonstrated clopidogrel-HTPR with the C-ADP cartridge (Tobin, Kinsella et al. 2013). Using this novel, scientifically valid longitudinal definition, the prevalence of aspirin-HTPR was much lower than that anticipated from a study employing a 'cross-sectional definition' in the early phase after TIA/stroke (24% *vs.* 60%, p=0.003), and there was a trend towards a lower prevalence of aspirin-HTPR in the late phase after symptom onset also (18% *vs.* 43%, p=0.3) (McCabe, Harrison et al. 2005). The number of patients included in this pilot study was small, and the reportedly more sensitive INNOVANCE® PFA P2Y cartridge was not available to assess clopidogrel-HTPR in CVD patients in this study.

A pilot randomised trial showed that 30 days treatment with aspirin-dipyridamole combination therapy may lead to enhanced inhibition of platelet function compared with aspirin alone in Japanese patients with ischaemic CVD (Serebruany, Malinin et al. 2004). A further longitudinal, randomised study comprehensively assessed platelet activation and function in type II diabetic patients in the late stages after TIA, allocated to receive aspirin-dipyridamole combination therapy, clopidogrel

monotherapy, or aspirin-clopidogrel combination therapy (Serebruany, Malinin et al. 2008). There were no significant differences in platelet function on the PFA-100 between the three treatment groups.

Another pilot longitudinal, observational study from the TRAP investigators revealed that 59% of CVD patients at approximately 14 days, and 56% at ≥90 days after symptom onset did not have **additional inhibition** of platelet function on the C-ADP cartridge when 200 mg of dipyridamole MR BD was added to aspirin (n= 52) (Tobin, Kinsella et al. 2011). Using the novel longitudinal definition alluded to above, these patients were deemed to have 'dipyridamole HTPR' on the PFA-100 C-ADP, but did not undergo simultaneous platelet function testing at low shear stress. (Tobin, Kinsella et al. 2011). However, this study illustrated that carefully-designed longitudinal studies enable identification of additional inhibition of platelet function with dipyridamole in 41-44% of patients with a whole blood platelet function analyser in response to stimulation with collagen and ADP that might not be identified at all in cross-sectional studies or in other whole blood assays (Heptinstall, Fox et al. 1986).

VerifyNow®

The VerifyNow® whole blood platelet function analyser employs a modified optical aggregometry paradigm to assess platelet function inhibition at low shear stress in a stirred solution in response to stimulation with different agonists. AA is used in the 'Aspirin cartridge' which is sensitive at detecting *ex vivo* aspirin-HTPR, and ADP, iso-thrombin receptor activating peptide, and PAR-4 activating peptide in the

'$P2Y_{12}$ cartridge' that may detect HTPR to $P2Y_{12}$ ADP-receptor antagonists (Accumetrics Inc.) (Kinsella, Tobin et al. 2013). Five studies in CVD employed the precursor to the VerifyNow, called the Ultegra® Rapid Platelet Function Assay (RPFA), including the study by Harrison *et al.* described above (Harrison, Segal et al. 2005). These data will be discussed first, followed by data from 6 studies that used VerifyNow.

Platelet function was assessed with the RPFA-Aspirin, PFA-100 C-ADP cartridge, and optical platelet aggregometry (5um ADP and 5 um epinephrine) in aspirin-free patients within 2-6 months after ischaemic stroke (n=40), and in patients within 6 months of stroke on aspirin (27-650 mg daily; n=40) (Serebruany, Malinin et al. 2004). Aspirin-treated stroke patients exhibited inhibition of platelet function on the RPFA (p=0.02), prolongation of C-ADP closure times (p=0.03), and inhibition of epinephrine-induced aggregation (p=0.0001) compared with unmatched stroke patients not on aspirin. However, the proportion of patients with HTPR was not reported in this study. In another pilot cross-sectional study using the RPFA, aspirin-HTPR was noted in 30% of 50 CVD patients who were on 100 mg of aspirin daily for 2 years (Bennett, Yan et al. 2008), but prospective assessment of the relationship between HTPR and recurrent vascular events was not performed.

Seok *et al* identified aspirin-HTPR in 12% of 88 Korean ischaemic stroke patients on 100 mg of enteric-coated aspirin daily with the RPFA (Seok, Joo et al. 2008). In patients in whom urinary thromboxane B_2 levels were also available, the prevalence of aspirin-HTPR was 25% on urine testing, suggesting that the RPFA was more sensitive at detecting the antiplatelet effects of aspirin than urinary thromboxane B_2. Clinical and laboratory follow-up were not performed.

Ozben *et al* reported a 33% prevalence of aspirin-HTPR on the RPFA in acute stroke patients on 100 mg of aspirin daily for ≥1 week (N =106) (Ozben, Ozben et al. 2011). The National Institutes of Health Stroke Scale (NIHSS) score was higher (p=0.006), and in-hospital (20% *vs*. 5.6%, p=0.038) and 2-year mortality rates (60.0 vs. 31.0%, p=0.004) were higher in patients with *vs*. those without aspirin HTPR. Aspirin-HTPR was also an independent predictor of 2-year mortality (odds ratio 3.1; p=0.037) (Ozben, Ozben et al. 2011). However, aspirin-HTPR was only assessed once, so it is uncertain whether these results apply to late phase CVD patients or not. Furthermore, one other limitation of this study is that 'all-cause mortality' was recorded rather than vascular death, as is often assessed in TIA and stroke studies.

Kinsella *et al*. showed that the prevalence of antiplatelet-HTPR was significantly lower on the VerifyNow than on the PFA-100 in a cross-sectional study in the late phase after TIA/ischaemic stroke (P<0.001) (Kinsella, Tobin et al. 2013). The authors identified aspirin-HTPR in 8% of patients on aspirin-dipyridamole combination therapy (75 mg of aspirin daily and 200 mg of dipyridamole MR BD), and clopidogrel-HTPR in 44% on 75 mg of clopidogrel monotherapy daily on the VerifyNow.

Further studies identified aspirin-HTPR in 21% of patients on 100-200 mg of aspirin monotherapy > 1 week after TIA/ischaemic stroke onset (Agayeva, Gungor et al. 2014), and clopidogrel-HTPR in 29% on 75 mg of clopidogrel daily within 1 week of TIA/ischaemic stroke with the VerifyNow (Maruyama, Takeda et al. 2011).

Aspirin-HTPR on the VerifyNow aspirin cartridge was identified in 6% of 101 Thai CVD patients on 81-325 mg of aspirin daily (Dharmasaroja and Sae-Lim 2014). During prospective follow-up over 17 months, there was no difference in the risk of recurrent transient ischaemic attack, ischaemic stroke, unstable angina, myocardial infarction, cardiac interventions, cardiovascular death, or all-cause mortality between patients with and without HTPR (p=0.06). The outcome measure in this study was more widely encompassing than that employed in other studies, thus limiting comparisons between studies. Of interest, data from simultaneous urinary 11-Dehydrothromboxane B_2 assays identified 40% of patients with aspirin-HTPR, again indicating that the VerifyNow is more sensitive at detecting the antiplatelet effects of aspirin (Seok, Joo et al. 2008).

Aspirin-HTPR was assessed in 66 patients with acute ischemic stroke who were on long-term aspirin therapy (81-325 mg/day), and clopidogrel-HTPR was assessed in these patients 26 and 64 hours after administering clopidogrel (300 mg loading dose, followed by 75 mg daily) (Sternberg, Ching et al. 2013). Whole blood samples were tested with the VerifyNow, Thrombelastograph Platelet Mapping System, and whole blood impedance aggregometry. Aspirin-HTPR was identified in 23% of patients at baseline, and clopidogrel-HTPR in 40% of patients at 26 hours and 26% at 64 hours on the VerifyNow. The prevalence of aspirin-HTPR on the VerifyNow in this study was similar to other studies in the early phase after stroke (Agayeva, Gungor et al. 2014). The prevalence of clopidogrel-HTPR was higher than that reported in the early phase after stroke (Maruyama, Takeda et al. 2011), but similar to another study in late phase CVD patients (Kinsella, Tobin et al. 2013).

Jover *et al.* assessed clopidogrel-HTPR in 18 TIA/stroke patients with the VerifyNow P2Y12 assay, INNOVANCE® PFA P2Y cartridge, 5 μmol/l ADP-induced optical aggregometry, and vasodilator-stimulated phosphoprotein seven and 90 days after commencing clopidogrel 75 mg daily as monotherapy or in combination with aspirin 100-300 mg daily (Jover, Rodriguez et al. 2014). Clopidogrel-HTPR was seen in 56% at day 7 and 61% at day 90 on the VerifyNow. On the INNOVANCE PFA P2Y, clopidogrel-HTPR was observed in 39% (7/18) at day 7 and appears to have increased to 56% (10/18) at day 90. Corresponding figures for clopidogrel–HTPR on optical aggregometry were 50% at day 7 and 90. This pilot cross-sectional study was the first to compare the VerifyNow P2Y12 and INNOVANCE PFA P2Y assays in early and late phase CVD. However, much larger studies are required to validate these findings, and to assess the impact of long-term compliance on the results, especially as the prevalence of HTPR unexpectedly increased over time.

Multiplate® assay

The Multiplate whole blood platelet aggregation assay is based on measurement of impedance at low shear stress as platelets adhere to 2 adjacent electrodes and aggregate to one another within a cuvette (Mueller, Dieplinger et al. 2007; von Pape, Dzijan-Horn et al. 2007). The extent of platelet adhesion and aggregation is recorded as the Area Under the Curve (AUC) up to 6 minutes after the addition of either arachidonic acid (AA) or ADP to measure the antiplatelet effects of aspirin or clopidogrel, respectively (Mueller, Dieplinger et al. 2007; von Pape, Dzijan-Horn et al. 2007). There are few data on the assessment of HTPR with the Multiplate assay

in CVD patients on aspirin or clopidogrel (Yoo NT 2012; Azmin S 2013; Łabuz-Roszak B 2015); two studies met criteria for inclusion in this review

One prospective, pilot, cross-sectional study included 89 patients within 72 hours of TIA/ischaemic stroke onset who had received at least 1 dose of 75 mg of aspirin, 33 of whom had been on long-term aspirin (dose unspecified) (Mannu, Macartney et al. 2015). Blood sampling was performed within 12-24 hours of the first 75 mg aspirin dose in hospital and >48 hours later. Thirty-two per cent had aspirin-HTPR on the Multiplate with no significant differences in measurements between time-points. There was no clear relationship between HTPR status and the risk of recurrent cardiovascular events or all-cause mortality at 1 year, but there were too few events to make any definitive conclusions, and events rates were calculated by chart review rather than by in-person or telephone assessment. Although this study shows that the Multiplate provides reproducible results in CVD patients in a university hospital setting, the short duration of low-dose aspirin therapy in the majority, and the absence of late-phase laboratory assessment of HTPR do not allow one to comment on the long-term prevalence of aspirin-HTPR in CVD (Mannu, Macartney et al. 2015).

A prior cross-sectional study, revealed HTPR in 42% of patients with recent ischaemic stroke (n=133) on 150 mg of aspirin daily for ≥7 days (Jastrzebska, Chelstowski et al. 2013). The aspirin dose was increased to 300 mg daily in a proportion of those with HTPR who 'qualified for increased treatment', but 67% of this subgroup had persistent aspirin-HTPR 7 days later. Baseline NIHSS scores were slightly higher in patients with than in those without HTPR (3.7 *vs*. 2.5, P<0.01), and the data suggested that increasing levels of CRP and VWF activity

enhanced platelet reactivity in the subgroup with HTPR (Jastrzebska, Chelstowski et al. 2013). The authors changed patients to a 'thienopyridine antiplatelet agent' if they had persistent aspirin-HTPR on 300 mg daily, but there was no clear clinical evidence-base behind this decision, long-term laboratory re-assessment was not subsequently performed, and clinical outcome events during short-term follow-up were not reported. Therefore, one cannot conclude that one should alter antiplatelet therapy in CVD patients based on these data.

3.4. Potential influence of pharmacogenetic factors on HTPR on antiplatelet therapy in CVD

The response to antiplatelet agents may be influenced by genetic factors (Johnson, Yanek et al. 2010; Kim, Suktitipat et al. 2013; Lewis, Ryan et al. 2013). However, pharmacogenetic studies in CVD have mainly focused on candidate gene analysis rather than on Genome Wide Association Studies (GWAS).

3.4.1. Data on aspirin

A recent study in 859 Chinese stroke patients, including one of the largest cohorts treated with aspirin, found that the rs1330344 single nucleotide polymorphism (SNP) of COX-1 (CC genotype) was associated with a higher incidence of nonfatal ischaemic stroke, myocardial infarction, and death from cardiovascular causes during follow-up compared with non-carriers of this genotype. The authors reported that this genotype may upregulate COX-1 RNA and protein expression, and in theory, enhance conversion of arachidonic acid to thromboxane A^2. However,

simultaneous assessment of HTPR was not reported, and replication of the genetic analysis in another population was not performed (Cao, Zhang et al. 2014).

3.4.2. Data on clopidogrel

Pharmacogenetic studies have linked alleles of cytochrome P450 genes, mainly CYP2C19 polymorphisms, with platelet reactivity in patients on clopidogrel (Shuldiner, O'Connell et al. 2009). These polymorphisms may affect metabolism of clopidogrel from its pro-drug to an active thiol derivative. In a meta-analysis of 42,016 patients on clopidogrel who had 3,545 major vascular events during follow-up, certain CYP2C19 alleles were associated with recurrent vascular events, including ischaemic stroke. Simultaneous assessment of platelet function/reactivity was only performed in 4 included studies, and indicated that patients with the CYP2C19 2*/2* genotype (associated with reduced drug metabolism) exhibited platelet hyper-reactivity on 600 mg of clopidogrel compared with the CYP2C19 1*/1* genotype (normal drug metabolism). However, different assays were used to assess Clopidogrel HTPR, and we have no evidence regarding the safety or efficacy of this dose of clopidogrel in CVD patients. Furthermore, when the overall meta-analysis was restricted to studies containing >200 patients, the association with CYP2C19 polymorphisms and outcome events was not significant. (Holmes, Perel et al. 2011).

In another recent study, CYP2C19 *2/*3 loss of function (LOF) alleles were associated with recurrent non-fatal ischaemic stroke, non-fatal MI or vascular death in a cohort of 625 Chinese ischaemic stroke patients on clopidogrel (Sun, Li et al.

2014). This CYP2C19 *2/*3 LOF genotype was associated with clopidogrel-HTPR on ADP-induced aggregometry (59%), and worse outcomes on the MRS at 3 and 6 months in another study in 259 Chinese ischaemic stroke patients on clopidogrel (Jia, Chen et al. 2013). A significant association between LOF alleles and worse outcome on MRS has been observed in another study in 211 stroke patients on clopidogrel, in which the CYP2C19 *2/*3 genotype was associated with higher platelet reactivity on ADP-induced platelet aggregation than patients without these LOF alleles (Qiu, Sun et al. 2015). However, the reason for the association with poor outcome is unexplained and needs validation and reassessment in larger studies.

3.5. Discussion

This comprehensive review has shown that the prevalence of *ex vivo* HTPR in patients after TIA or ischaemic stroke on commonly prescribed antiplatelet therapy varies according to the definitions and platelet function devices employed. The relatively limited, available literature indicates that the prevalence of HTPR in CVD varies between 3-62% with aspirin monotherapy (Helgason, Bolin et al. 1994; von Lewinski, Riggert et al. 2009), 8-61% with clopidogrel monotherapy (Fukuoka, Furuya et al. 2011; Meves, Overbeck et al. 2012; Jover, Rodriguez et al. 2014), and 56-59% when dipyridamole is added to aspirin (Tobin, Kinsella et al. 2011) in the early, subacute, or late phases after TIA/stroke onset. These summary figures for clopidogrel-HTPR exclude data from 2 studies that confirmed that the standard C-ADP cartridge on the PFA-100 is not sensitive at detecting the antiplatelet effects of clopidogrel if one uses a cross-sectional, case-control definition of HTPR (Grau, Reiners et al. 2003) (Kinsella, Tobin et al. 2013). Most studies in CVD employed

cross-sectional definitions of HTPR which may underestimate the effects of antiplatelet therapy on platelet function in individual patients compared with novel longitudinal definitions, in which patients act as their own baseline controls (Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013). However, one has no evidence as yet that novel longitudinal definitions of HTPR are more clinically informative at predicting recurrent vascular events during follow-up after TIA/stroke than more commonly applied cross-sectional definitions of HTPR.

There are some conflicting data about the consistency of HTPR measurements on optical platelet aggregometry over time (Helgason, Bolin et al. 1994) (Gengo, Rainka et al. 2008). In general, the prevalence of aspirin-HTPR appears higher on the 'moderately high-shear stress' PFA-100 C-EPI system that assesses platelet adhesion and aggregation than e.g. on 'low shear stress' platelet aggregometry assays (Harrison, Segal et al. 2005; McCabe, Harrison et al. 2005; von Lewinski, Riggert et al. 2009; Kinsella, Tobin et al. 2013). None of the studies published to date have been adequately powered to definitively comment on whether *ex vivo* HTPR status on platelet function testing in CVD patients predicts the risk of recurrent vascular events. Evidence pertaining to the relationship between antiplatelet-HTPR status and functional outcome, stroke severity and mortality on antiplatelet therapy following TIA or stroke is emerging, but available data from small-medium sized studies need to be validated in larger studies (Boncoraglio, Bodini et al. 2009; Lai, Chen et al. 2012; Jia, Chen et al. 2013; Yi, Zhou et al. 2013; Qiu, Sun et al. 2015). Furthermore, with the exception of 3 studies (Schwammenthal, Tsabari et al. 2008; Boncoraglio, Bodini et al. 2009; Yi, Zhou et al. 2013), duration of clinical follow-up has been relatively short.

No adequately powered studies have comprehensively assessed the impact of pharmacogenetic factors on platelet reactivity/HTPR in diverse geographical populations of TIA or stroke patients on antiplatelet therapy outside China. Most small-medium sized studies in CVD were performed in the era before GWAS, and findings have not been replicated. Different international consortia, such as the International Stroke Genetics Consortium (ISGC) (http://www.strokegenetics.org/), or the International Clopidogrel Pharmacogenomics Consortium (ICPC) (https://www.pharmgkb.org/page/icpc) are in a position to investigate the relationship between pharmacogenetic factors and HTPR in CVD in collaboration with translational platelet scientists and vascular neurologists / stroke physicians with expertise in this area.

In summary, assessment of *ex vivo* HTPR at high and low shear stress, preferably in the physiological milieu of whole blood, has the potential to play a significant role in facilitating optimal, 'individualised antiplatelet treatment' in CVD patients. However, at present, one cannot justify altering antiplatelet therapy in individual TIA or stroke patients in routine clinical practice based on *ex vivo* measurements of HTPR or based on specific genetic polymorphisms outside the setting of a research study or clinical trial. Large, adequately-sized, prospective, multicentre collaborative studies are urgently needed to address this critical public health issue to determine whether comprehensive assessment of HTPR at high and low shear stress with a range of user-friendly, whole blood platelet function testing platforms, in conjunction with pharmacogenetic data, improves our ability to predict the risk of recurrent vascular events in CVD patients. Such data from existing and emerging

platelet function testing platforms (http://www.plateletsolutions.co.uk ; Fox, May et al. 2009; Chan, Armstrong et al. 2011; Dovlatova, May et al. 2015) should improve our understanding of the mechanisms responsible for HTPR, and hopefully enhance secondary prevention in TIA or ischaemic stroke patients requiring long-term antiplatelet treatment.

Table 3. 1 Comparison of characteristics of commonly available platelet function technology platforms

(Some data adapted from Michelson AD [Michelson 2009); (Lim et al. 2015)

Platelet Function Test	Principle employed	Advantages	Disadvantages
Bleeding Time (Rodgers and Levin 1990)	*In vivo* screening test	Cheap; physiological	Invasive; not sensitive or reproducible; not specific for particular antiplatelets or platelets alone
Optimal Aggregometry (Helgason, Bolin et al. 1994; Chamorro, Escolar et al. 1999)	Responsiveness to Agonists	Specific	**PRP only** and as outlined in text above
Impedence Aggregometry (Fong, Cheng-Ching et al. 2011; Sternberg, Ching et al. 2013)	Responsiveness to Agonists	**Whole blood test;** several agonists	Trained personnel required; labour intensive; high blood volume; semi-quantitative; expensive
Aggregometry and Luminescence (Cattaneo 2009; Cattaneo, Hayward et al. 2009)	Aggregation and ADP release	Informative re platelet pathways affected	Trained personnel required; labour intensive; expensive
Laser Platelet Aggregometer (PA- 200®) (Yamamoto, Ishii et al. 1993; Ozaki, Satoh et al. 1994)	Platelet micro-aggregates	Sensitive; studies hyper-reactivity	**PRP only**; no widespread experience
Thromboelastography (TEG®)/Thromboelastometry (Trentalange and Walts 1991; Sternberg, Ching et al. 2013)	Global Haemostasis	**Whole blood**; may predict bleeding	Measures clot properties only; not specific for effects of particular antiplatelets or platelets alone
Glass Filterometer (O'Brien and Salmon 1987)	Shear induced platelet reactivity	**Whole blood;** user friendly; reproducible	Requires platelet counter; not widely used; results not predictive of MI or stroke risk in one study
Clot Signature Analyser (CSA) (Gorog and Ahmed 1984)	Global haemostasis, high shear platelet function	**Whole blood;** global haemostasis	Not platelet specific; sensitivity at detecting effects of all common antiplatelet agents unproven
Haemodyne (Carr, Martin et al. 2003)	Platelet contractile force	**Whole blood** or PRP; rapid; simple	Measures clot properties only; not specific for effects of particular antiplatelets or platelets alone
HemoSTATUS® (Coiffic, Cazes et al. 1999)	Platelet procoagulant function	**Whole blood;** simple	Insensitive to aspirin and GpIb function
Thrombotic Status Analyser (TSA) (Gorog and Kovacs 1995)	Measure antiplatelet and thrombolytic effects at high shear stress	**Whole blood**; Simple	Little widespread experience
Cone & Plate Analyser (CPA) (Varon, Lashevski et al. 1998; Levy-Shraga, Maayan-Metzger et al. 2006)	High shear stress induced platelet adhesion/aggregation	**Whole blood;** physiological; small volume	Requires image analysis; not widely available; limited experience
ICHOR (Plateletworks®) (Carville, Schleckser et al. 1998)	Platelet counting pre- and post-platelet activation	**Whole blood;** rapid; simple, point-of-care; small blood volume	Need preparation of reagents; need access to analyser within minutes of venepuncture, so impractical in many routine clinical settings; indirectly measures aggregation inhibition
Platelet Function Analyser (PFA-100®) (Alberts, Bergman et al. 2004; Kinsella, Tobin et al. 2013)	Moderately high shear stress-induced adhesion/aggregation	**Whole blood**; rapid; user-friendly; reproducible; may detect antiplatelet effects of aspirin and clopidogrel, and additional effects of dipyridamole over aspirin	Fixed shear rate; fixed dose agonists; some cartridges expensive, VWF dependent, one pipetting step required
Verify Now® (Sternberg, Ching et al. 2013; Agayeva, Gungor et al. 2014)	Platelet aggregation at low shear; Aspirin effect; P_2Y12 antagonists; GpIIb/IIIa-dependent aggregation	**Whole blood**; user-friendly; No specific expertise required once trained; reproducible; may detect antiplatelet effects of aspirin, clopidogrel	2 ml blood volume per cartridge; some cartridges are expensive; no specific assays identified for dipyridamole

Multiplate® Impedance Aggregometry (Jastrzebska, Chelstowski et al. 2013; Mannu, Macartney et al. 2015)	Platelet aggregation at low shear; Responsiveness to panel of agonists	**Whole blood**; User-friendly; No specific expertise required once trained; may detect antiplatelet effects of aspirin and clopidogrel; reproducible; easily available	Specialised equipment; costs; pipetting steps required; no specific assays identified for dipyridamole
Flow Cytometry	Platelet glycoprotein expression; platelet activation markers & platelet reactivity in response to agonist stimulation	**Whole blood** or PRP; Sensitive and specific; flexible; centralised assays available to detect aspirin and clopidogrel effects (**Platelet Solutions UK**)	Trained personnel required; labour intensive; expensive equipment and reagents required on site unless centralised assays performed, no specific assays identified for dipyridamole
Cellix I Diagnose platform	Platelet adhesion and aggregation	**Whole blood**; reproducible; variable shear rates and concentrations of agonists; multiple simultaneous agonists	Not yet widely available
Serum thromboxane B_2 or urinary 11-dehydro-thromboxane B2 / Creatinine ratio (Dharmasaroja and Sae-Lim 2014)	Thromboxane biosynthesis	'Standard assay' for inhibition of thromboxane biosynthesis by aspirin	ELISA technique; **serum or urine**; indirect measure of effects of aspirin; not platelet-specific, requires specialised equipment and trained personnel; very time consuming; expensive

Table 3. 2 Prevalence of ex vivo high on treatment platelet reactivity in CVD patients on antiplatelet therapy

Markers of platelet function and reactivity (↑ = increased; ↔= not significantly different, ↓=decreased) WB, Whole Blood, PRP, platelet-rich plasma; PPP, Platelet-poor plasma; TIA, transient; ASA, aspirin ;CP, Clopidogrel; DP, Dipyridamole; AA, Arachidonic acid; ADP, Adenosine Diphosphate; HTPR, high on treatment platelet reactivity.

Author	Patient Population	Device	Agonists	HTPR Definition	% HTPR Results of study	Antip Agent a
Helgason et al 1993 Cross-sectional	N= 113 prior stroke N = 24 acute stroke	Classical Aggregometry	500 µM AA 5 µM ADP 5µM EPI 0.8 µg/ml COL	incomplete inhibition of platelet aggregation on 1300 mg	4.2% ASA HTPR acute stroke 2.7% ASA HTPR chronic	ASA 325mg –
Helgason et al 1994 Cross-sectional	N = 306 stroke patients	Classical aggregometry	500 µM AA 5 µM ADP 5µM EPI 0.8 µg/ml COL	incomplete inhibition of platelet aggregation on 1300 mg	26% ASA HTPR initial dose 8% ASA HTPR 1300mg dose	ASA > 325 mg
Chamorro et al 1999 Case Control	N = 39 total N = 18 stroke recurrence on aspirin N = 25, no stroke recurrence on aspirin	Classical aggregometry	1.4 µmol/l AA 1-2 µmol/l ADP 10 µmol/l EPI	Non Defined	↑ aggregation stroke recurrence vs non stroke recurrence (p < 0.001) ↑ aggregation 300 mg vs 600mg	ASA 300mg –
Harrison et al 2005 Cross-sectional	N = 100	Optical Aggregometry	1 mg/ml AA 10 µmol/L ADP	> 20% aggregation with 1 mg/mL AA >70% aggregation with 10 µmol/L ADP	5% ASA HTPR	ASA 75mg –
Serebruany et al 2008 Randomised single blind Pilot study	N=60	Light Aggregometry	5 µmol/L ADP 5 µg/mL COL	Non Defined	↓ clopidogrel on ADP (P=0.001) ↓ ASA + clopidogrel on Collagen (P= 0.001) ↔ ASP + ER D	ASA 81 mg CLO 75 mg ER DP + ASP

Sztriha et al 2008 Retrospective	N = 241	Optical Aggregometry	10 µmol/l EPI 2µg/ml COL	Non defined	↔ recurrent episodes vs non-recurrence	ASA 100mg
Gengo et al 2008 Cross sectional	N = 653	Impedance aggregometer	1 µg/ml of COL 0.5 mM AA	> 10 ohms on 1 µg/ml of COL > 50% response 5µg/ml COL > 6 ohms 0.5 mM AA	14% (89) ASP HTPR AA 17% (109) ASP HTPR COL 20% (129) ASP HTPR COL+ AA 66% (57) HTPR + Recurrent event (OR 14.25)	ASA 81mg
Fong et al 2011 Retrospective Analysis	N = 465	LTA	10mM ADP 0.5mg/ML AA	ASA HTPR > 70% 10mM ADP ASA HTPR >20% 0.5mg/ML AA CLO HTPR >40% 10mM ADP	28% (120) ASA HTPR 28% (83) CLO HTPR 9.3% (25) ASP & CLO HTPR	ASA 81mg CLO 75 mg
Fukuoka et al 2011 Cross Sectional	N = 465	Turbidometry Screen Filtration	1µmol/L ADP 4µmol/L ADP	CLO HTPR > 34% 1µmol/L ADP ASP HTPR > 66 % 4µmol/L ADP	CLO HTPR 8.3% on 1µmol/L ADP CLO HTPR 18.1% 4µmol/L ADP	CLO 75mg
Meves et al 2012 Cross Sectional	N = 737	Impedance	2 µmol/ml COL 0.5mM AA 5µM ADP	 ASA HTPR > 0 ohms AA CLO HTPR > 5 ohms ADP	HTPR: 18.4% with IV 500 mg ASA, 32.5% with oral 200 mg ASA, 35.9% with oral 100 mg ASA daily and 45.7% on CLO 75mg	ASA 100mg CLO 75mg
Depta et al 2012 Cross Sectional	N = 324 TIA = 74 ISCH STROKE = 250	Optical AGG	0.5% mg/ml AA 10 µM ADP 10 µM ADP	ASA HTPR >20% 0.5% mg/ml AA >70% 10 µM ADP CLO HTPR >40% 10 µM ADP	ASA HTPR 43% CLO HTPR and 35% 23% (73) Changed anti-platelets	ASA 75mg CLO 75 mg
Yi et al 2013 Cross Sectional Prospective	N = 634	Optical AGG	10 uM ADP 0·5 mg/ml AA	AR □ > 70% 10 uM ADP or > 20% with 0·5 mg/ml AA. ASR > 70% 10 uM ADP or >20% with 0·5 mg/ml AA	Aspirin resistance (AR) was detected in 129 patients (20·4%) and "aspirin semi-resistance" (ASR) in 28 patients (4·4%) ↑ MI, Vascular events in AS + AR (p<0.001)	ASA 100-2
Meves et al 2014 Cross Sectional	N = 737	Impedance	0.5mM AA	ASP HTPR > 0 ohms AA	HTPR was 28% in the CVD group, 18.1% in the CAD group and 21.6% in the PAD	ASA 100mg, 200 500mg IV

Lago et al 2014 Observational	N = 56 TIA = 8 ISCH STROKE = 48	Optical	3 μM ADP	Not Defined	ASA + CLO ↑ 13% VS ASP (p=0.008)	ASA 100-300 400mg IV CLO 75 MG ASA + CLO
Yi *et al.* 2016 (i) Prospective	N = 426 AIS	LTA	10 uM ADP 0·5 mg/ml AA	AR □ > 70% 10 uM ADP or > 20% with 0·5 mg/ml AA. ASR > 70% 10 uM ADP or >20% with 0·5 mg/ml AA	ASA HTPR 24.4% CLO HTPR 35.9% Dual ASA + CLO HTPR 19.2%	ASA 100 mg CLO 75 mg ASA + CLO

Table 3. 3 Prevalence of ex vivo high on treatment platelet reactivity in CVD patients on antiplatelet therapy on the VerifyNow, unless otherwise specified that the Ultegra-RPFA* was used

Markers of platelet function and reactivity (↑= increased; ↔= not significantly different, ↓=decreased).C-ADP, collagen-adenosine diphosphate; C-EPI, collagen-epinephrine; CT, closure time; CVD, cerebrovascular disease; VerifyNow, VerifyNow system; WB, Whole Blood, PRP, platelet-rich plasma; PPP, Platelet-poor plasma; TIA, transient; ASA, aspirin ;CP, Clopidogrel; DM, Dipyridamole; AA, Arachidonic acid; ADP, Adenosine Diphosphate; ARU, Aspirin Response Units; PRU, *P2Y12 Reaction Units;* dTXB2, 11-Dehydrothromboxane B2; HTPR, high on treatment platelet reactivity
*Ultegra RPFA used

Author and study design	**Patient population**	**Control Population**	**VerifyNow Aspirin CT**	**VerifyNow P2Y12 CT**	**High on Treatment Platelet Reactivity**	**Anti-platelet Agent**	**Sample type**
Agayeva et al. (2015) Prospective observational	Ischemic stroke n= 335 78 ASA treatment group 257 no–ASA treatment group	N/A	Sufficient platelet inhibition on ASA ARU <550 n=62 Insufficient platelet inhibition on ASA ARU ≥550 n = 16	N/A	16/78 (21%) on ASA monotherapy	ASA	WB
Bennett et al. (2008) * Pilot Cross-sectional	Ischemic stroke n=50	N/A	ASA non responders median ARU=576 (range 550 - 626) ASA sensitive median ARU=466 (range 385 – 547)	N/A	15/50 (30%)	ASA	WB

Dharmasaroja (2014) Prospective longitudinal	Ischemic stroke n=101		Mean ARU = 446 ± 50 ARU >550 insufficient antiplatelet effect	N/A	40% by Urinary dTXB2 criteria 6% by ARU criteria Poor correlation between tests (r = 0.135 P = 0.190)	ASA	WB Urine
Jover et al. (2014) Case-control	Ischemic stroke/TIA n=18			HTPR = PRU >235	HTPR = 40%(day 7) 42% (day 90)	CP	WB
Kinsella et al. (2013) pilot cross-sectional observational	Ischemic stroke/TIA Late stable phase (≥3 months) n=76	N/A	ASA +DP (n=51) Mean ARU= 476 (range 350-660) CP (n=25) Mean ARU= 648 (range 546-674)	ASA+DP (n=51) Mean PRU=325 (range 233-428) CP (n=25) Mean PRU=204 (range 35-372)	HTPR=4 /51 (8%) on ASA+ DP measured by ASA Cartridge HTPR=11/ 25 (44%) on CP measured by P2Y12 cartridge	CP ASA+ DP	WB
Maruyama et al. (2011) observational	ischemic stroke/ carotid artery stenting n=77 CP only treatment group n=62 CP + Cilostazol combination Treatment group n=15		N/A	↓PRU CP+ Cilostazol group(170.2 ± 89.4) than CP only group (113.3±72.8) p=0.021 % inhibition was↑ in CP+ Cilostazol group (64.9% ±22.7%) than CP only group (41.7%±28.0 %; p=0.005	18 /62 (29%) in CP only group.	CP	WB

Ozben et al. (2011) * Longitudinal Case-control	Acute ischemic stroke n=106		ASA non-responders: Mean ARU= 595 ± 43 ASA sensitive: Mean ARU=487 ± 39 8/27 (29.6%) Aspirin resistance in patients with prior ischemic stroke	N/A	35/106 (33%) Higher NIHSS score in patients with HTPR (15 ± 3) than without ASA HTPR (12 ± 5) (p = 0.006) In-hospital mortality rate in patients with HTPR vs without ASA HTPR (20% vs. 5.6%) p = 0.038 2-year mortality rates rate in patients with HTPR vs without ASA HTPR (60.0 vs. 31.0%) p = 0.004	ASA	WB

Seok et al. (2008) * Case-control	Ischemic stroke n=88		ARU ≥550 insufficient antiplatelet effect	N/A	11/88 (12%) HTPR by ARU criteria 18/88 (25%) HTPR by Urinary dTXB2 criteria 4/88 (4.5%) HTPR by both criteria poor correlation between tests (*r* =−0.115, *p* = 0.34)	ASA	WB Urine
Serebruany et al. (2004)* Observational Case-control	Ischemic stroke n=120 ASA free after ischemic stroke n=40 Patients receiving ASA post-stroke n = 40 ASA free controls with multiple risk factors for vascular disease n = 40	Healthy controls	ASA free after ischemic stroke: Mean ARU=665± 61 Patients receiving ASA post-stroke: Mean ARU= 394±47# ASA free controls: Mean ARU=641± 56 #P<0.05 vs both ASA free after ischemic stroke & ASA free controls groups	N/A	Patients receiving ASA post-stroke: significant inhibition of epinephrine-induced aggregation (p=0.0001) ↑ CT measured by PFA-100 (p=0.03) ↓ARU (p=0.02)	ASA	WB PRP

Sternberg et al. (2013) Observational Case-control	Acute ischemic stroke n=66	N/A	ASA non-responders: Mean ARU=482± 8.4 (range 388-664)		22.7% ASA poor responders 26% CP poor responders 53.8% ASA poor responders also responded poorly to CP as measured by Verify Now	ASA CP	WB

Table 3. 4 Prevalence of ex vivo HTPR/'non-responsiveness' in CVD patients on antiplatelet therapy using PFA-100

Markers of platelet function and reactivity (↑= increased; ↔= not significantly different, ↓=decreased).C-ADP, collagen-adenosine diphosphate; C-EPI, collagen-epinephrine; CT, closure time; CVD, cerebrovascular disease; PFA-100, Platelet Function Analyser-100; WB, Whole Blood; PRP, platelet-rich plasma; PPP, platelet-poor plasma; TIA, transient; ASA, aspirin ;CLO, Clopidogrel; DP, Dipyridamole; AA, Arachidonic acid; ADP, Adenosine Diphosphate

Author	Patient population	Control Population	PFA-100 C-EPI CT	PFA-100 C-ADP CT	Platelet Aggregometry	As Non-re Ness / Res
Alberts et al. 2004 Prospective Case-control	Stroke/TIA Acute phase n=129	Not specified	Significant difference in mean CT when comparing ASA dose and preparation low-dose ASA- 183 sec high-dose ASA -233 sec enteric-coated ASA-173 sec uncoated ASA-235 sec (*P*<0.01 for comparisons)	N/A	N/A	48/129 (37% EPI c Higher p patients rec than 325 mg 28%; p
Boncoraglio et al. 2009 Longitudinal Case-control	Stroke/TIA/ vascular cognitive impairment n=129	N/A	Not specified	N/A	N/A	26/129 (20. C-EPI
Godeneche et al 2009 Cross sectional	Acute ischaemic stroke patients N=100	N/A	ANR defined as C-EPI CT < 187s	C-ADP in ANR patients group = 73 +/- 10s	N/A	15/100 (15% EPI c C-ADP CT ↓ HTN indepe of ANR (↔ betwee gene polym S
Grau et al 2003 Case control	lacunar or 'atherothrombotic' ischaemic stroke late phase n=31	healthy subjects who no vascular risk factor or arterial vascular disease n=21	↑ under combinination therapy vs Aspirin (P=0.0009) or Clopidogrel (P=0.0074)	↑ under combination therapy vs aspirin (P0.0009) or Clopidogrel (P0.0074)	N/A	5/31 (16. car 29/31 (94%) mono 21/29 (72% combinatio ADP
Grundmann *et a*l *2003 Cross-sectional*	Ischemic stroke/ (TIA) symptomatic/ asymptomatic patients n=53	Not specified	↓symptomatic Vs asymptomatic (p<0.01)	↔symptomatic Vs asymptomatic	23/53 (66%) Inhibition Symptomatic m ↑C-EPI CT	1 (3 Sympto

Harrison et al 2005 Cross-sectional	Stroke/TIA n=100	normal volunteers n=6	↓ ASA non responders vs ASA responsive group (*P*=0.009)	Not specified	12/99 (12%) with AA-induced aggregation 14/98 (14%) with ADP-induced aggregation	17 of 10 22/ PFA-100
Lai et al. 2012 Prospective, Case-control	ischemic stroke acute phase n=269	N/A	N/A	N/A	N/A	83/2 PFA-100
Lago et al 2014 Observational	N = 56 total TIA = 8 ISCH STROKE = 48	N/A		N/A	ASA + CLO group achieved a 13% significant reduction in ADP induced aggregation	C-EPI C only gro ASP and A
McCabe et al. 2005 Longitudinal Observational case–control	Stroke/TIA in territory of 70% carotid stenosis/ occlusion n = 11 n = 55 early phase n = 45 convalesent phase post Stroke / TIA	Healthy subjects with no cerebro-vascular disease n = 23	↔ early phase ($P = 0{\cdot}3$); ↑ late phase ($P < 0{\cdot}001$)	↔	No late phase patients defined as aspirin on PFA-100 were non responders on Aggregometry	(7/1 phase phase C-E
Serebraury et al. 2004 Randomised trial	ischemic stroke with atherosclerotic morphology of at least 50% n=40	Placebo control for ASA	↑CT ASA+ DM group at 30 days (P=0.04)	↑CT ASA+ DM group at 30 days (P=0.04)		
Serebruany et al 2005 Randomised case control	Ischemic stroke convalescent phase n=71	N/A	Not specified	↑ ASA Baseline vs 3 months (203±46→ 214±45) ASA + CP Baseline (197±38→ 249±41)	ASA + CP significant inhibition of platelet aggregation in response to ADP (P = 0.00001) or collagen (P= 0.021	No

Serebraury et al. 2008 randomized, single-blind pilot study	TIA patients with type 2 diabetes mellitus N=60	N/A	↑in Clopidogrel Monotherapy treated arm (P = 0.01)		Clopidogrel monotherapy inhibition of ADP-induced platelet aggregation (P = 0.001) ASA + Clopidogrel inhibition of collagen-induced platelet aggregation (P = 0.001)	Not s
Von Lewinski et al. 2009 retrospective analysis	CVD patients n=142	healthy subjects, no medication n=51	↑ Controls vs ASA alone (P < 0.01) or in combination (P =0.02)	N/A	ASA non-responsiveness 27-33% by collagen-induced aggregation Clopidogrel non responsiveness 44% by ADP-induced aggregation	58-62%

4. Value of platelet function/reactivity testing at predicting risk of recurrent vascular events and outcomes after TIA or ischemic stroke: A Systematic review and meta-analysis.

4.1. Abstract:

Importance: The value of testing for 'high on-treatment platelet reactivity (HTPR)' to predict outcomes in TIA/ischemic stroke patients on antiplatelet therapy is unclear.

Objectives: Assess the prevalence of *ex-vivo* antiplatelet-HTPR and relationship between antiplatelet-HTPR status and recurrent vascular events/outcomes in patients with ischemic cerebrovascular disease (CVD).

Data Sources: MEDLINE, EMBASE and Cochrane Library were searched for completed manuscripts in English from database' establishment until May 2019.

Study Selection: Patients with TIA/ischemic stroke, ≥18 years, treated with commonly prescribed antiplatelet therapy, who had platelet function/reactivity testing and any prospective follow-up data on recurrent stroke/TIA, myocardial infarction, vascular death or other cerebrovascular outcomes.

Data Extraction and Synthesis: Two investigators independently extracted data. Study quality was assessed with the ROBINS-I Scale. Data were pooled using random-effects meta-analysis.

Main Outcomes Measures: The primary outcome was the composite risk of recurrent stroke/TIA, myocardial infarction or vascular death. Secondary outcomes were risk of recurrent stroke/TIA, or severe stroke (NIHSS >16) or disability/impairment (modified Rankin scale ≥3) during follow-up.

Results: Antiplatelet-HTPR prevalence was 3-65% with aspirin, 8-56% with clopidogrel and 1.8-35% with aspirin-clopidogrel therapy. Twenty studies (4,989 patients) were included in our meta-analysis. There was a higher risk of the composite outcome (OR 2.93, 95%CI: 1.90-4.51) and recurrent ischemic stroke/TIA (OR 2.43, 95%CI: 1.51-3.91) in patients with *vs*. those without antiplatelet-HTPR on any antiplatelet regimen. The risk of the composite outcome (OR 3.13, 95%CI: 1.77-5.56) and recurrent ischemic stroke/TIA (OR 2.26, 95%CI: 1.19–4.30) was also higher in patients with *vs*. those without Aspirin-HTPR, with high heterogeneity between studies (I^2 ≥84%, P< 0.001). Clopidogrel-HTPR status did not predict risk of subsequent composite vascular outcomes or ischemic stroke/TIA, but the number of included studies was much smaller, with moderate heterogeneity between studies (I^2 ≥66.1%, P≤ 0.051). The risk of having a severe stroke was higher in those with *vs*. those without antiplatelet-HTPR (OR 2.65, 95%CI: 1.00-7.01).

Conclusions and Relevance: These data suggest that identification of antiplatelet-HTPR predicts the risk of recurrent vascular events and outcomes in CVD patients, especially on aspirin. Given the moderate-high heterogeneity between studies, further prospective, multi-center studies are needed to address this issue.

4.2. Introduction

An important proportion of patients with ischemic cerebrovascular disease (CVD) are not protected from recurrent vascular events with commonly prescribed 'non-monitored' antiplatelet therapy. Because the risk of recurrent events is highest early after a non-cardioembolic TIA/ischemic stroke, early institution of an effective preventive antiplatelet regimen is very important (Rothwell, Buchan et al. 2006).

Monitoring effects of antiplatelet therapy with reliable *ex vivo* platelet function/reactivity tests has the potential to facilitate precision-based medical treatment of CVD patients (Collins and Varmus 2015). Prior data suggesting that *ex vivo* antiplatelet-HTPR increases the risk of subsequent vascular events are mostly derived from patients with ischemic heart disease (IHD) (Grotemeyer, Scharafinski et al. 1993; Eikelboom, Hirsh et al. 2002; Gum, Kottke-Marchant et al. 2003; Mason, Jacobs et al. 2005; Bennett, Yan et al. 2008; Krasopoulos, Brister et al. 2008), but data in this cohort are conflicting (Aradi, Komocsi et al. 2010; Combescure, Fontana et al. 2010; Sofi, Marcucci et al. 2010; Pettersen, Seljeflot et al. 2012; Reny, Berdague et al. 2012). A prior meta-analysis found a higher incidence of stent thrombosis, myocardial infarction or death in patients with antiplatelet-HTPR compared with those with lower on-treatment platelet reactivity on the VerifyNow following percutaneous coronary intervention (PCI) (Brar, ten Berg et al. 2011). However, altering antiplatelet therapy based on *ex vivo* platelet function testing with the VerifyNow did not improve 'vascular outcomes' in two large trials in IHD patients (Price, Berger et al. 2011; Collet, Cuisset et al. 2012). A recently published meta-analysis of 10 randomized clinical trials, including a total

of 4213 patients, assessed the efficacy and safety of intensifying clopidogrel therapy based on HTPR status in patients undergoing PCI (Aradi, Komocsi et al. 2013). Intensifying antiplatelet therapy based on HTPR testing was associated with reduced cardiovascular mortality and stent thrombosis after PCI (P=0.02), with no difference in the risk of major hemorrhagic complications between 'intensified' and 'standard treatment' groups (P=0.44). Due to the heterogenous etiology of TIA and ischemic stroke (Adams, Bendixen et al. 1993) and the higher risk of intracerebral cerebral haemorrhage after stroke than after an acute coronary syndrome, one cannot simply extrapolate data on *ex vivo* HTPR from IHD patients to those with CVD. This area of translational research has received much less attention in CVD patients, as outlined in prior systematic reviews (Lim, Coughlan et al. 2015) (Fiolaki, Katsanos et al. 2017).

The aims of this updated systematic review and innovative meta-analysis were to determine the potential role of *ex vivo* platelet function/reactivity testing in predicting (a) the risk of recurrent vascular events and (b) 'neurological outcomes' following TIA or ischemic stroke.

4.3. Methods

4.3.1. Search Strategy

The systematic review and meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher, Liberati et al. 2009). This study protocol is registered with the

International Prospective Register of Systematic Reviews (PROSPERO) (Registration No. CRD42018104210).

MEDLINE (OVID and PubMed), EMBASE (via OVID) and The Cochrane Library (Cochrane Database of Systematic Reviews, Cochrane Central Register of Controlled Trials (CENTRAL), Cochrane Methodology Register) were searched for completed, peer-reviewed manuscripts in English from the establishment of these databases until 1st May 2019. The search strategy encompassed subject headings or thesaurus terms, and a comprehensive, relevant text-word strategy utilizing truncations and 'wild-cards'. Searches were combined using Boolean operators 'and', 'or' and 'not'. The following search terms were used in different combinations: transient ischemic attack, TIA, stroke, platelet function, platelet reactivity, platelet aggregation, platelet aggregometry, antiplatelet resistance, high on-treatment platelet reactivity, aspirin, clopidogrel, dipyridamole, and three commonly-available whole blood platelet function/reactivity testing platforms at the time of the review (Platelet Function Analyser-100 (PFA-100®), VerifyNow®, Multiplate®). In addition, reference lists of included papers and systematic reviews were critically evaluated to search for articles not identified with the above search strategy.

4.3.2. Study Selection and Data Extraction

We used the following **inclusion criteria**:

1) Studies including patients with TIA or ischemic stroke, ≥18 years, treated with aspirin or clopidogrel monotherapy, aspirin-dipyridamole or aspirin-clopidogrel

combination therapy, who had platelet function/reactivity testing with aggregometry or the above commonly-available whole blood platelet function/reactivity platforms;

2) Any prospective follow-up **after** platelet function/reactivity testing;

3) As clopidogrel may potentially have less clinical efficacy in 'poor' or 'intermediate metabolizers' and greater efficacy in 'extensive metabolizers' e.g. those without CYP2C19 loss-of-function alleles,[Wang, Zhao et al. 2016; Simon and Danchin 2017] we included pharmacogenetic studies with concurrent antiplatelet-HTPR data.

We **excluded** studies assessing platelet function *in vitro* or *ex vivo* 'platelet activation status', pharmacogenetic studies not linked to antiplatelet-HTPR testing, or reports in which it was unclear whether hemorrhagic stroke patients were included. Studies which did not prospectively collect data on the risk of recurrent vascular events or outcomes during follow-up **after *ex vivo* platelet reactivity/function testing** were excluded. We also excluded studies purely focused on subgroups of patients with moderate-severe symptomatic or asymptomatic carotid atherosclerotic stenosis because this is the subject of a separate systematic review in preparation. However, some included studies on CVD patients overall did of course incorporate data on some patients with recently symptomatic carotid stenosis.

The majority of studies assessing antiplatelet-HTPR in CVD used a 'cross-sectional, case-control' definition of HTPR where patients' results at a single time point were compared with those from a group of healthy controls or the manufacturer's normal range.(Lim, Coughlan et al. 2015) Prospective longitudinal studies which assessed alteration of platelet function/reactivity *ex vivo* in matched samples from individual CVD patients who were tested prior to and following commencement or

modification of their antiplatelet regimen are limited (Grau, Reiners et al. 2003; Raman and Jilma 2004; Serebruany, Malinin et al. 2004; Serebruany, Malinin et al. 2005; Serebruany, Steinhubl et al. 2005; Serebruany, Malinin et al. 2008; Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013). Our group has proposed the novel concept of 'longitudinal antiplatelet-HTPR status', which we defined as failure to alter a patient's platelet function/reactivity data compared with their own 'baseline value' before undergoing a change in antiplatelet therapy by more than twice the coefficient of variation of the assay (Tobin, Kinsella et al. 2013). Therefore, data pertaining to cross-sectional and longitudinal definitions of HTPR in CVD were analysed as both have the potential to be clinically informative (Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013).

Two independent reviewers (STL and SYL) screened the title and/or abstract of retrieved citations. Any discrepancies were resolved by consensus between reviewers. If the abstract suggested the article met our inclusion criteria, the full-text article was reviewed by these two reviewers who extracted the following data on pre-specified forms: authors; journal; year of publication; geographical location of the study; study design; inclusion and exclusion criteria; baseline clinical and demographic data; sample size; clinical indication for antiplatelet therapy; prescribed antiplatelet regimens and doses; type of platelet function test/platform utilised and definition of antiplatelet-HTPR employed; duration of follow-up; comparison between those with *vs*. those without antiplatelet-HTPR. Each manuscript was critically-appraised by the first and supervising author who finally adjudicated on any disagreements between reviewers. Available data on the **primary outcome** were collected: the composite risk of recurrent stroke, TIA, myocardial infarction or vascular death. **Secondary outcomes** were: (a) the risk of

having a recurrent ischemic stroke or TIA; (b) severe stroke (NIHSS score > 16) **or** disability/impairment (defined as a modified Rankin scale score [MRS] ≥ 3) during follow-up because antiplatelet-HTPR could potentially contribute to progressive ischemia or infarction following stroke onset.

It is important to note that because different inclusion and exclusion criteria were used for the comprehensive review outlined in chapter 3 and this systematic review and meta-analysis, we anticipated that there would be slight differences in the prevalence ranges of antiplatelet-HTPR quoted in the 2 chapters (see below).

4.4. Statistical Methodology

We used standard descriptive statistical methodology for the systematic review. VT coordinated the meta-analyses using the STATA/IC 15.1 statistical package. We calculated unadjusted odds ratios (ORs) for each individual study based on reported binary data. In studies requiring OR calculations from binary data which included arms with zero events, 0.5 was added to all cells. These outcomes were pooled on the log-scale using the DerSimonian-Laird random-effects model with an inverse-variance calculation method, and the pooled effect exponentiated with resulting ORs. A secondary analysis utilizing a fixed-effects model was performed to complement the random-effects approach. Heterogeneity was assessed using the I^2 statistic. To explore possible sources of heterogeneity, we undertook subgroup sensitivity analyses based on factors which might influence outcomes between patients with and without antiplatelet-HTPR: antiplatelet regimen (aspirin, clopidogrel, aspirin-clopidogrel, aspirin-dipyridamole combination therapy); aspirin dose (≤100 mg/day, >100 mg/day); geographical location/'likely ethnicity'

(arbitrarily defined as ‘Non-Asian’ versus ‘Asian’ study populations) based on Country of origin of respective studies. Furthermore, studies were categorized and analysed according to methods used to assess HTPR status, including ‘platelet aggregation under low shear stress’, ‘platelet adhesion and aggregation under high shear stress’ and ‘other methods’. We also performed ‘random-effects meta-regression analyses’ of data based on common variables, such as the effect of year of study publication, patient age and sex on the risk of subsequent stroke/TIA, MI or vascular death.

4.5. Quality Assessment and Risk of Bias

The Risk Of Bias In Non-randomized Studies-of Interventions (ROBINS-I) tool (Sterne, Hernan et al. 2016) was employed by two independent reviewers (STL and SYL) to assess quality and risk of bias of included studies. The ROBINS-I domain encompassing ‘deviation from interventions’ was not relevant to this review.

4.6. Results

The systematic database search identified 34 studies for our systematic review, 20 of which met inclusion criteria for both qualitative and quantitative synthesis in our meta-analysis (Figure 4.1). The meta-analysis was performed on pooled data from 4,989 patients (1507 patients with HTPR and 3482 without HTPR), 56.7% of whom were men, with a mean age of 65.8 years (standard deviation: 4.9 years). The mean duration of follow up was 14.9 months for all studies combined.

4.6.1. Systematic Review of the Prevalence of Antiplatelet-HTPR

Most studies used 'cross-sectional, case-control' definitions of antiplatelet-HTPR (Lim, Coughlan et al. 2015), and prevalence varied according to the device used, definition employed (Helgason, Tortorice et al. 1993; McCabe, Harrison et al. 2005) and doses of prescribed therapy (Meves, Overbeck et al. 2012; Lim, Coughlan et al. 2015). The prevalence of antiplatelet-HTPR across all studies ranged from 3-65% with aspirin, 8-56% with clopidogrel, and 1.8-35% with aspirin and clopidogrel in patients on dual therapy (Tables 4.1 and 4.2). In our systematic review, thirteen independent studies found a clear association ($P< 0.05$, Table 4.3), and eleven studies found no clear independent association between antiplatelet-HTPR status and the risk of recurrent vascular events (Table 4.4). Twelve studies revealed an association between antiplatelet-HTPR status and more severe strokes, early neurological deterioration or adverse outcomes ($P < 0.05$; Table 4.5). One study found a relationship between clopidogrel-HTPR status and CYP2C19*2 and CYP2C19*3 pharmacogenetic profiles, and revealed that clopidogrel-HTPR, but not CYP2C19 genotype, was associated with an increased risk of recurrent ischemic stroke/TIA, cardiac events or vascular death (Han, Lv et al. 2015). Four studies showed an association between CYP2C19 genotype and the risk of clopidogrel-HTPR (Jia, Chen et al. 2013; Han, Lv et al. 2015; Yi, Lin et al. 2016; Lin, Han et al. 2018). One study suggested that patients with a 'CYP2C19 extensive metaboliser genotype' were more likely to have good functional outcomes (MRS $\leq$2) at 3 and 6 months follow-up.(Jia, Chen et al. 2013)

4.6.2. Quality Assessment and Risk of bias

Fourteen of 20 included studies were considered to have a "very low risk" of bias, with 3 studies potentially influenced by "missing data" and 4 studies had some domains categorised as "sound for a non-randomized study but not comparable to a rigorous randomized trial". The overall risk of bias was deemed to be low in 18/20 (90%) included studies (Figure 4.11).

4.6.3. Meta-analysis of Data on HTPR Status and Risk of Composite Vascular Outcomes

There was an increased risk of the composite outcome of recurrent stroke/TIA, myocardial infarction or vascular death in patients with *vs*. those without antiplatelet-HTPR on any antiplatelet regimen (OR=2.93, 95%CI: 1.90-4.51 (Figure 4.2), with high heterogeneity between studies (I^2 = 80.7%, p<0.001). Subgroup analysis by antiplatelet regimen demonstrated a significant risk of the composite outcome in patients with *vs*. those without aspirin-HTPR (OR=3.13, 95%CI: 1.77-5.56) and dual antiplatelet-HTPR (OR=3.14, 95% CI: 1.86-5.31) (Figure 4.3). Patients with clopidogrel-HTPR did not have a significantly higher risk of the composite outcome, but the number of included studies was very small (OR=1.98, 95%CI: 0.89-4.4). There was high heterogeneity between aspirin studies (I^2 = 84%, p<0.001), moderate heterogeneity between clopidogrel studies (I^2 = 67.3%, p=0.047), and low heterogeneity between dual antiplatelet studies (I^2 = 0%, p=0.78). Separate subgroup analysis of outcomes in studies on lower dose aspirin (≤ 100mg daily) and higher dose aspirin (>100 mg daily) revealed that there was an increased risk of the composite outcome in patients with *vs*. those without aspirin-

HTPR on lower dose aspirin (OR=3.38, 95% CI: 1.77-6.45), but not on higher dose aspirin (OR=2.24, 95% CI: 0.46-10.99) (Figure 4.4). Antiplatelet-HTPR on any regimen was associated with a higher risk of the composite outcome in 'Asian' and 'Non-Asian' populations, with no difference between these subgroups (Figure 4.5). However, there was high heterogeneity in each subgroup. Studies employing the principle of platelet aggregometry (e.g. conventional aggregometry, VerifyNow and Multiplate) showed a higher risk of composite outcomes in those with *vs*. those without antiplatelet-HTPR (OR=3.27, 95%CI: 2.02-5.31; Figure 4.12). Studies which assessed platelet adhesion/aggregation under high shear stress (PFA-100) did not show a significant association between HTPR status and risk of the subsequent composite outcome, but the number of subjects included in this analysis was limited (N=521 in total; Figure 4.12).

4.6.4. Meta-analysis of Data on HTPR Status and Risk of Recurrent Ischemic stroke/TIA

Meta-analysis of studies which included data on risk of recurrent ischemic stroke/TIA showed a significantly higher risk of ischemic stroke/TIA recurrence in patients with *vs*. those without antiplatelet-HTPR on any antiplatelet regimen (OR=2.43, 95%CI: 1.51-3.91) (Figure 4.6). However, there was considerable heterogeneity between studies (I^2 = 81.6%, p < 0.001). Analysis of specific antiplatelet regimens showed that aspirin-HTPR (OR=2.26, 95%CI: 1.19-4.30) and dual antiplatelet-HTPR (OR=4.78, 95%CI: 2.65-8.62) were associated with a higher risk of recurrent ischemic stroke/TIA. However, there was no significant association between clopidogrel-HTPR and risk of subsequent ischemic stroke/TIA (OR=1.98,

95%CI: 0.89-4.42). There was high heterogeneity between studies assessing aspirin-HTPR (I^2 = 84.9%, p < 0.001), moderate heterogeneity between clopidogrel-HTPR studies (I^2 = 66.1%, p=0.05) and low heterogeneity between studies of dual antiplatelet therapy (I^2 = 0%, p=0.38) (Figure 4.7). Aspirin-HTPR was associated with an increased risk of subsequent ischemic stroke/TIA in patients on lower dose aspirin (OR=2.70, 95%CI: 1.30-5.59), but not in those on higher dose aspirin (Figure 4.8). There was high heterogeneity between studies of lower dose aspirin (I^2 = 88.1%, p < 0.001), but low heterogeneity in studies with higher dose aspirin (I^2 = 0%, p=0.99). There was a higher risk of subsequent ischemic stroke/TIA in patients with antiplatelet-HTPR in the 'Asian' subgroup' (OR=2.48, 95%CI: 1.56-3.93), but the differences were not significant in the 'Non-Asian' subgroup (OR=2.02, 95%CI: 0.62-6.61), with high heterogeneity in each subgroup (Asian: I^2 =77.2%; Non-Asian I^2: 80.1%; P < 0.001) (Figure 4.9).

4.6.5. Meta-analysis of data on the relationship between HTPR status and stroke severity or disability during follow up

The risk of having a severe stroke during follow up (NIHSS >16) was higher in those with *vs*. those without HTPR (OR=2.65, 95%CI: 1.00-7.01) (Figure 4.10), but there were insufficient data to comment on MRS outcomes in those with *vs*. those without HTPR. There was moderate heterogeneity between studies (I^2 = 63.2, P=0.099).

4.6.6. Meta-regression Analysis

Meta-regression analysis revealed that the only common variable which influenced the relationship between antiplatelet-HTPR status and the risk of the composite outcome was the 'year of publication', with older studies more likely to reveal a significant relationship between antiplatelet-HTPR status and outcomes **(P=0.01)**. However, *post-hoc* analysis after excluding data from Grotemeyer *et al* (1993), to focus on the era of more modern secondary preventive therapy from 2005 onwards, negated the impact of year of publication on our results (P=0.7). Importantly, the higher risk of composite outcomes with antiplatelet-HTPR overall, or aspirin-HTPR in particular, persisted after *post-hoc* meta-analyses which excluded Grotemeyer's data (Figures 4.13 and 4.14).

4.7. Discussion

Our comprehensive systematic review revealed a wide range of prevalence of antiplatelet-HTPR of 3-65% with aspirin, 8-56% with clopidogrel, 1.8-35% with either aspirin or clopidogrel in patients on aspirin-clopidogrel combination therapy, and 56-59% with dipyridamole when added to aspirin in the early, subacute or late phases TIA/ischemic stroke (Lim, Coughlan et al. 2015). The highest absolute prevalence of aspirin-HTPR was observed in a cross-sectional study assessing platelet adhesion/aggregation with the PFA-100 under moderately-high shear stress(Alberts, Bergman et al. 2004); the highest prevalence of clopidogrel-HTPR was noted with a modified aggregometry paradigm (VerifyNow)(Jover, Rodriguez

et al. 2014) (Table 4.1). Cross-sectional definitions may underestimate effects of antiplatelet therapy in individuals compared with novel longitudinal definitions (Lim, Coughlan et al. 2015)·(Tobin, Kinsella et al. 2013), but most data in this review were derived from studies employing traditional cross-sectional definitions. Variability in HTPR prevalence rates between studies and devices emphasises the importance of ideally assessing platelet reactivity with more than one device in future studies to clarify which testing platforms are most likely to predict outcomes.

Our meta-analysis indicates that CVD patients with antiplatelet-HTPR on any regimen, based on HTPR definitions employed in individual studies, had 2-3 times the risk of experiencing the composite outcome or recurrent ischemic cerebrovascular events during prospective follow-up compared with patients without antiplatelet-HTPR. These findings also applied to patients on aspirin monotherapy, but there was high heterogeneity between studies. Our outcome data on aspirin are broadly in keeping with a prior systematic review and meta-analysis which reported an increase in the relative risk of recurrent ischemic stroke/TIA in patients with *vs*. those without aspirin-HTPR (Fiolaki, Katsanos et al. 2017). However, not all recurrent vascular events were recorded during prospective follow-up **after** HTPR testing, and the value of HTPR status in predicting risk of the composite outcome was not analysed in that study (Fiolaki, Katsanos et al. 2017). In contrast to the findings by Fiolaki *et al* (Fiolaki, Katsanos et al. 2017), the presence of clopidogrel-HTPR did not significantly predict the risk of composite vascular or ischemic cerebrovascular outcomes using different studies in our meta-analysis. However, our clopidogrel-HTPR findings should be interpreted with caution because they might reflect a type II error due to the lower number of patients on

clopidogrel with prospective data available for analysis, and there was moderate heterogeneity between studies. Larger prospective studies assessing the value of clopidogrel-HTPR at predicting outcomes after TIA/ischemic stroke are warranted.

Pre-planned subgroup analyses revealed that the relationship between aspirin-HTPR and risk of recurrent vascular outcomes overall or cerebrovascular outcomes pertained to studies on 'lower dose' (≤100 mg/day) but not 'higher dose' aspirin (>100mg/day). This highlights the need to conduct adequately-powered, multi-center studies assessing the predictive value of aspirin-HTPR status in CVD patients in routine clinical practice on a range of aspirin doses. Most patients included in this review received lower dose aspirin, in keeping with recommendations from meta-analysis of clinical trials (Antithrombotic Trialists 2002). However, because a minority of studies assessed patients on higher dose aspirin, one should not conclude that assessment of aspirin-HTPR status is not warranted in patients on higher dose aspirin because the 95% CIs of the analyses of data from lower and higher dose aspirin overlapped.

Our data indicate that the potential value of antiplatelet-HTPR status in predicting the risk of recurrent composite vascular outcomes applies to both 'Asian' and 'non-Asian' study populations. However, the absence of information on the precise ethnic background of study patients is a limitation of this review and precludes further comment. HTPR data were predictive of the composite outcome in studies using aggregometry or modified aggregometry techniques, but not in studies assessing platelet adhesion or aggregation at high shear stress. It must be acknowledged that the limited number of outcome data from prospective studies

with the PFA-100 could also have led to a type II error, and thus warrants further study.

An emerging body of evidence indicates that the presence of aspirin-HTPR in particular might have an adverse effect on baseline stroke severity, early deterioration, poorer functional outcome or higher mortality during follow-up (Table 4.5) (Englyst, Horsfield et al. 2008; Schwammenthal, Tsabari et al. 2008; Bugnicourt, Roussel et al. 2011; Ozben, Ozben et al. 2011; Lai, Chen et al. 2012; Zheng, Churilov et al. 2013; Coignion, Poli et al. 2015; Kim, Heo et al. 2015; Oh, Yu et al. 2016). These studies do not prove that antiplatelet-HTPR is directly responsible for the pathogenesis of more severe strokes at presentation or poorer outcomes during follow-up because the dose and duration of therapy varied between studies, but might partly reflect 'secondary platelet hyper-reactivity' following larger strokes which is not inhibited by prescribed doses of aspirin or clopidogrel. We identified an increase in the risk of having a persistently severe stroke during follow-up in those with *vs*. those without antiplatelet-HTPR. However, there were insufficient data to perform separate subgroup analyses on aspirin or clopidogrel, respectively due to the limited number of studies in which these dichotomous NIHSS outcome data were included. Future meta-analyses should explore the impact of antiplatelet-HTPR on dynamic NIHSS changes post-stroke. These preliminary findings are informative and indicate that assessment of the combined risk of recurrent vascular events **or** 'adverse functional outcomes' could enhance the statistical power of future studies.

Our study had some **limitations**. The meta-analysis was limited by fundamental constraints inherent in some studies, e.g. small sample size and the discernible clinical heterogeneity between individual studies clearly outlined above, and we did not conduct an individual patient data meta-analysis. There were differences in definitions of HTPR and timing of measurement of HTPR status following index TIA/stroke between studies. Further analyses are warranted to determine whether time from symptom onset influences results because prior studies have shown a higher absolute prevalence of aspirin-HTPR in the early *vs*. late phase after TIA/ischemic stroke (McCabe, Harrison et al. 2005). We could not entirely control for the possibility of positive publication bias if groups did not publish findings if their data were not predictive of recurrent events/outcomes. However, this comprehensive review included a wide range of positive and negative predictive studies, including those not powered or designed to predict outcomes, thus minimising selection bias in our own systematic review process. In addition, assessment of studies with the Robins-I tool revealed that the majority (90%) had a low risk of bias.

4.8. Conclusion

In conclusion, monitoring antiplatelet-HTPR status with platelet function/reactivity assays, in combination with pharmacogenetic testing (Lim, Coughlan et al. 2015), clearly has the potential to predict the risk of recurrent vascular events and functional outcomes in CVD patients on commonly-prescribed antiplatelet regimens, especially in those on aspirin. Given the moderate-high heterogeneity

between studies, further prospective, adequately-powered, multi-center studies in diverse geographical populations are urgently needed to address this issue. Such information would facilitate progression to a definitive interventional trial to determine whether altering therapy in individual patients with antiplatelet-HTPR reduces the risk of recurrent vascular events, adverse functional outcomes or improves survival following TIA/ischemic stroke. However, the current evidence-base does not yet support routine alteration of antiplatelet therapy in clinical practice based on *ex vivo* antiplatelet-HTPR testing outside of a research study or clinical trial.

Figure 4. 1 Flow chart summarising search strategy used in line with PRISMA statement

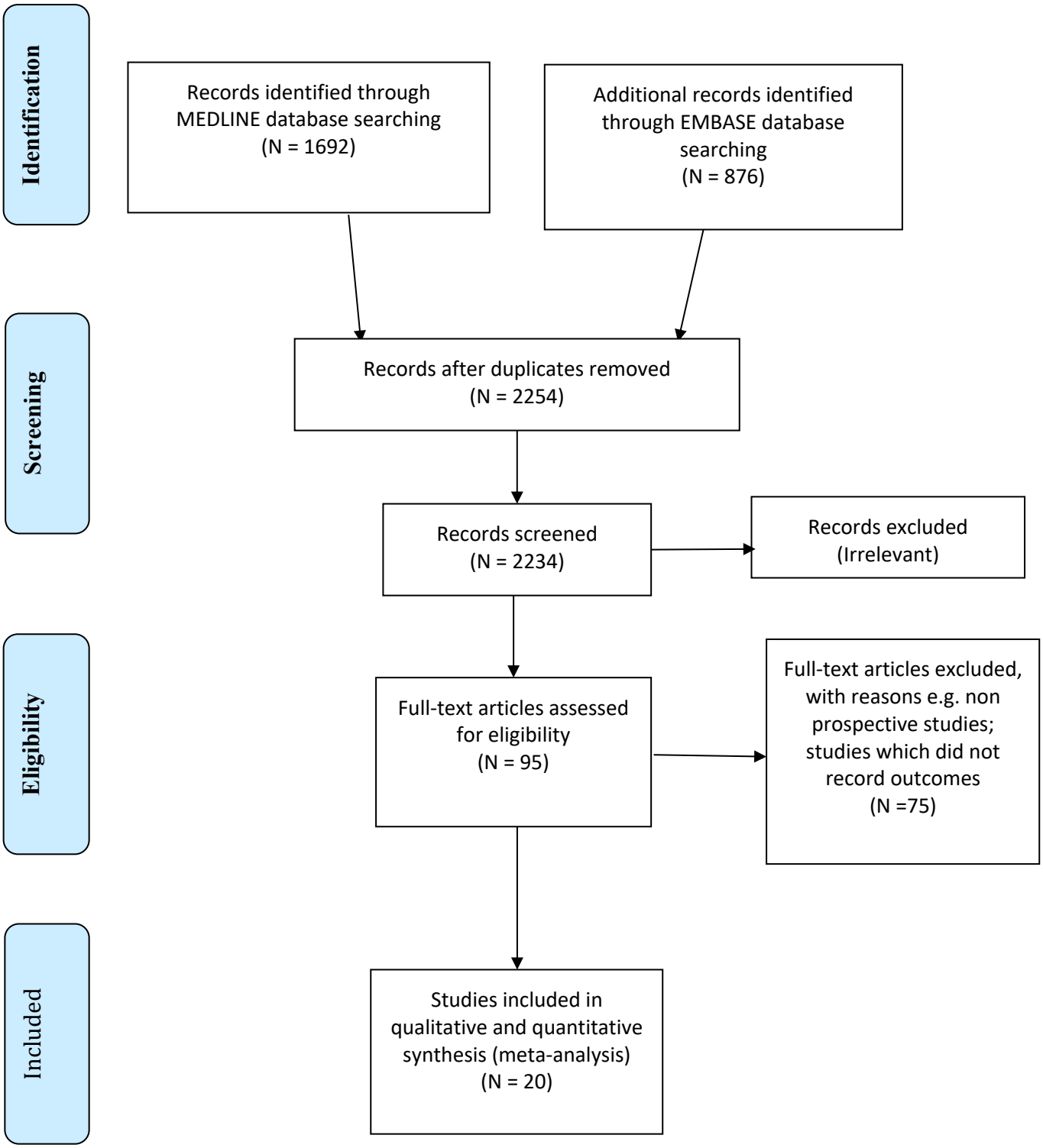


Figure 4. 2 Combined analysis of the composite risk of subsequent ischemic stroke/TIA, myocardial infarction or vascular-related death in those with vs. those without antiplatelet-HTPR on any antiplatelet regimen

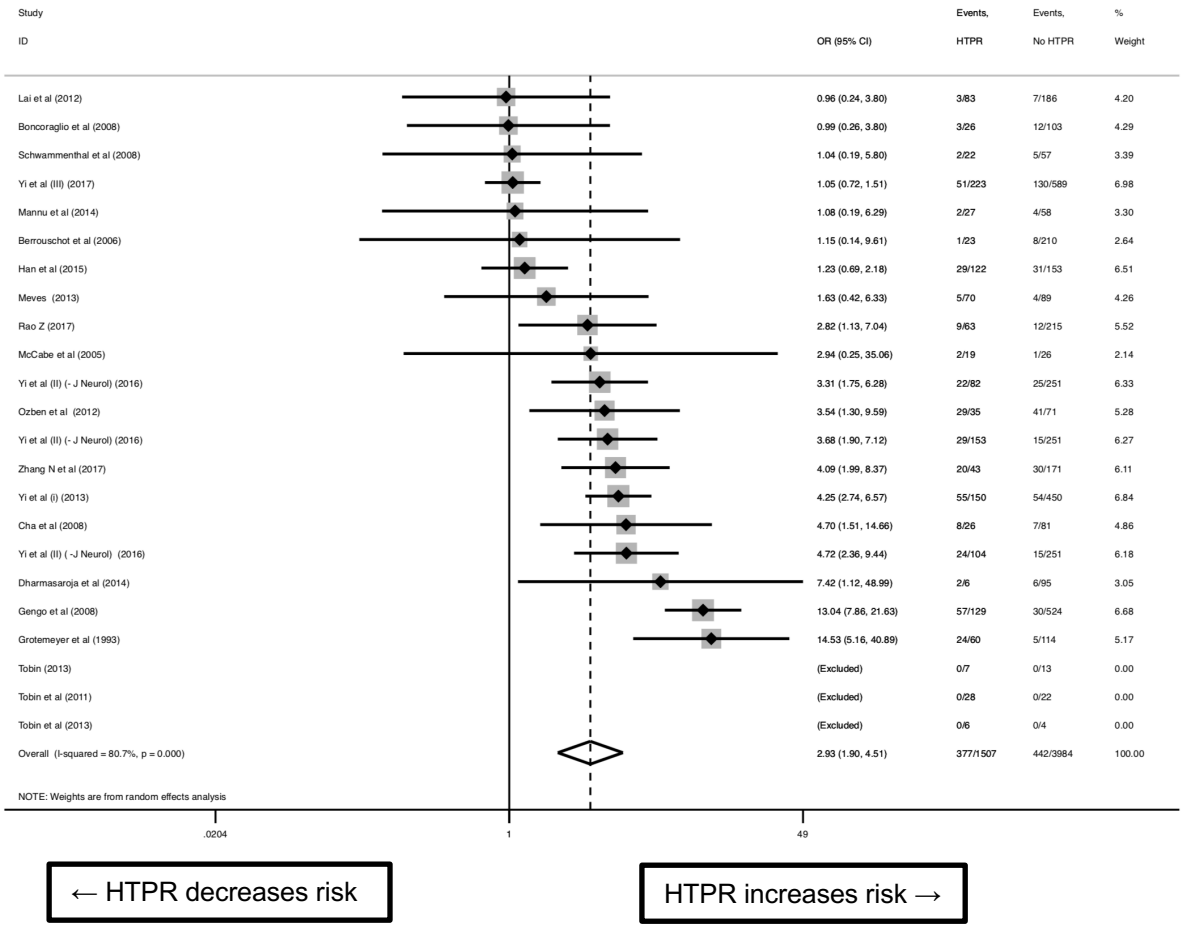


Figure 4. 3 Subgroup analysis based on antiplatelet regimen on the composite risk of subsequent ischemic stroke/TIA, myocardial infarction or vascular death in those with vs. those without HTPR on aspirin, clopidogrel or dual antiplatelet therapy

(Tobin *et al*. 2011 study included patients changing from aspirin to aspirin-dipyridamole combination therapy, but no outcome events were observed during follow-up. All other 'dual antiplatelet therapy' studies were on aspirin-clopidogrel)

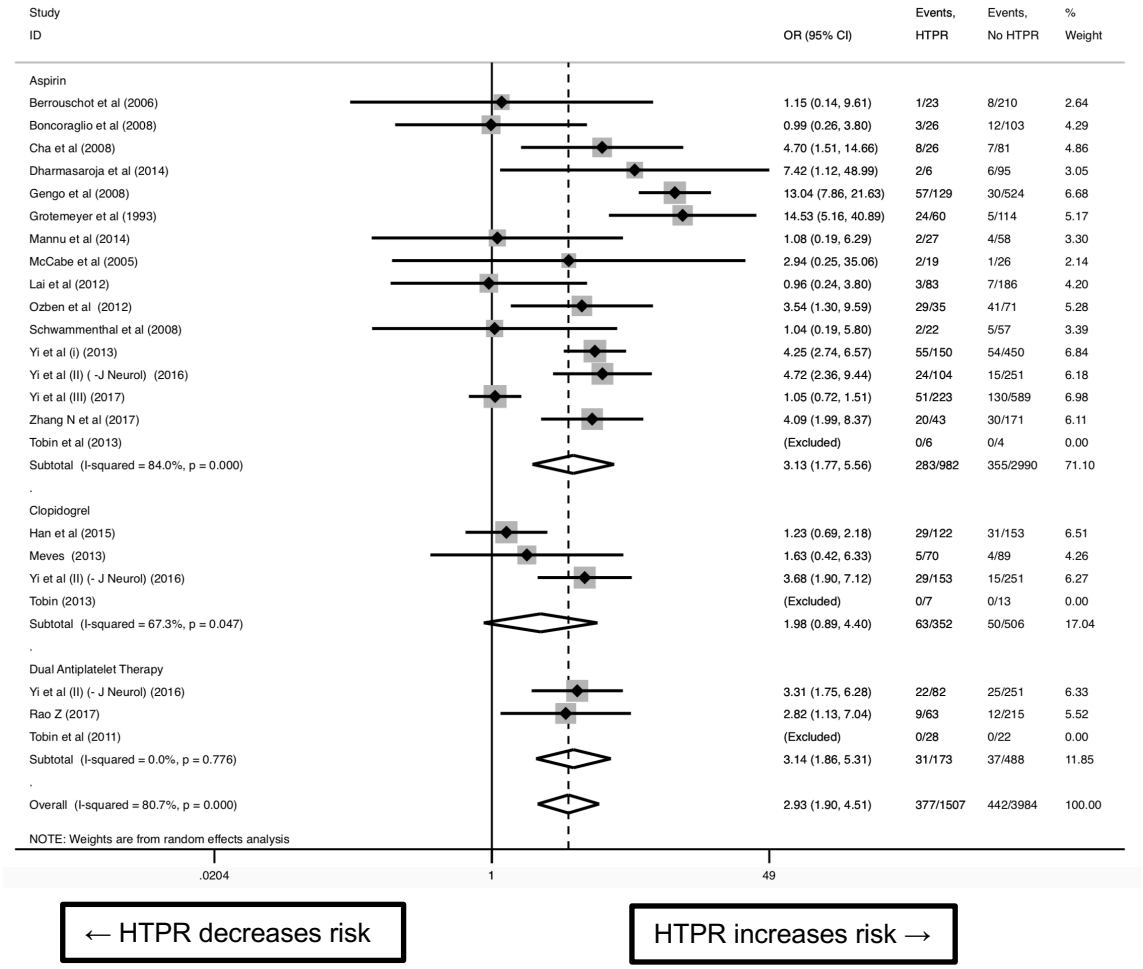


Figure 4. 4 Subgroup analysis based on aspirin dose on the composite risk of subsequent ischemic stroke/TIA, MI or vascular death in those with vs. those without aspirin-HTPR on 'Lower dose' (≤ 100 mg daily) and 'Higher dose' (> 100 mg daily) aspirin.

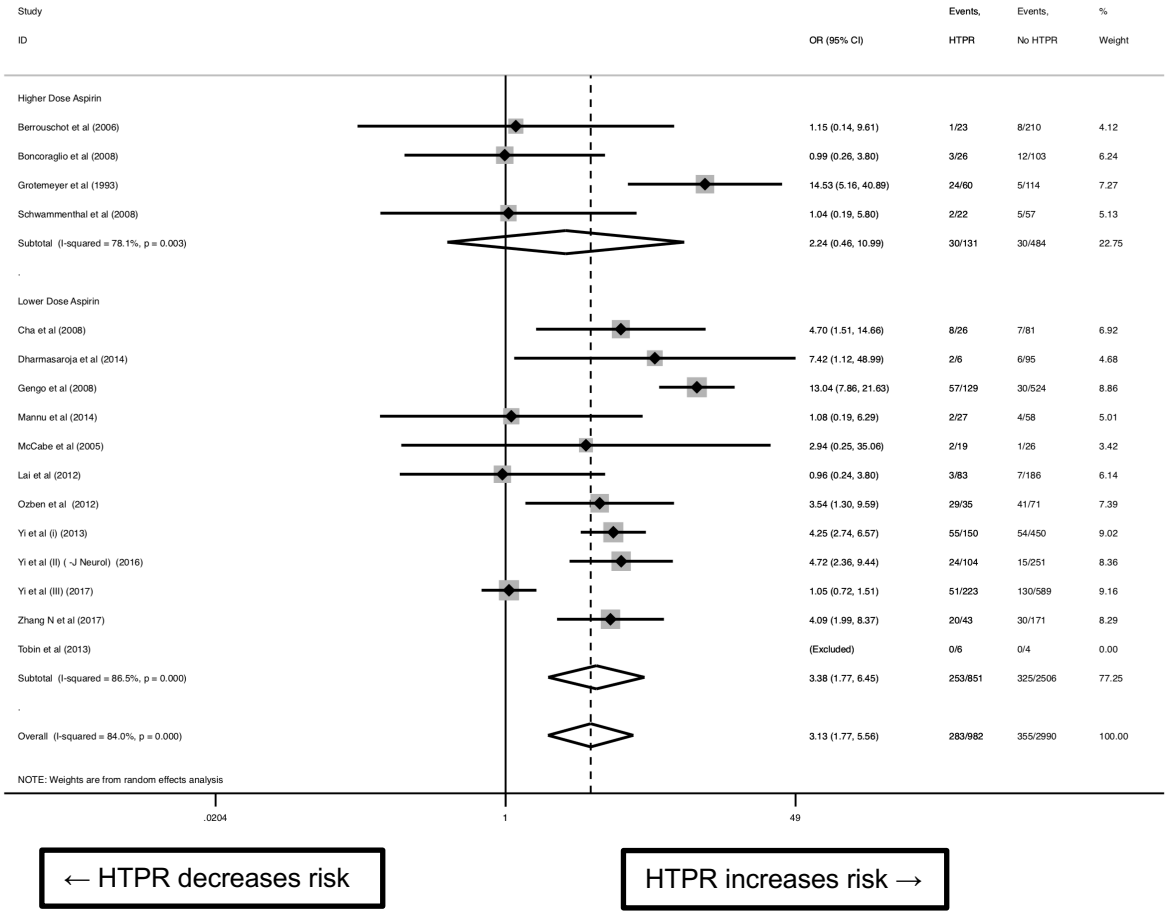


Figure 4. 5 Subgroup analysis based on geographical study location on the composite risk of subsequent ischemic stroke/TIA, myocardial infarction or vascular death in those with vs. those without antiplatelet-HTPR

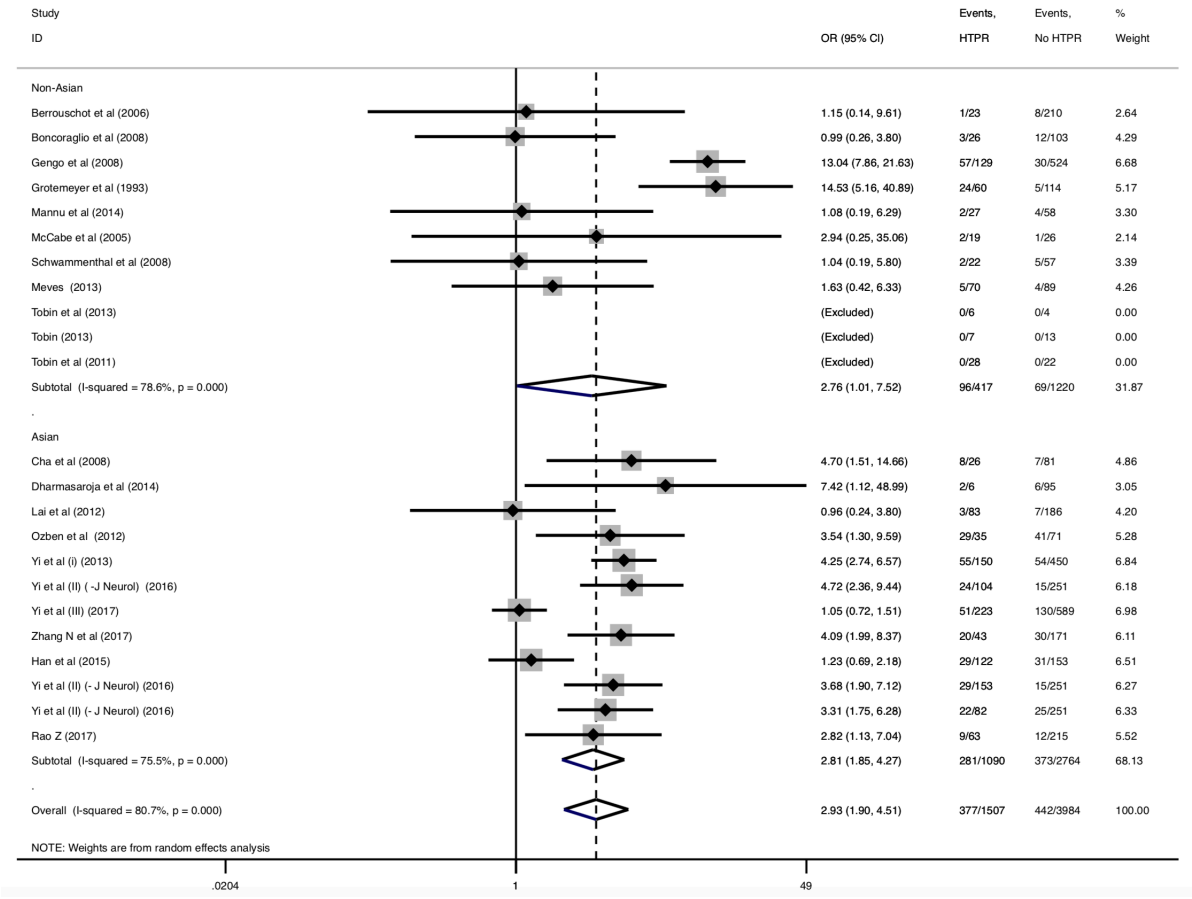


Figure 4. 6 Combined analysis of the risk of recurrent ischemic stroke/TIA in those with vs. those without antiplatelet-HTPR on any antiplatelet regimen (aspirin, clopidogrel or dual antiplatelet therapy) during follow-up

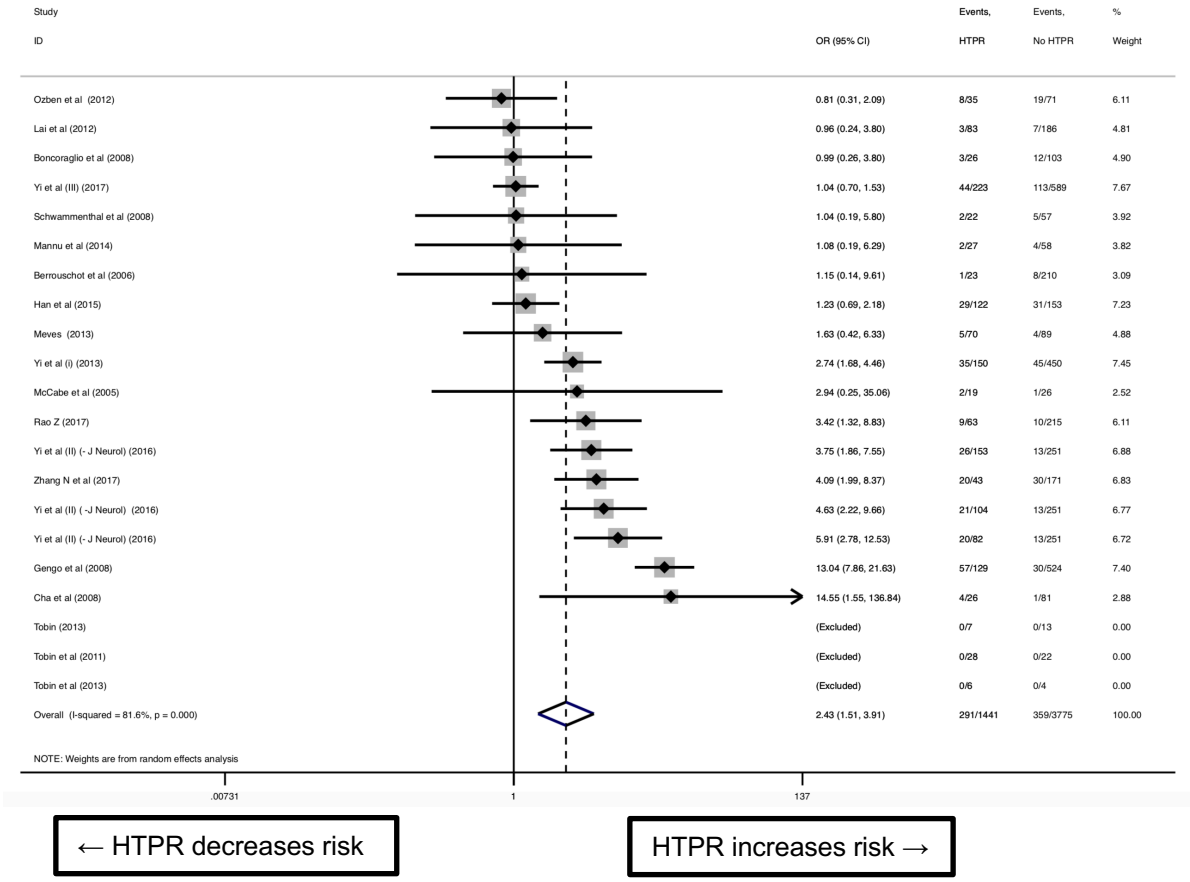


Figure 4. 7 Subgroup analysis of the risk of recurrent ischemic stroke/TIA in those with vs. those without HTPR on various antiplatelet regimens (Aspirin, Clopidogrel, Dual Antiplatelet therapy)

(Tobin *et al*. 2011 study included patients changing from aspirin to aspirin-dipyridamole combination therapy, but no outcome events were observed during follow-up. All other 'dual antiplatelet therapy' studies were on aspirin-clopidogrel)

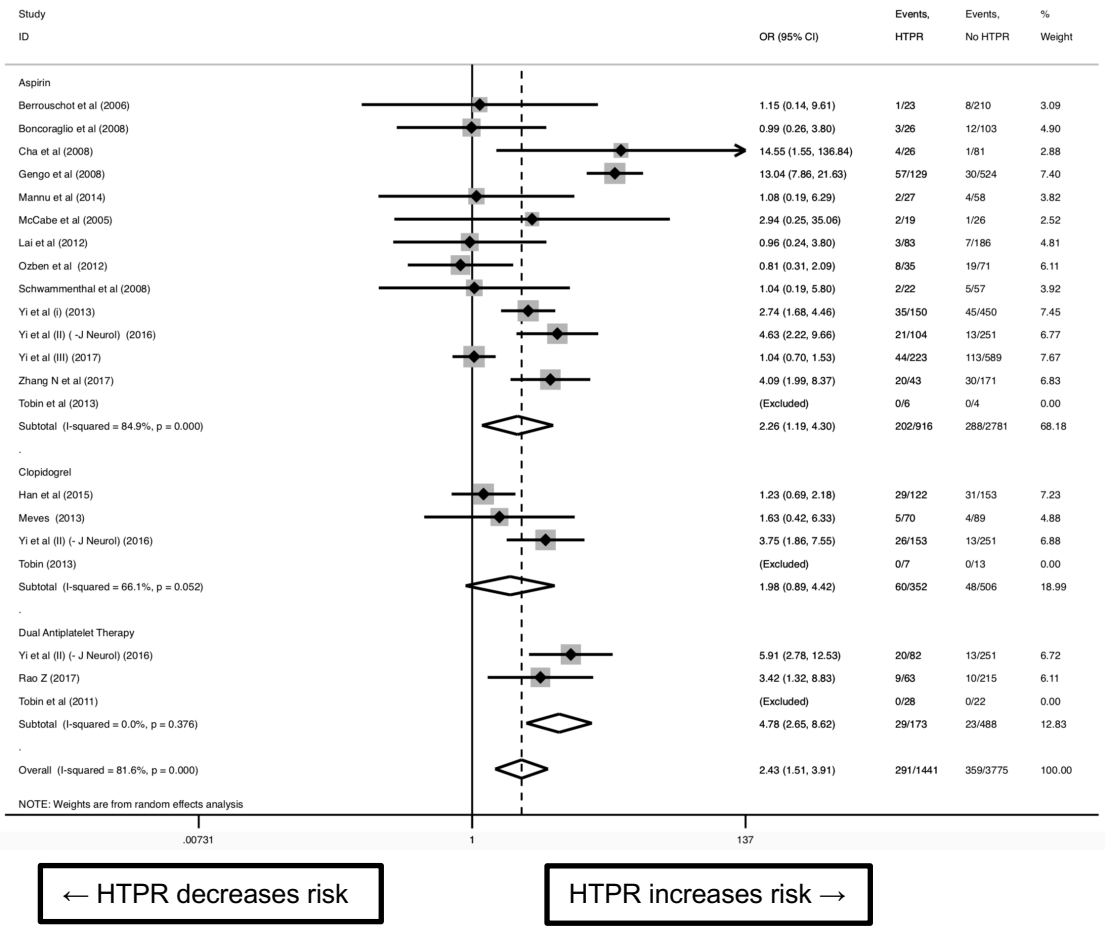


Figure 4. 8 Subgroup analysis on the risk of recurrent ischemic stroke/TIA in those with vs. those without aspirin-HTPR on 'Lower dose' (≤ 100 mg daily) and 'Higher dose' (> 100 mg daily) aspirin

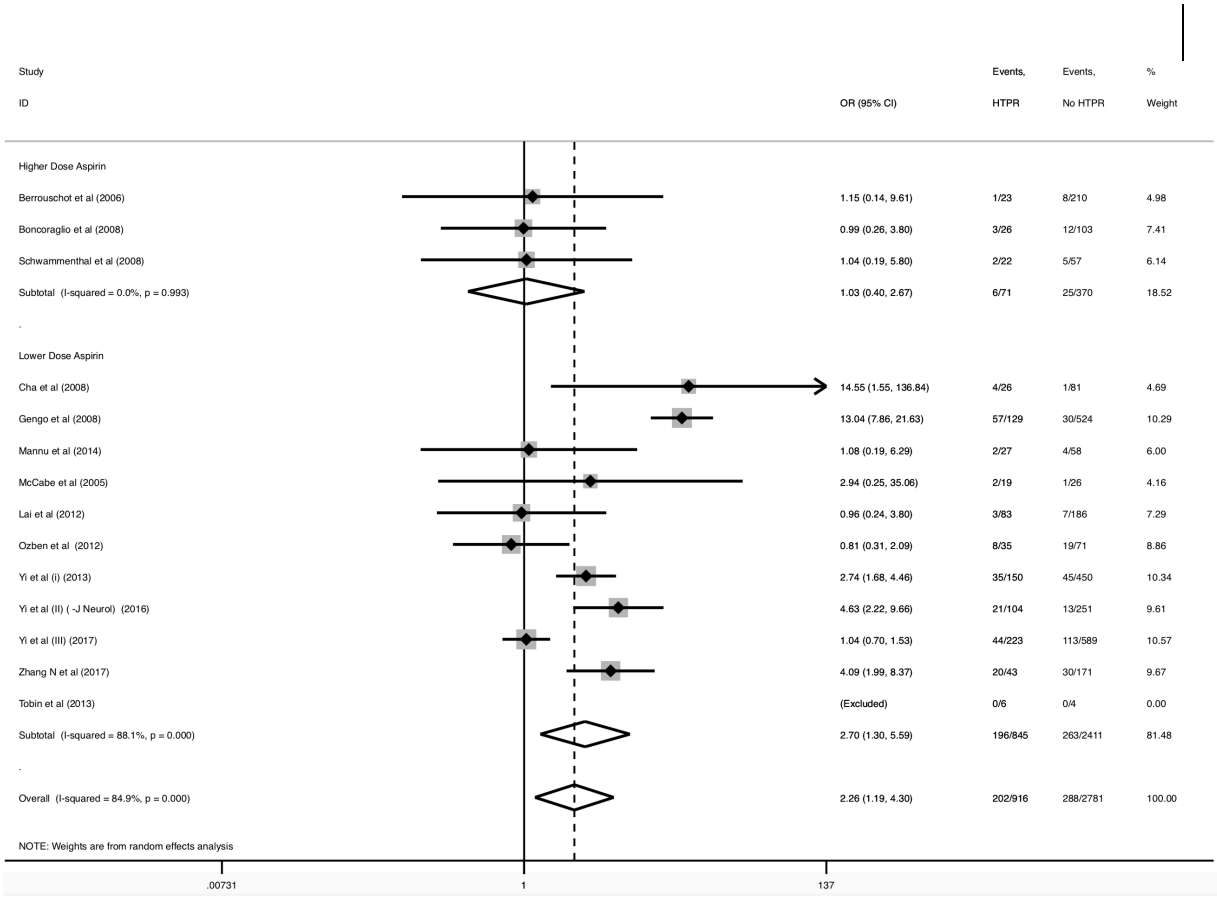


Figure 4. 9 Subgroup analysis of studies based on 'geographical location' on the risk of subsequent ischemic stroke/TIA in those with vs. those without antiplatelet-HTPR on aspirin, clopidogrel or dual antiplatelet therapy during follow-up

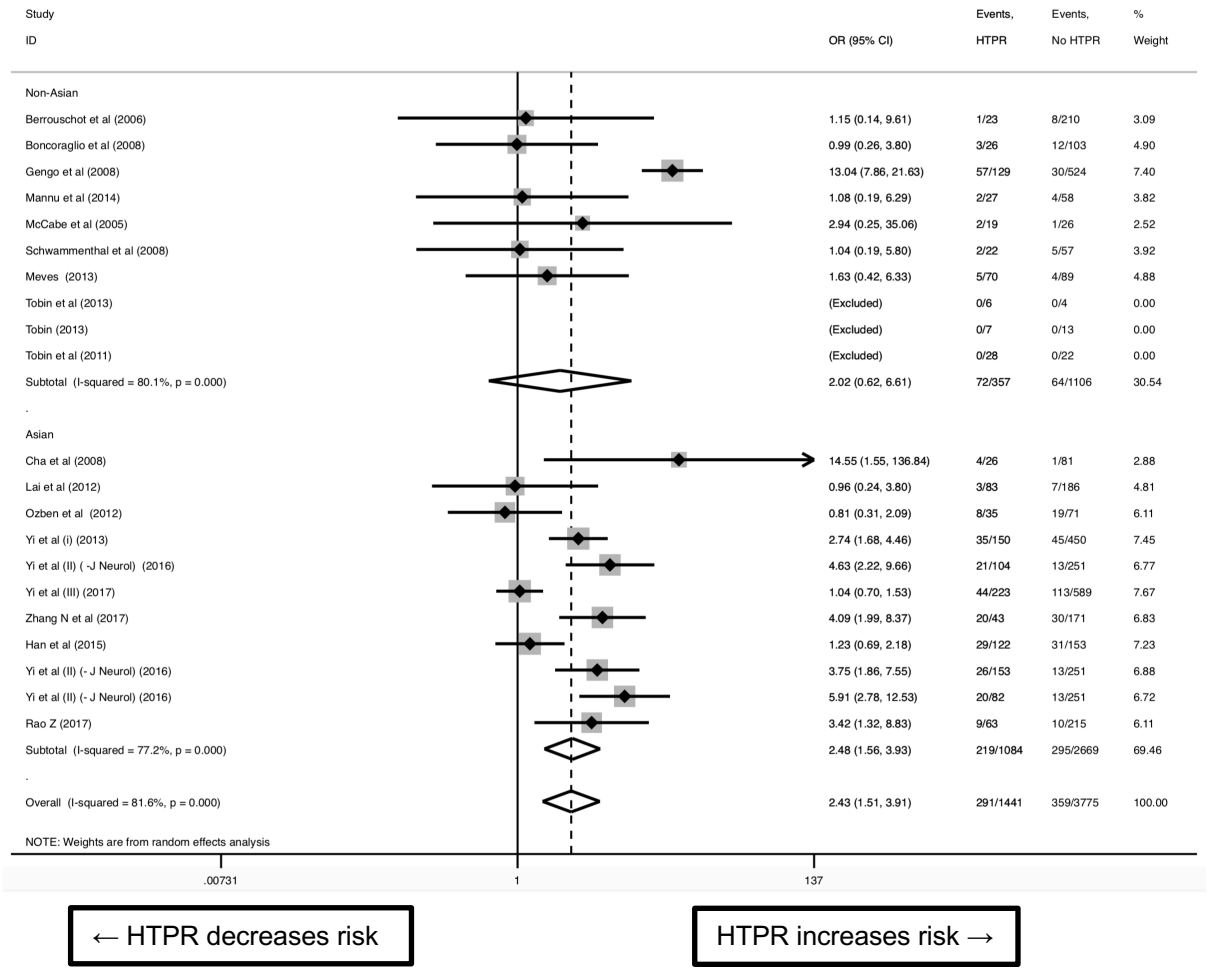


Figure 4. 10 Analysis of the odds of having a severe disabling stroke (defined as having an NIHSS > 16) in those with vs. those without antiplatelet-HTPR on aspirin or clopidogrel

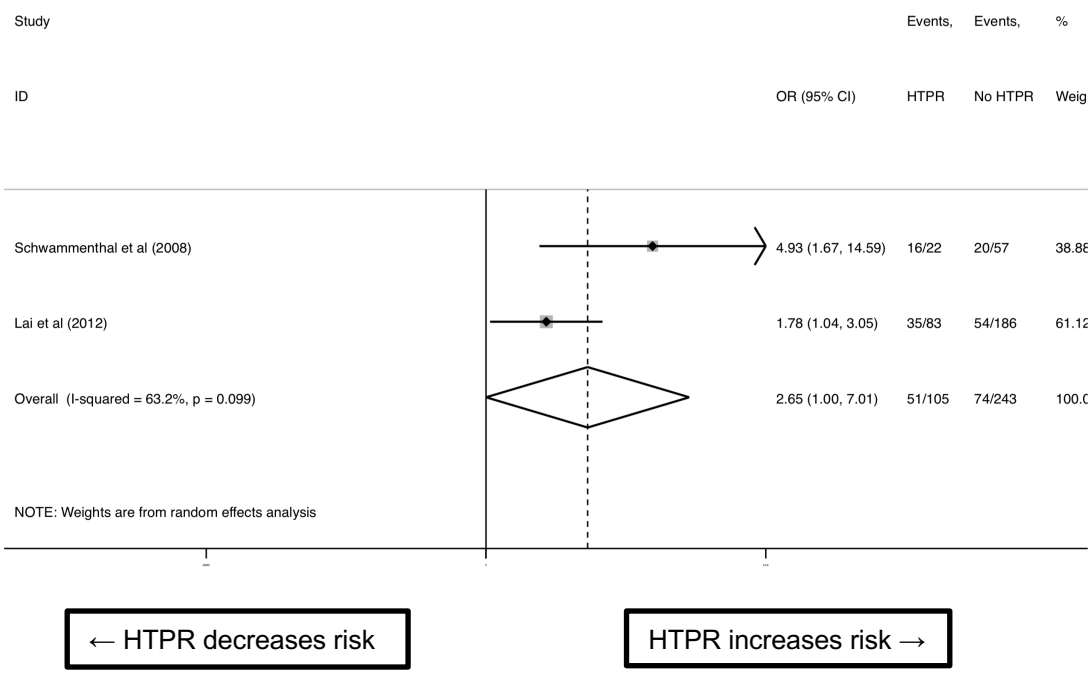


Table 4. 1 Prevalence of Antiplatelet-HTPR using 'cross-sectional' definitions

Testing Platform	Principle Employed	Aspirin 75-1300 mg/d	Clopidogrel 75-150 mg/d	Dual Antiplatelet Therapy
Optical Aggregometry	Aggregation and change in light transmission in response to agonists at low shear stress	3-45% (Helgason, Tortorice et al. 1993; Cha, Jeon et al. 2008; Halawani, Williams et al. 2011; Depta, Fowler et al. 2012; Lim, Coughlan et al. 2015)	28-50% (Fong, Cheng-Ching et al. 2011; Depta, Fowler et al. 2012; Lim, Coughlan et al. 2015; Lin, Han et al. 2018)	9.3% HTPR to both Aspirin and Clopidogrel on a combination of Aspirin 81mg daily and Clopidogrel 75mg daily (Fong, Cheng-Ching et al. 2011) 19.2% HTPR to both Aspirin and Clopidogrel on a combination of Aspirin 200mg daily for 14 days followed by 100mg daily, and Clopidogrel 75 mg daily (Yi, Wang et al. 2016)
Impedance Aggregometry	Aggregation and change in impedance in response to agonists at low shear stress	14-36% (Berrouschot, Schwetlick et al. 2006; Gengo, Rainka et al. 2008; Meves, Overbeck et al. 2012; Lim, Coughlan et al. 2015)	8-46% (Fukuoka, Furuya et al. 2011; Meves, Overbeck et al. 2012; Lim, Coughlan et al. 2015)	N/A
PFA-100	Moderately high shear stress-induced platelet adhesion/aggregation	15-65% (Grau, Reiners et al. 2003; Grundmann, Jaschonek et al. 2003; Alberts, Bergman et al. 2004; Harrison, Segal et al. 2005; McCabe, Harrison et al. 2005; Boncoraglio, Bodini et al. 2009; Godeneche, Sorel et al. 2009; Lai, Chen et al. 2012; Tobin, Kinsella et al.	35-41% (Tobin, Kinsella et al. 2013; Jover, Rodriguez et al. 2014)	N/A

		2013; Freitas-Silva, Goncalves et al. 2015; Derle, Ocal et al. 2016)		
VerifyNow	Modified optical platelet aggregation paradigm in response to agonists at low shear stress	6-33% (Bennett, Yan et al. 2008; Seok, Joo et al. 2008; Jeon, Song et al. 2010; Ozben, Ozben et al. 2011; Kinsella, Tobin et al. 2013; Sternberg, Ching et al. 2013; Zheng, Churilov et al. 2013; Agayeva, Gungor et al. 2014; Dharmasaroja and Sae-Lim 2014; Kim, Heo et al. 2015; Oh, Yu et al. 2016; Cheng, Xie et al. 2017; Wang, Chen et al. 2019)	21-56% (Maruyama, Takeda et al. 2011; Bagoly, Sarkady et al. 2013; Kinsella, Tobin et al. 2013; Nordeen, Patel et al. 2013; Sternberg, Ching et al. 2013; Jover, Rodriguez et al. 2014; Han, Lv et al. 2015; Rath, Jorgensen et al. 2018; Rath, Rye Jorgensen et al. 2018; Wang, Chen et al. 2019)	29.7% HTPR in the Clopidogrel 75mg daily + Aspirin 100mg daily group (Wang, Chen et al. 2019) 12.5% HTPR in the Ticagelor 90 mg twice daily and Aspirin 100mg daily group (Wang, Chen et al. 2019)
Multiplate	Platelet impedance aggregometry in response to agonists at low shear stress	9.1-42% (Yoo NT 2012; Azmin S 2013; Jastrzebska, Chelstowski et al. 2013; Łabuz-Roszak B 2015; Mannu, Macartney et al. 2015; Rosafio, Lelli et al. 2017)	22-40% (Meves, Schroder et al. 2014; Lundstrom, Wallen et al. 2015; Rosafio, Lelli et al. 2017)	34.5% HTPR to both Aspirin and Clopidogrel on a combination of Aspirin 100mg daily and Clopidogrel 75 mg daily (Rosafio, Lelli et al. 2017)
Thrombo-elastography (TEG®)	Monitoring of rate and quality of overall clot formation.	3.6% (Rao, Zheng et al. 2017)	17.3% (Rao, Zheng et al. 2017)	1.8% (Rao, Zheng et al. 2017)

Table 4. 2 Prevalence of antiplatelet-HTPR on the PFA-100® using cross-sectional and longitudinal definitions

(Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013)

Testing Platform	Aspirin 75-300 mg/day	Clopidogrel 75 mg/day	Dipyridamole MR 200mg BD
PFA-100 (Cross-sectional)	15-65% (C-EPI) (Grau, Reiners et al. 2003; Grundmann, Jaschonek et al. 2003; Alberts, Bergman et al. 2004; Harrison, Segal et al. 2005; McCabe, Harrison et al. 2005; Boncoraglio, Bodini et al. 2009; Godeneche, Sorel et al. 2009; Lai, Chen et al. 2012; Tobin, Kinsella et al. 2013; Derle, Ocal et al. 2016)	33-39% (INNOVANCE (P2Y) (Tobin, Kinsella et al. 2013; Jover, Rodriguez et al. 2014)	N/A
PFA-100 (Longitudinal)	18-24% (C-EPI) (Tobin, Kinsella et al. 2013)	35-41% (C-ADP) (Tobin, Kinsella et al. 2013)	56-59% (C-ADP)(Tobin, Kinsella et al. 2011)

Figure 4. 11 ROBINS-I risk of bias assessment

(Sterne, Hernan et al. 2016)

Authors	Confounding	Selection	Measurement of Intervention	Missing Data	Measurement of Outcomes	Reported Results	Overall
Berrouschot *et al*. 2006							
Boncoraglio *et al*. 2008							
Cha *et al*. 2008							
Dharmasaroja *et al*. 2014							
Gengo *et al*. 2008							
Grotemeyer *et al*. 1993				?			
Mannu *et al*. 2014							
McCabe *et al*. 2005							
Lai *et al*. 2012							
Ozben *et al*. 2012							
Schwammenthal *et al*. 2008							
Tobin *et al*. 2011							
Tobin *et al*. 2013							
Yi *et al*. (I) 2013							
Yi *et al*. (II) 2016							
Yi *et al*. (III) 2017							
Zhang N *et al*. 2017				?			
Han *et al*. 2015							
Meves *et al*. 2013				?			
Rao et al. 2017							

 "Comparable to a well-performed randomized trial"

 "Sound for a non-randomized study", but not comparable to a rigorous randomized trial"

 Presence of "important problems"

 "Too problematic to provide any useful evidence on the effects of intervention"

? **No Information**: Insufficient information to determine risk of bias

Overall risk of bias: Equal to the most severe level of bias found in any domain

Figure 4. 12 Subgroup analysis based on the methodology of HTPR measurement on the composite risk of subsequent ischemic stroke/TIA, myocardial infarction or vascular death in those with vs. those without antiplatelet-HTPR

('Platelet adhesion' refers to assessment of adhesion/aggregation with the PFA-100 under moderately high shear stress conditions)

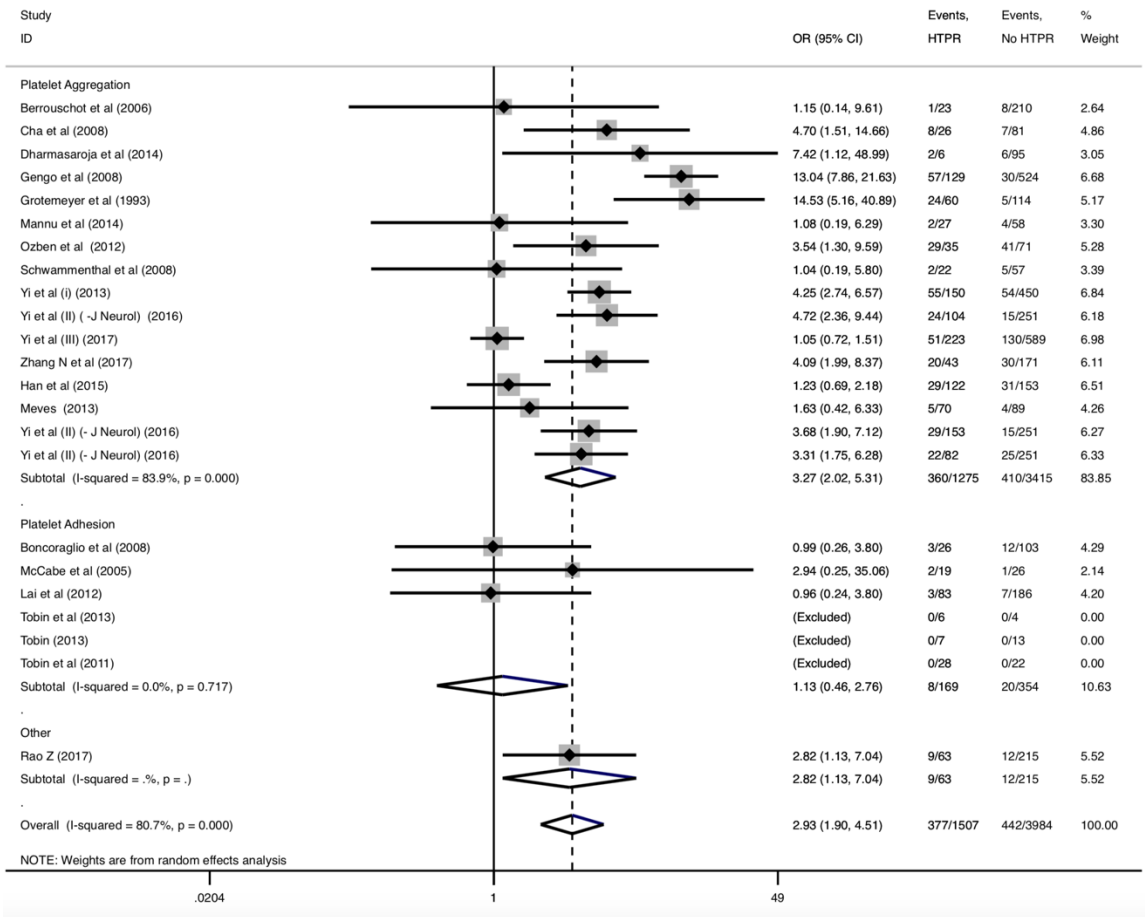


Figure 4. 13 Post-hoc combined analysis of the composite risk of subsequent ischemic stroke/TIA, myocardial infarction or vascular-related death in those with vs. those without antiplatelet-HTPR on any antiplatelet regimen after excluding 1993 data from Grotemeyer et al. to focus on the era of more modern secondary preventive therapy after 2005

[Of note, the risk of the composite outcome on any antiplatelet regimen in this *post-hoc* analysis remained significantly higher in those with *vs*. those without antiplatelet-HTPR (OR 2.69, 95% CI: 1.75-4.14)].

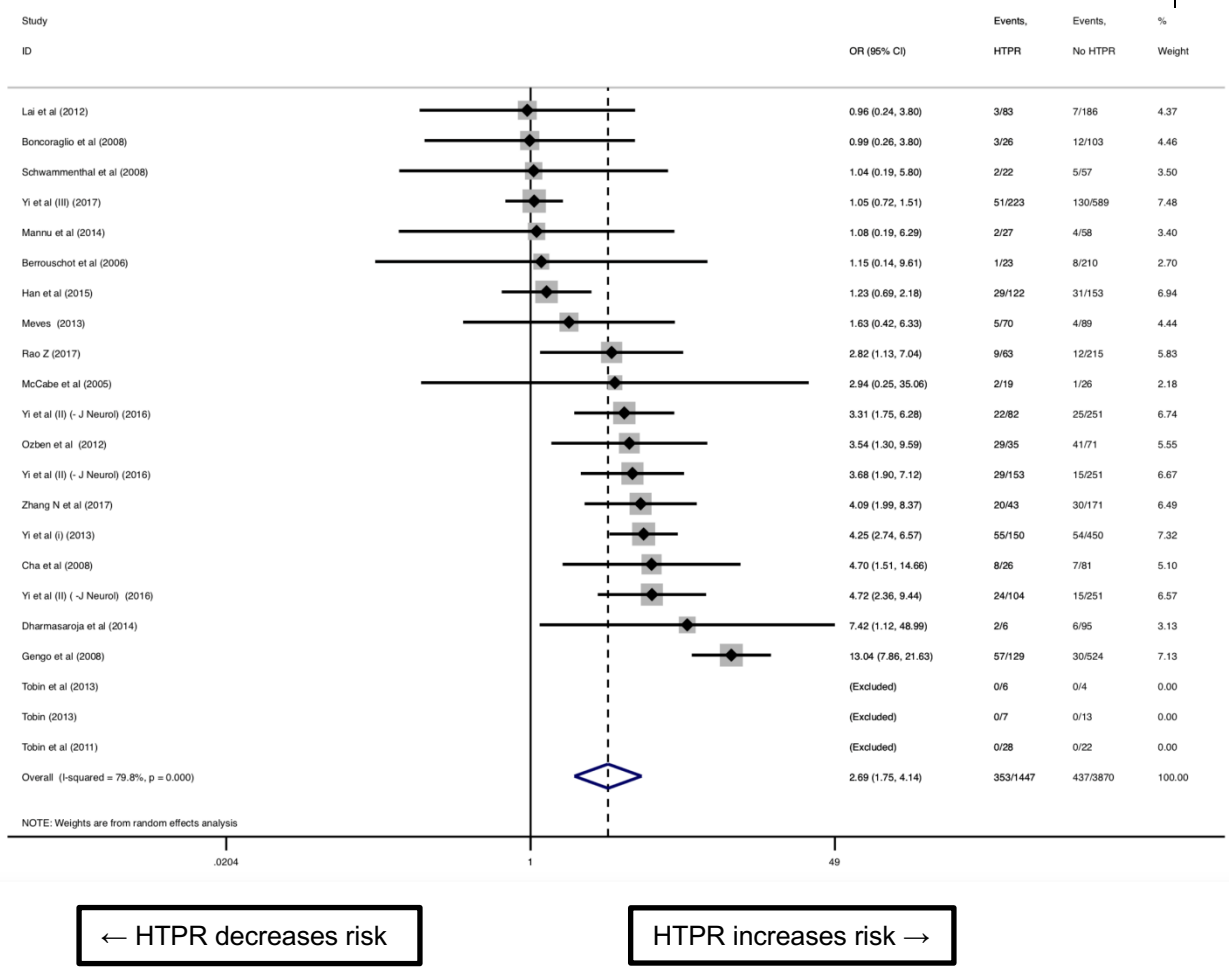


Figure 4. 14 Post-hoc subgroup analysis, based on prescribed antiplatelet regimens, on the composite risk of subsequent ischemic stroke/TIA, myocardial infarction or vascular death in those with vs. those without HTPR on aspirin, clopidogrel or dual antiplatelet therapy after excluding 1993 data from Grotemeyer et al. to focus on the era of more modern secondary preventive therapy after 2005

[Of note, the risk of the composite outcome on aspirin in this *post-hoc* analysis remained significantly higher in those with *vs*. those without aspirin-HTPR (OR 2.79, 95% CI: 1.56-4.99)].

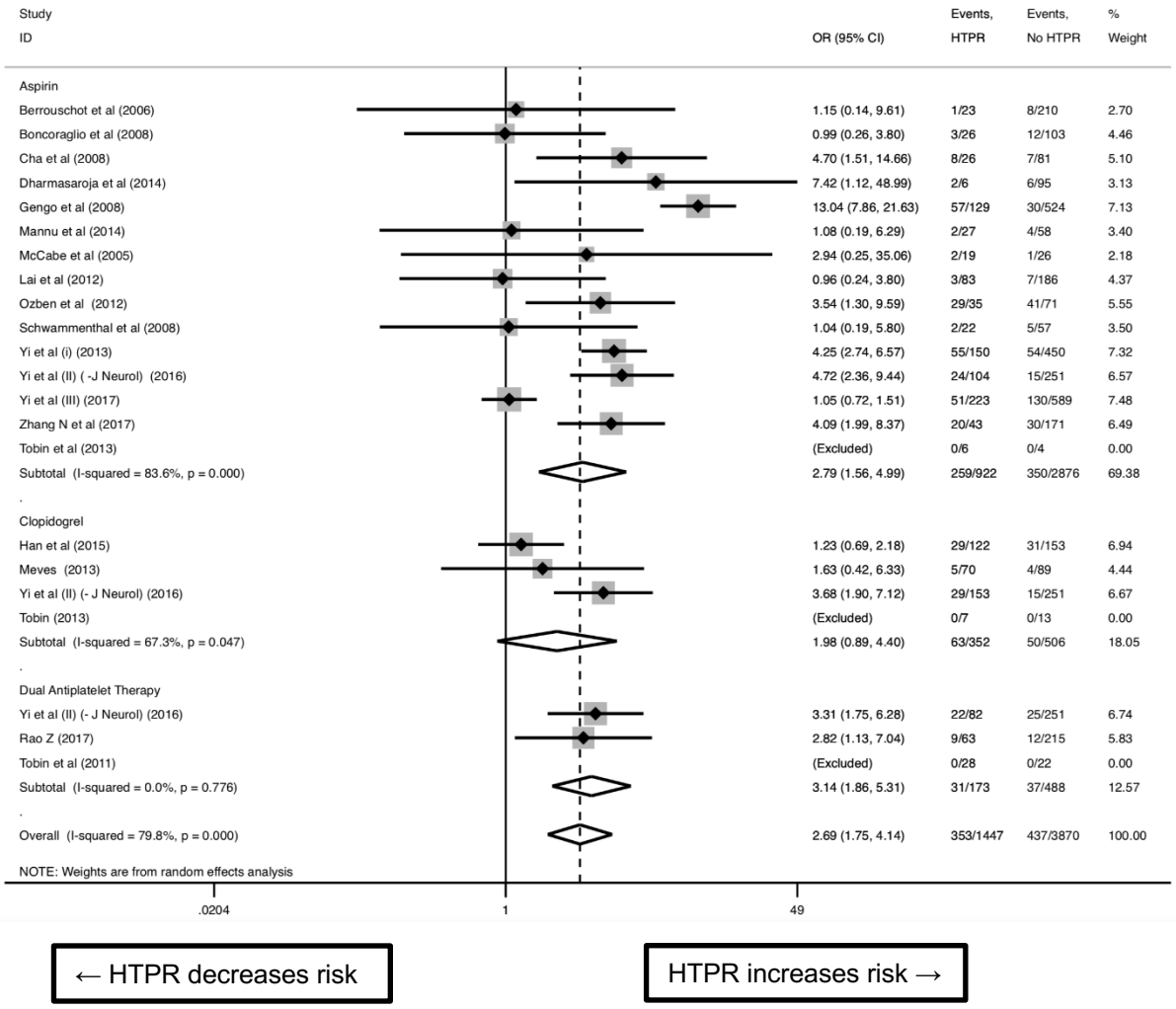


Table 4. 3 Studies indicating an association between antiplatelet-HTPR status and risk of recurrent stroke or vascular events

Author / Country / Study Type	Patient population / Treatment Regime	Method	Method Principle	Outcome
Grotemeyer *et al*. 1993 / Germany (Prospective)(Grotemeyer, Scharafinski et al. 1993)	174 stroke patients on Aspirin 1500 mg daily	TOA-PL-100 / Platelet Reactivity Index	Platelet Aggregation under low shear stress	16 patients (9.2%) had an MI and 13 (7.5%) had recurrent s without Aspirin-HTPR (40% *vs*. 4.4%; P < 0.0001)
Gengo *et al*. 2008 / USA (Prospective)(Gengo, Rainka et al. 2008)	653 TIA/acute ischemic stroke (AIS) patients on Aspirin 81-325 mg daily	Impedance Aggregometry	Platelet Aggregation under low shear stress	OR for Aspirin-HTPR in patients who had *vs*. those who di 23.7; P < 0.05). Prevalence of Aspirin-HTPR higher in pati while taking aspirin (P < 0.05)
Cha *et al.* 2008 / China (Prospective)(Cha, Jeon et al. 2008)	107 AIS patients on Aspirin 100 mg od	Optical Aggregometry	Platelet Aggregation under low shear stress	Aspirin-HTPR was associated with a higher risk of the com related deaths (OR 1.1; 95% CI: 1.01 to 1.20; P = 0.026)
Jeon *et al.* 2010 / Korea (Prospective)(Jeon, Song et al. 2010)	117 AIS patients given a loading dose of 500mg of Aspirin and maintenance dose of 100 mg of Aspirin daily	VerifyNow	Fully automated measurement of Platelet Aggregation under low shear stress	Aspirin-HTPR was associated with asymptomatic DWI-pos stroke (OR 6.01; 95% CI 1.29-28.09; p = 0.023) but not wit (p=0.234)
Yi *et al.* 2013 / China (Prospective)(Yi, Zhou et al. 2013)	634 stroke patients on Aspirin 100mg daily	Light Transmission Aggregometry	Platelet Aggregation under low shear stress	Recurrent stroke, MI, 'PAD' or death more common in pati up of 19.4 months (31% *vs*. 12%; P < 0.001)
Freitas-Silva *et al.* 2015 / Portugal (Prospective)(Freitas-Silva, Goncalves et al. 2015)	87 AIS patients on Aspirin 100mg daily	PFA-100	Platelet adhesion/aggregation under high shear stress	Higher risk of developing new stroke over 24 months using HTPR (P = 0.005)
Han *et al*. 2015 / China (Prospective)(Han, Lv et al. 2015)	345 within 14 days of AIS on Clopidogrel 75 mg daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	Recurrent stroke, TIA and vascular death more common in (OR 2.149, P = 0.001)
Kim *et al*. 2015 (i) / Korea (Prospective)(Kim, Heo et al. 2015)	367 acute ischemic stroke patients on 100mg of Aspirin daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	More likely to have new asymptomatic ischemic lesions on HTPR (OR 2.0, P = 0.047)
Yi *et al.* 2016 (i) / China (Prospective)(Yi, Lin et al. 2016)	375 AIS patients on 75 mg of Clopidogrel daily	Light Transmission Aggregometry / CYP SNP Genotyping	Platelet Aggregation under low shear stress	CYP SNP and clopidogrel-HTPR were associated with an i MI or 'all cause death' (HR 1.98, P = 0.006) after a period
Yi *et al.* 2016 (ii) / China (Prospective)(Yi, Lin et al. 2016)	535 AIS patients on either 75 mg Clopidogrel daily or combination of Aspirin 200 mg daily and Clopidogrel 75 mg daily	Light Transmission Aggregometry	Platelet Aggregation under low shear stress	Clopidogrel-HTPR was associated with an increased risk of death' (HR 2.82, P < 0.01) after a period of 6 months follow
Yi *et al.* 2016 (iii) / China (Prospective)(Yi, Wang et al. 2016)	426 AIS patients on either 100 mg of Aspirin daily, 75mg of Clopidogrel daily or both.	Light Transmission Aggregometry	Platelet Aggregation under low shear stress	Concomitant Aspirin-HTPR and Clopidogrel-HTPR associa 2.45, 95% CI: 1.86–8.67; P < 0.01). Concomitant Aspirin a deterioration (HR 2.68, 95% CI: 1.36 – 7.23, P < 0.01)
Rao et al. 2017 / China (Prospective)(Rao, Zheng et al. 2017)	278 AIS patients on Aspirin 100mg daily and Clopidogrel 75 mg daily	TEG	Monitoring of rate and quality of clot formation	Patients with antiplatelet-HTPR overall had a higher risk of arterial disease, and 'all cause death') at 3 months *vs*. those modelling showed that the presence of antiplatelet-HTPR in 7.91, P = 0.006)

Zhang *et al.* 2017 / China (Prospective)(Zhang, Wang et al. 2017)	214 AIS patients on 100mg Aspirin daily	Ultegra Rapid Platelet Function Analyser / VerifyNow	Platelet Aggregation under low shear stress	Aspirin-HTPR was associated with a higher risk of stroke recu follow up

Table 4. 4 Studies with no clear association between Antiplatelet-HTPR & risk of recurrent stroke or vascular events

Author / Country/ Study Type	Patient population / Treatment Regimen	Method	Method Principle	Outcome
Berrouschot *et al.* 2006 / Germany (Prospective)(Berrouschot, Schwetlick et al. 2006)	291 TIA or early phase stroke patients on Aspirin 300mg daily for secondary prevention assessed at 24hrs, 3, 6 and 12 months after initiation of treatment	Aggregometry	Platelet aggregation	Aspirin-HTPR noted in Recurrent stroke in 1/23
McCabe *et al*. 2005 / United Kingdom (Prospective, Observational case-control)(McCabe, Harrison et al. 2005)	57 early phase (≤4 weeks) & 46 late phase (≥3 months) patients on Aspirin 75-300 mg daily after TIA/stroke	PFA-100	Platelet adhesion / aggregation under high shear stress	33/55 (60%) of patients on the C-EPI cartridge. 1/26 (3.8%) without Asp
Boncoraglio *et al.* 2009 / Italy (Prospective Case-control)(Boncoraglio, Bodini et al. 2009)	129 Stable CVD patients with TIA or Stroke within preceding 1-12 months on Aspirin 75mg-300mg daily	PFA-100	Platelet adhesion /aggregation under high shear stress	Risk of recurrent ischem HTPR during mean follo
Tobin *et al.* 2011 / Ireland (Prospective)(Tobin, Kinsella et al. 2011)	52 TIA or early phase ischemic stroke patients changing from Aspirin monotherapy (median daily dose = 150 mg) to Aspirin (median daily dose at 14 days = 75mg) & Dipyridamole MR (median dose at 14d = 200mg bd) (Assessed at baseline, 14d & 90d)	PFA-100	Platelet adhesion/ aggregation under high shear stress	No patients had stroke re
Lai *et al.* 2012 / Taiwan (Prospective)(Lai, Chen et al. 2012)	269 patients with AIS on Aspirin 100mg daily	PFA-100	Platelet adhesion/ aggregation under high shear stress	Recurrent ischemic strok *vs*. 3.8%, P = 1.0)
Tobin *et al.* 2013 / Ireland (Prospective)(Tobin, Kinsella et al. 2013)	26 TIA or early ischemic stroke patients changing from 'No Medication' to Aspirin monotherapy (median dose at 14d = 300mg); 22 patients changing from Aspirin (median baseline dose = 75mg daily) to Clopidogrel monotherapy (median dose at 14d = 75mg daily); (Assessed at baseline, 14d and 90d)	PFA-100	Platelet adhesion/ aggregation under high shear stress	No patients had recurren (range 93-244d) in patie on Clopidogrel monothe
Dharmasaroja *et al.* 2014 / Thailand (Prospective)(Dharmasaroja and Sae-Lim 2014)	101 Stable Ischemic Stroke patients on 81-325 mg Aspirin daily	VerifyNow Urinary 11-dehydro-thromboxane B_2 ($DTxB_2$)	Fully automated measurement of platelet aggregation under low shear stress	No significant differenc infarction, cardiac interv between patients with ar
Meves *et al*. 2014 /Germany (Prospective)(Meves, Schroder et al. 2014)	159 AIS patients on Clopidogrel 75mg daily	Multiplate	Monitors changes in impedance caused by platelet aggregation in response to platelet agonists at low shear stress	Nine patients had recurr patients with (N=5, 7.1%
Mannu *et al*. 2014 / United Kingdom (Observational, 'semi-prospective' as outcome events calculated by **chart review only**)(Mannu, Macartney et al. 2015)	N=89 within 72 hours of acute TIA/ischemic stroke after at least 1 dose of Aspirin 75mg daily; unspecified dose of long-term Aspirin	Multiplate	Monitors changes in impedance caused by platelet aggregation in response to platelet agonists at low shear stress	No clear relationship betw mortality at 1 year (4/21 v
Rath et al. 2018 / Denmark (Prospective)(Rath, Jorgensen et al. 2018)	142 patients with a history of prior TIA/Stroke on Clopidogrel 75mg daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	No clear relationship betw or vascular death over a 2 HTPR was too small for r

Wang *et al.* 2019 / China (Prospective)(Wang, Chen et al. 2019)	280 AIS or TIA patients on combination therapy with Aspirin 100 mg daily and Ticagrelor 90 mg twice daily; 290 AIS or TIA patients on combination therapy with Aspirin 100 mg daily and Clopidogrel 75 mg daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	The prevalence of Antiplatel than in the Aspirin-Ticagrelo difference in the risk of recu haemorrhagic stroke, TIA, M comparison of outcomes betv treatment subgroup were not

Table 4. 5 Studies indicating that Antiplatelet-HTPR status is associated with more severe strokes, early neurological deterioration or adverse outcomes

Author/ Country/Study Type	Patient population / Treatment Regime	Method	Method Principle	Stroke Severity / Functional Outcome
Schwammenthal *et al.* 2008 / Germany (Observational)(Schwammenthal, Tsabari et al. 2008)	105 AIS patients on Aspirin 100-325mg daily	Optical Aggregometry	Platelet aggregation	More severe strokes, more unfavorable clinical course 0.05 for all). Could not reliably comment on whether A events (N=7) during median follow-up of 11.5 months
Englyst *et al* 2008 / UK (Case-control)(Englyst, Horsfield et al. 2008)	45 AIS patients on Aspirin 75 mg daily	Thrombo-Elastography (TEG)	Monitoring of the rate and quality of clot formation	MRS worse at 72 hours in patients with *vs*. without As
Bugnicourt *et al.* 2010 / France (Prospective)(Bugnicourt, Roussel et al. 2011)	85 AIS patients on Aspirin 160mg daily	PFA-100	Platelet adhesion / aggregation under high shear stress	Aspirin-HTPR associated with early neurological dete Health Stroke Scale (NIHSS) ≥4 points in the first 72 h
Lai *et al.* 2012 / Taiwan (Prospective)(Lai, Chen et al. 2012)	269 patients with AIS on Aspirin 100mg daily	PFA-100	Platelet adhesion / aggregation under shear stress	Patients with Aspirin-HTPR were less likely to have fa score ≤ 2 at 30d: 47% *vs*. 60%, P = 0.047)
Coignion *et al*. 2015 / France (Prospective)(Coignion, Poli et al. 2015)	287 patients with AIS on Aspirin or Clopidogrel (dose not specified)	Multiplate	Monitors changes in impedance caused by platelet aggregation in response to platelet agonists at low shear stress	Patients with HTPR had higher baseline NIHSS scores
Ozben *et al.* 2011 / Turkey (Case Control)(Ozben, Ozben et al. 2011)	106 AIS patients on Aspirin 100mg daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	Mortality rates higher in patients with Aspirin-HTPR (P = 0.004); Independent predictor of 2-year mortality:
Zheng *et al.* 2013 / Australia (Prospective)(Zheng, Churilov et al. 2013)	90 AIS patients on Aspirin 100mg daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	Aspirin-HTPR associated with worse baseline NIHSS CT (P < 0.001)
Kim *et al.* 2015 (ii) / Korea (Prospective)(Kim, Heo et al. 2015)	349 AIS patients on 100mg Aspirin daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	Early neurological deterioration & increase in NIHSS change in Aspirin-HTPR status over time (42% *vs*. 22
Oh *et al*. 2016 / Korea (Prospective Observational)(Oh, Yu et al. 2016)	310 AIS patients on 100mg-200mg Aspirin daily	VerifyNow	Fully automated measurement of platelet aggregation under low shear stress	Aspirin-HTPR in 27.7% of patients. HTPR group had points *vs*. the non-HTPR group (P < 0.001) and a large
Yi *et al.* 2016 (ii) / China (Prospective)(Yi, Lin et al. 2016)	535 AIS patients on either 75 mg Clopidogrel daily or combination Aspirin 200 mg daily and Clopidogrel 75 mg daily	Light Transmission Aggregometry	Platelet aggregation under low shear stress	As stated above, Clopidogrel-HTPR was associated wi ischaemic stroke, MI or 'all cause death' (HR 2.82, P < Furthermore, Clopidogrel-HTPR was associated with h months (P < 0.001)
Yi *et al.* 2016 (iii) / China (Prospective)(Yi, Wang et al. 2016)	426 AIS patients on either 100 mg of Aspirin daily, 75mg of Clopidogrel daily, or both.	Light Transmission Aggregometry	Platelet Aggregation under low shear stress	As stated above, concomitant Aspirin-HTPR and Clop ischemic stroke after 90 days (HR 2.45, 95% CI: 1.86- Clopidogrel-HTPR was associated with early neurolog
Lin *et al.* 2018 / China (Prospective)(Lin, Han et al. 2018)	375 AIS patients (144 on Clopidogrel monotherapy 75 mg daily; 231 on 200mg of Aspirin daily and 75 mg of Clopidogrel daily)	Light Transmission Aggregometry / CYP SNP genotyping	Platelet Aggregation under low shear stress	Aspirin and Clopidogrel-HTPR was associated with a < 0.001)

5. General Methods

The ‘general methods’ section in this PhD thesis was adapted from Chapter 4 of Dr Stephen Murphy‘s PhD thesis entitled ‘Markers of Platelet Activation and Function and their relationship to Cerebral Micro-Embolic Signals in Symptomatic and Asymptomatic Carotid Stenosis’ (Murphy SJX, School of Medicine, Trinity College Dublin; 2017). Dr Murphy successfully defended his PhD at TCD in 2016 and his degree was officially awarded in 2017. Dr Murphy and I worked in very close collaboration with each other, in independent but linked projects under Prof. McCabe’s supervision, to recruit, assess, perform analysis of samples in the research laboratory and follow-up patients in our respective studies. Therefore, the wording of the general methodology section of both our theses was prepared and agreed by both of us who collaborated to finalise the wording of the following text under Prof. McCabe’s close supervision.

5.1. Study Participants

5.1.1. Ethical Approval

This study was fully approved by St. James’s Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2011/35/03). Written informed consent was obtained from all patients (or proxy consent from their relatives / next of kin, where appropriate). Detailed information on the purpose and nature of the study, as well as the procedures involved was provided to all participants both verbally and via written information sheets.

5.1.2. Recruitment

The specific inclusion and exclusion criteria for all patients and 'control participants' involved in each study described in this thesis are outlined in the respective results chapters.

5.1.3. Initial Clinical Assessment, Timing and Follow-up

Patients underwent detailed clinical assessment and were classified as having had a TIA or ischaemic stroke when this was confirmed by their Consultant Neurologist or Stroke Physician after clinical investigation according to ESO recommendations (European Stroke Organisation Executive and Committee 2008). The diagnosis was also confirmed in all cases by me as a clinically experienced Research SpR in Vascular Neurology under the supervision of Prof McCabe, or by Prof McCabe himself. All patients had appropriate, detailed neurovascular blood tests performed, including FBC, ESR, CRP, coagulation profile, renal and liver profiles, fasting lipids, fasting glucose, HbA1C, TFTs, vitamin B12, plasma folate and homocysteine. CT of brain and/or MRI of brain (with FLAIR, T2-weighted, T1-weighted, DWI, and T2* sequences, unless MRI was contra- indicated), colour Doppler ultrasound of carotid and vertebral arteries, and extracranial CTA or MRA, ECG, 24 hour Holter monitoring, transthoracic echocardiography (TTE) or trans-oesophageal echocardiography (TOE) with bubble and Valsalva were performed in all patients, as deemed appropriate by the treating physician. The underlying mechanism responsible for TIA or Stroke was categorised according to the TOAST classification (Adams, Bendixen et al. 1993) and ASCOD classification (Amarenco, Bogousslavsky et al. 2013).

Patients underwent detailed clinical and laboratory assessment with venepuncture before (**baseline**), 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after starting or changing antiplatelet therapy . In addition, clinical follow-up to assess the longer-term risk of recurrent vascular events and outcome measures was performed using a validated in-person or telephone questionnaire at ≥ 1 year after symptom onset.

5.2 Sample Collection and Separation Methods

5.2.1 Sample Collection

After resting for at least 20 minutes to minimise platelet activation *in vivo*, careful venepuncture was performed on all patients using a standardised procedure. Blood was taken from a free- flowing vein using a sterile 21G Butterfly® needle (Venisystems TM, Abbott, Ireland) and a Vacutainer® system with a luer adaptor (Becton Dickinson Vacutainer® Systems, U.K.). A tourniquet was applied to the arm and was released during collection of the first 3 ml of blood that was drawn into a 3 ml sterile Vacutainer® tube containing sodium citrate and subsequently discarded. The next two 2 ml sterile 3.2% buffered sodium citrate-anticoagulated samples were taken for analysis of platelet function with the VerifyNow® system (Accriva Diagnostics). Six further samples were taken in sterile 3 ml Vacutainers® containing 3.2% buffered sodium citrate. Gentle inversion was used in each case to ensure thorough mixing of the anticoagulant with the blood sample. The first and second of these citrate-anticoagulated samples were used for whole blood flow cytometric analysis and for measurement of platelet function with the platelet function analyser (PFA-100®, Dade-Behring, Germany). The next 3 citrate-anticoagulated samples were used for the preparation of platelet-poor-plasma (PPP),

as outlined below. The final sample was used for measurement of the platelet count, MPV and PDW in citrate. Subsequently, one 3 ml double-walled Vacutainer® tube containing 'recombinant hirudin anticoagulant' was taken for analysis of platelet function on the Multiplate® system. Thereafter, three 3ml sterile Vacutainer® tubes containing K_2 EDTA were obtained. This first sample was used to measure the full blood count (FBC), including measurement of the mean platelet volume (MPV) and platelet distribution width (PDW). This FBC was performed between 2 hours and 4 hours after venepuncture, because EDTA-induced platelet swelling is maximal in the first 1-2 hours, very minimal between 2 and 4 hours, and increases slowly in the first 24 hours after blood sampling (Trowbridge, Slater et al. 1984). The two remaining EDTA tubes containing whole blood were stored at -70 to -80°C for future, planned pharmacogenetic studies. A 3.5ml tube containing lithium-heparin was then taken for storage of serum, and two 6ml tubes containing K_2-EDTA were drawn to prepare serum for storage and for ABO blood grouping. Finally, two additional 6ml serum tubes were taken and stored for later analysis of serum thromboxane B_2 levels, and other serum markers which might affect the results of our other assays, but only plasma markers were analysed for this thesis.

5.2.2. Sample separation and storage

All plasma samples were separated by centrifugation within 60 minutes of venepuncture and were frozen at -70 to -80°C within 90 minutes of venepuncture.

Platelet Poor Plasma (PPP)

PPP was prepared from three 0.105M (3.2%) buffered sodium citrate-anticoagulated blood samples within one hour of venepuncture. The samples were centrifuged at 2250 x G for 15 minutes at room temperature. A plastic Pasteur pipette was used to carefully aspirate the upper two-thirds of each sample into 12 x 75 mm polypropylene sample tubes. These were then centrifuged again at 2250 x G for 15

minutes. The double-spun PPP was recovered from the upper two-thirds of these samples and aliquoted into three polypropylene tubes (Sarstedt®, Germany) which were immediately frozen at -70 to -80°C. The bottom third of each sample was also stored, but this was not considered to be 'double-spun PPP'.

5.3. Flow Cytometry

5.3.1 General Principles

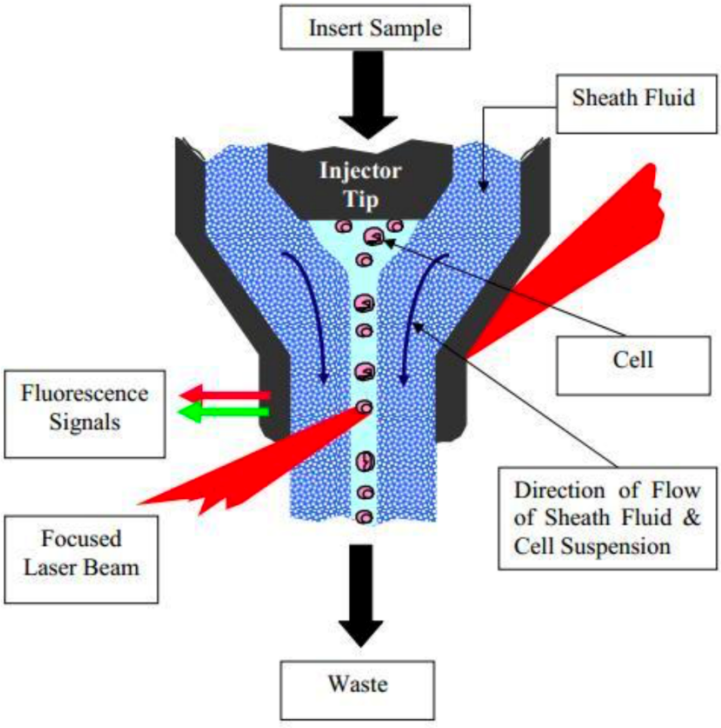
The flow cytometer used in this study was a Coulter® EPICS® XL-MCL (Beckman Coulter United Kingdom Ltd.) which was available to our research group at the time of this thesis; the information below applies to the operation of this model.

Flow cytometry is a method used for sensing cells or particles as they flow in a liquid stream through a laser beam (Macey and Idziorek 1994). The signals produced by the flow provide information about the cell. A cell suspension is inserted into the flow cell through which sheath fluid (ISOTON II®, Beckman Coulter, U.K.) is pumped. This sheath fluid is filtered with a 0.2µm filter and contains both bacteriostatic and fungistatic agents. It is also transparent and does not fluoresce in response to 488 nm laser light. The sheath fluid exerts a constant pressure on the cell suspension, and with a low flow rate of 10µl/min, the cells are aligned in single file in a process known as 'hydrodynamic focusing'.

The cells pass in single file past the 488 nm argon ion laser that is used in the Coulter® EPICS® XL-MCL, scattering the light in different directions. The degree of light scatter in the forward direction (forward scatter [**FS**]) is directly proportional to the size of the cell, with larger cells scattering the light more (Figure 5.1). The laser light also enters the cell and is reflected and refracted by internal structures and granules of the cell producing side scatter (**SS**) light. This side scatter is directly proportional to the granularity of the cell. In order to define particular cells or cellular features, they can be labelled with fluorochrome-linked antibodies, or stained with fluorescent dyes. These fluorochromes or fluorescent dyes absorb

laser light energy and emit fluorescence at different colour wavelengths. The FS sensor is positioned behind the sample stream, with the SS and other fluorescence sensors positioned at 90° to the laser beam and sample stream. In order to split the beam, a series of dichroic mirrors direct the different components of the transmitted light to the appropriate sensors. A series of filters remove the unwanted wavelengths of light. The sensors are called photomultiplier tubes (PMTs). They are a class of vacuum tube which amplify transmitted light and convert it into a voltage pulse whose magnitude is proportional to the amount of light incident on the tube. Smaller cells or particles generate smaller voltage pulses, whereas larger cells yield larger pulses. A logarithmic-amplification process may be used for amplification of the pulses. This allows more accurate scaling of the signals because smaller pulses will be made much larger, whereas larger pulses are amplified to a lesser degree, thus accentuating differences between two small pulses (Macey and Idziorek 1994).

Figure 5. 1 Diagrammatic Representation of a Flow Cell
(Redrawn from reference: Beckman Coulter United Kingdom Ltd, 1999) -
(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)



Flow cytometry can be used to study platelet activation in whole blood, platelet rich

plasma (PRP), or after separation of platelets from plasma (washed platelets) (Abrams and Shattil 1991). Whole blood flow cytometry was used in this thesis because this method has the advantages of analysing platelets in the physiological milieu of whole blood, and is therefore less susceptible to artefactual *in vitro* platelet activation and potential loss of platelet subpopulations than methods which use PRP or washed platelets (Michelson 1996).

The fluorochromes and fluorescent dye (Thiazole Orange [TO]) used for whole blood flow cytometric analysis in this thesis are listed in Table 5.1. The fluorochromes are conjugated to monoclonal antibodies to allow the detection of specific cell surface antigens. Monoclonal antibodies are preferred to polyclonal antibodies in whole blood flow cytometry because they result in less non-specific antibody binding, and can more reliably saturate all specific epitopes on the platelet surface (Michelson 1996). All of the monoclonal antibodies used in this thesis were purchased in conjugated form from the manufacturers (Beckman Coulter, UK).

Table 5. 1 Fluorochromes / Fluorescent Dye Used In Flow Cytometry

Fluorochrome / Fluorescent Dye	**Emission Wavelength (nm)**	**Fluorescence**	**Fluorescence Detector**
Fluorescein Isothiocyanate (FITC)	525	Green	FL1
Thiazole Orange (TO)	533	Green	FL1
Phycoerythrin (PE)	575	Orange	FL2
R-phycoerythrin-Cy5 (RPE-Cy5)*	670	Red	FL3

*(Michelson 1996) RPE-Cy5 is a combination of two fluorochromes [R-phycoerythrin [RPE] and Cyanine 5 [Cy5]) that are covalently coupled to one another. The argon ion laser excites the RPE at 488 nm, and the emitted light energy excites the Cy5 closely bound to the RPE molecule. The Cy5 then fluoresces at 670 nm and the FL3 detector detects this fluorescence.

The degree of monoclonal antibody binding to, or fluorescent dye uptake by a cell sample can be expressed as the percentage of cells staining positive (percent positive) for a particular antibody, or as mean particle fluorescence intensity (MFI) (Michelson 1996). Some degree of non-specific fluorescence is present in all blood samples due to a combination of sample auto-fluorescence and non-specific staining of the cell with a given monoclonal antibody or fluorochrome (Schmitz, Rothe et al. 1998). To control for this non-specific fluorescence, 'matched isotype control' monoclonal antibodies were used for all monoclonal antibody-based assays throughout this thesis because this was accepted practice at the planning phase and during the main phase of data collection in our study, and was in keeping with prior, peer-reviewed and fully validated protocols in our laboratory. Although these antibodies were conjugated to the same fluorochrome being tested, they did not recognise the target antigens on platelets (Schmitz, Rothe et al. 1998).

To quantify the degree of non-specific fluorescence in the TO assay, a matched 'control sample' that was incubated in the absence of the fluorescent dye was used (see below). For every assay, the 'antibody positive population' was determined using an analysis marker placed to the right of a histogram of 'fluorescence versus intensity' from a matched control sample identified on the Coulter® EPICS® XL-MCL (Michelson 1996) (see below). The results were expressed as percent positive platelets as this method is both simpler than MFI, and is independent of the variation in signal amplification which can be caused by voltage/gain changes in the PMT over time because the isotype control signal changes in proportion with the test sample (Michelson 1996). This method also has the advantage of being very sensitive at detecting an increase in antigen expression by a small sub-population of cells with a heterogeneous staining pattern (Schmitz, Rothe et al. 1998). However, it must be noted that antibody positive platelets may have very little antigen expressed on their surface. For example, although 10% of circulating platelets may express a particular surface antigen, if each platelet expresses only 10% of the maximal level of the antigen, then the average increase in platelet antigen expression is only 1% per platelet. MFI is therefore the preferred method of data expression if the goal is to determine the total amount of platelet surface antigen expression (Michelson 1996), or if minor decreases in the expression of a ubiquitous surface marker are being investigated (Schmitz, Rothe et al. 1998). In this thesis, all measurements will

be expressed as percent positive platelets as small changes were predicted in the expression of platelet activation markers between groups. The voltage and gain settings were not changed during this study.

5.3.2. Quality Control

The optical alignment of the Coulter® EPICS® XL-MCL flow cytometer's laser and the fluidics of the system were calibrated every day before use with commercially-available fluorescent beads (Flow-Check® Fluorospheres, Beckman Coulter, U.K.). These beads consist of 10 μm fluorescent spheres and emit light within the wavelength range of 525 nm to 700 nm when excited by the 488nm argon ion laser. Before use, the flow cytometer was calibrated, with 10,000 events being collected for each sample tested at a rate of < 200 events per second. A half peak coefficient of variation of ≤ 2.0 on the relevant histograms was deemed acceptable (Tobin 2011).

5.3.3. Platelet surface markers

GpIb (CD42b) – Principle

As described above, flow cytometry can be used to distinguish platelets from red and white blood cells by the characteristic pattern of forward and side scatter they generate. This scatter is dependent on their size and granularity, respectively. The GpIbα (CD42b) subunit of the GPIb- IX-V complex is the predominant receptor for von Willebrand Factor (VWF:Ag) on platelets (Clemetson and Clemetson 1995; Ruggeri 1997; Escolar and White 2000). With the exception of patients with Bernard-Soulier syndrome, in whom this receptor may be deficient, if one stains platelets with an 'activation-independent' platelet-specific antibody directed against GPIb and conjugated to PE, the flow cytometer threshold can be set to detect only PE-positive particles i.e. GpIb-positive platelets and platelet-derived microparticles

(Abrams and Shattil 1991). Our method for detecting GpIb-positive platelets was adapted from the whole blood flow cytometric protocol described by Shattil et al (Shattil, Cunningham et al. 1987). GpIb expression can also be used as a marker of platelet activation because there is some degree of GpIb-IX-V receptor 'internal redistribution' from the platelet surface membrane to the membranes of the surface-connected open canalicular system upon platelet activation. This can reduce the accessibility of anti-GpIb-IX-V monoclonal antibodies to their epitopes on platelets, and this reduction in receptor expression can be quantified. However, the reduction in surface expression of GpIb-IX-V is highly time-dependent, decreasing within 30 seconds of platelet activation before reaching a nadir at approximately 5 minutes and returning to baseline over the next 45 minutes. As immediate fixation of the samples was not performed before labelling with monoclonal antibodies, the reduction in GpIb binding was not quantified in this thesis.

Reagents

- Anti-IgG1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25 ug/ml)
- Anti-CD42b-PE mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 1.56 ug/ml)
- Hepes buffered solution (HBS) - made up with NaCl 0.145mol/L, KCl 5 mmol/L, MgSO4 1 mmol/L and HEPES 10mmol/L, dissolved in distilled water and pH adjusted to 7.4 (Chronos et al, 1994). The solution was filtered with a 0.2 μm filter prior to storage at 4°C, but allowed to reach room temperature before use
- Fixative: 0.2% formalin saline solution (Sigma Aldrich, USA) – made up of 0.25 ml of 40% formaldehyde solution diluted 1:200 with 50 ml of 0.9% NaCl. The fixative was freshly prepared each day, and filtered with a 0.2 μm filter to remove any debris.

Method

In order to equalise the concentration of the anti-IgG1-PE isotype control antibody

(6.25 ug/ml) with the concentration of the anti-CD42b-PE monoclonal antibody (1.56 ug/ml), 5 µl of the anti-IgG1-PE control antibody was diluted 1:4 with 15 µl of HBS before use in the assay. 5µl of citrate anticoagulated whole blood was aliquoted into the control and test polypropylene tubes. respectively within 45 minutes of venepuncture. 5 µl of diluted anti-IgG1-PE antibody was added to the isotype control sample, and 5 µl of anti-CD42b-PE antibody to the test sample. After adding 40 µl of HBS to each sample, the samples were gently mixed, covered and incubated at room temperature for 20 minutes. The samples were then fixed with 1 ml of fixative; flow cytometric analysis began within 45 minutes of fixation. A flow cytometry protocol was designed in which only cells in the control sample with a particular forward and side scatter profile consistent with platelets were analysed. A gate was manually positioned around the platelet cloud on the 'log FS' versus 'log SS' histogram, and the gating settings were saved and used for subsequent platelet activation studies on the same sample. We analysed 10,000 platelet events in all assays using a low flow rate, unless otherwise stated. The non-specific fluorescence of the anti-IgG1-PE isotype control monoclonal antibody was measured on a histogram that plotted the platelet count versus log fluorescence detected in FL2. The 'positive analysis function' was set at 0.5% for this and all other assays performed as part of the 'panel set up' on the flow cytometer. This allowed the percentage of 'antibody-positive' platelets in the test sample to be determined by measuring those platelets with a fluorescence intensity exceeding that of 99.5% of the control sample. By including 0.5% of the matched control sample histogram in the calculation of percent positive cells, one avoided excluding weakly positive cells. In order to confirm that the cells within the gate in the test sample were platelets, the % GpIb binding in the test sample was then measured in FL2; the majority of cells within the gate were considered to be platelets if the % GpIb binding was ≥ 95%. If the % GpIb binding was < 95%, the process was repeated until ≥ 95% GpIb positivity was obtained for the test sample. The gating settings around the platelet cloud were then saved and used for analysis of all the other platelet activation markers described below. This allowed single labelling of platelets with the fluorochrome-conjugated monoclonal antibody of interest.

Figure 5. 2 Scatterplot and Histogram from a GpIb Control Sample

(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

Figure 5.2.I Scatterplot of log FS versus log SS from a GpIb Control sample identifies platelets within region 'B' by their characteristic light scatter profiles; gate 'A' is manually positioned around the platelet cloud

Figure 5.2.II Histogram of cell count (Count) versus log fluorescence detected in FL2 from an anti-IgG1-PE isotype control sample to calculate the degree of non-specific background fluorescence of the control sample. The positive analysis function includes 0.5% of the control sample in region 'E'

Figure 5.2.I

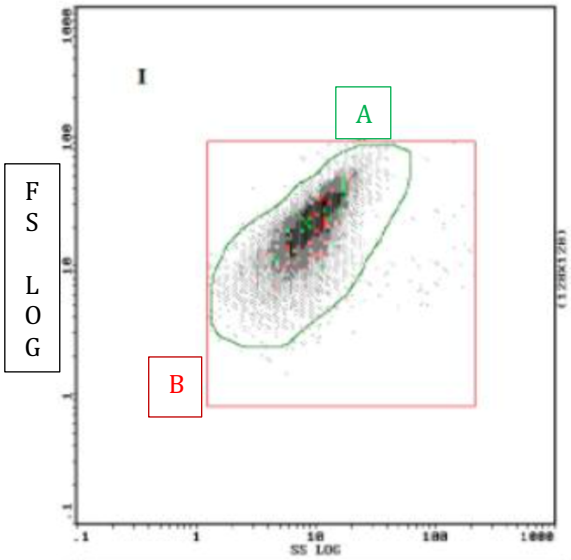


Figure 5.2.II

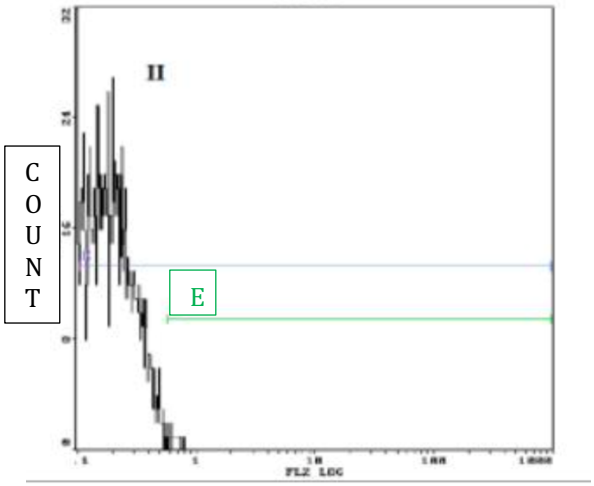


Figure 5. 2.III Scatterplot of log FS versus log SS from a GpIb test sample (Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

The position of gate 'A' is identical to that in the control sample in Figure 5.2.a

Figure 5.2.IV: Histogram of cell count versus log fluorescence detected in FL2 from the GpIb test sample confirming that 99.2% of cells within the region of interest were GpIb-positive

Figure 5.2.III

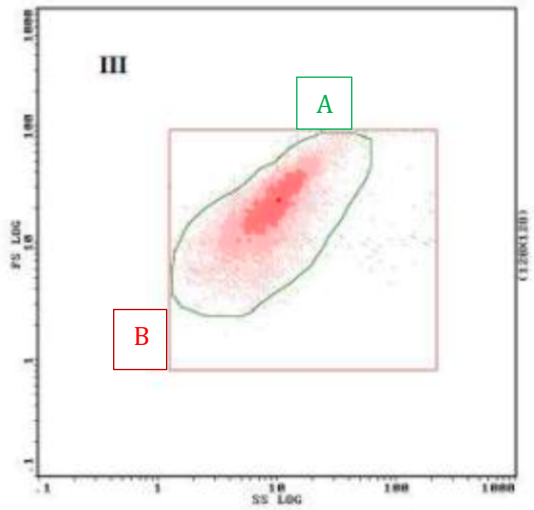
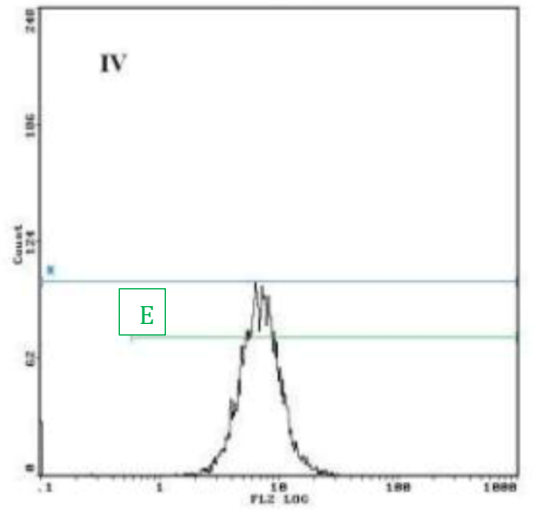


Figure 5.2.IV



CD62P and CD63 - Principle:

'Activation-dependent' monoclonal antibodies bind minimally, or not at all, to unstimulated platelets, but bind specifically and saturably to activated platelets (Abrams and Shattil 1991). CD62P is only expressed on the platelet surface membrane after alpha or dense-granule secretion and the CD63 antigen is expressed on the platelet surface after its release from lysosomes or dense granules. The use of specific CD62P- and CD63-targeted activation- dependent monoclonal antibodies conjugated to a fluorochrome allows quantification of the sample fluorescence via whole blood flow cytometry and evaluation of the levels present on the platelet surface.

CD62P

Because CD62P is ultimately shed into the circulation by proteolysis (Frijns, Kappelle et al. 1997), whole blood flow cytometric analysis of platelets was only used to quantify CD62P expressed on the platelet surface.

Reagents

- Anti-IgG1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25 ug/ml)
- Anti-CD62P-PE mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25ug/ml)
- HBS
- Fixative: 0.2% formalin saline solution

Method

The experimental methodology for the CD62P assay was identical to that used for the GPIb assay with the exception that 5µl of anti-IgG1-PE antibody was substituted as an additive to the isotype control sample, and 5 µl of anti-CD62P-PE antibody was added to the test sample. The platelet cloud was identified on the log SS versus log FS histogram using the gating settings from the GpIb assay. The non-specific fluorescence of the control sample was calculated, and the % CD62P positivity was then measured in FL2.

Figure 5. 3 Scatterplot and Histogram from a CD62P Test sample

(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

Figure 5.3.I: Scatterplot of log FS versus log SS from a CD62P test sample

Figure 5.3.II: Histogram of cell count versus log fluorescence detected in FL2 from the same sample; flow cytometric analysis showed that 9.5% of platelets in this patient's sample expressed CD62P

Figure 5.3.I

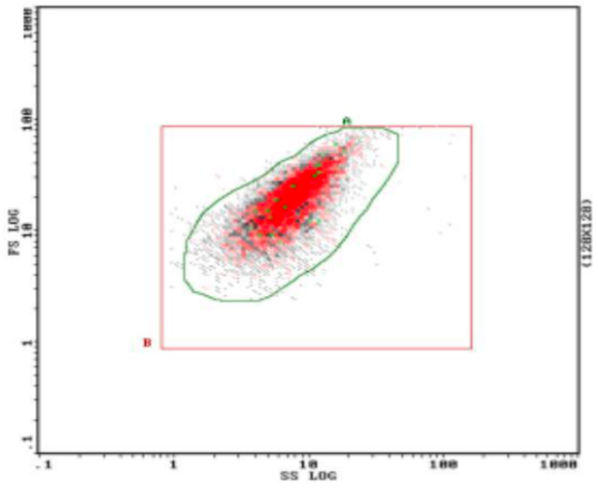
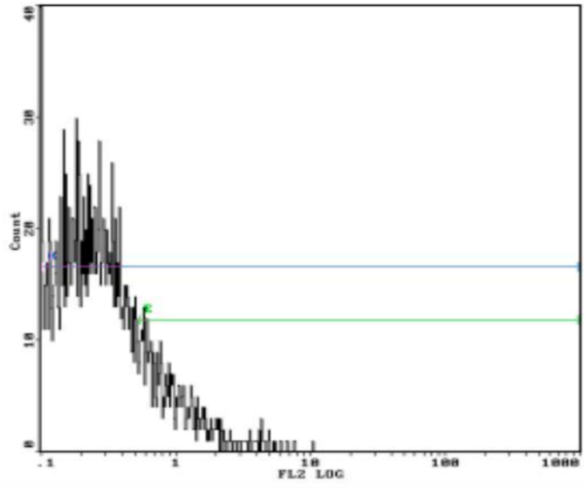


Figure 5.3.II



CD63

Reagents

- Anti-IgG1-FITC isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 50 ug/ml)
- Anti-CD63-FITC mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 50 ug/ml)
- HBS
- Fixative: 0.2% formalin saline solution

Method

The experimental methodology for the CD63 assay was also identical to that used for the GPIb assay with the exception that 5µl of anti-IgG1-FITC antibody was substituted as an additive to the isotype control sample, and 5 µl of anti-CD63-FITC antibody was added to the test sample. The platelet cloud was identified on the log SS *vs*. log FS histogram using the gating settings from the GpIb assay. The non-specific fluorescence of the control sample was calculated, and the % CD63 positivity was then measured in FL1.

Figure 5. 4 Scatterplot and Histogram from a CD63 Test Sample

(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

Figure 5.4.I: Scatterplot of log FS versus log SS from a CD63 test sample

Figure 5.4.II: Histogram of cell count versus log fluorescence detected in FL1 from the same sample; flow cytometric analysis showed that 11.3% of platelets in the region of interest in this patient sample expressed CD63

Figure 5.4.I

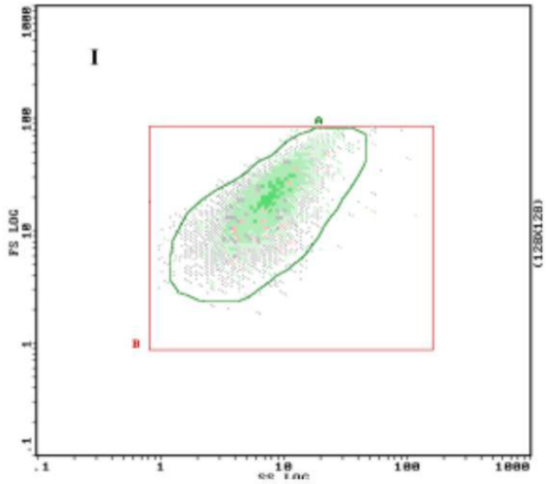
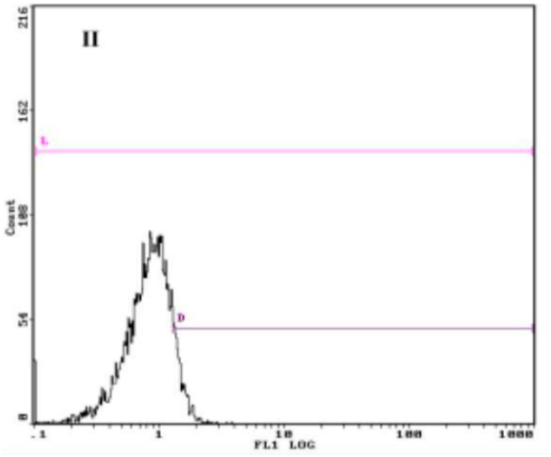


Figure 5.4.II



5.3.4. Reticulated Platelets

Principle

Megakaryocytes fragment in the bone marrow and release platelets into the peripheral blood steam or potentially the pulmonary circulation (Harrison, Robinson et al. 1997) Freshly released, immature platelets contain a residual amount of megakaryocyte-derived mRNA – first described by Ingram and Coopersmith in 1969 (Ingram and Coopersmith 1969), and were termed 'reticulated platelets', a name derived from reticulated red cells. They were reported to be larger in size and have an increased mean density compared with normal platelets (Ingram and Coopersmith 1969). They have also been shown to be unstable and undergo rapid degredation within the circulation in animal studies (Ault and Knowles 1995). As a result of this, measurement of the percentage of reticulated platelets *in vivo* or *ex vivo* has the potential to be a useful marker of the platelet production/turnover that is postulated to occur in patients with increased platelet activation.

The reagent used to stain for residual RNA in this thesis was Thiazole Orange (TO), a fluorescent dye that was originally synthesised to quantify red cell reticulocytes. It readily permeates live cell membranes without the need for a permeabilisation step, and fluoresces at 533 nm on binding to nucleic acids, with a greater affinity for RNA than DNA (Michelson 1996; Robinson, Harrison et al. 1998). TO can be used to label reticulated bloods cells in the circulation that can be quantified by whole blood flow cytometry, but there is no 'gold standard' reference method available and no standardised control against which the results obtained can be compared (Harrison, Robinson et al. 1997; Robinson, Mackie et al. 1998). However, it has been shown that high concentrations of TO can non-specifically label dense granules, whereas low concentrations of the dye do not result in non-specific labelling (Robinson, Harrison et al. 1998; Robinson, Mackie et al. 1998; Robinson, MacKie et al. 2000). For the purpose of this study, a low concentration of TO was used.

Reagents

- Retic-COUNT™ (Becton Dickinson, San Jose, USA)
- Isoton II® (Beckman Coulter, UK)

Method

Previous experiments by our group have shown that the uptake of Thiazole Orange by platelets is stable and reproducible if sample processing begins within 30 to 60 minutes after venepuncture. At the same time, it was established that that the percentage of reticulated platelets remains stable if the sample is stored in the fridge at 4°C, and processing begins between 1 and 6 hours after venepuncture (McCabe DJ, Personal communication). In this study, experiments were performed on non-refrigerated samples.

One ml of Isoton II® alone was aliquoted into the control tube, and a 1:10 dilution of Retic-COUNT™ was performed by adding 900 µl of Isoton II® to 100 µl of Retic-COUNT in the test sample tube (Robinson et al, 2000A). 5 µl of citrate anticoagulated whole blood was then added to the control and test tubes, respectively, between 30 and 60 minutes after venepuncture. The samples were covered and incubated in the dark for exactly 30 minutes, then centrifuged at 1200 x G for 3 minutes. In order to prevent further incubation of the test sample with Retic-COUNT, the supernatant was immediately discarded and the sediment pellet re-suspended in 1ml of Isoton II® within an hour of venepuncture. The platelet cloud in the control sample tube was identified on a scatterplot of log FS versus log SS which had been saved on the flow cytometer, as described previously. The non-specific fluorescence of the control sample was calculated, and the % of TO-positive (reticulated) platelets in the test sample was then measured in FL1.

Figure 5. 5 Scatterplot of Log FS vs. SS from TO Control and Test Samples
(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

Figure 5.5.I: Scatterplot of log FS *vs.* log SS from a TO control sample identifies platelets within region B by their characteristic light scatter profiles; gate 'A' is manually positioned around the platelet cloud;

Figure 5.5.II: Scatterplot of log FS *vs.* log SS from a TO test sample shows that the TO-positive platelets (blue cells) are amongst the largest and most granular in the platelet cloud.

Figure 5.5.I

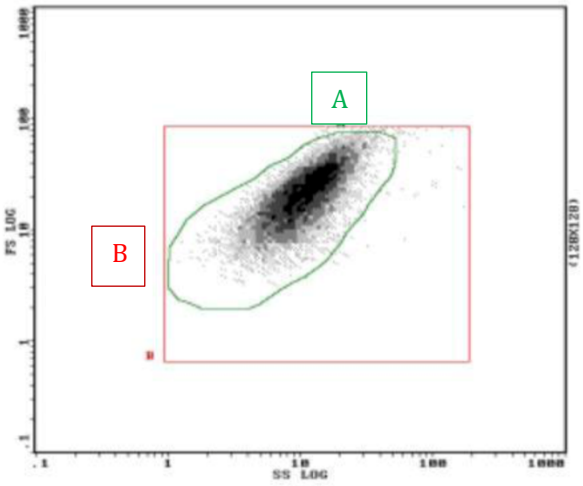
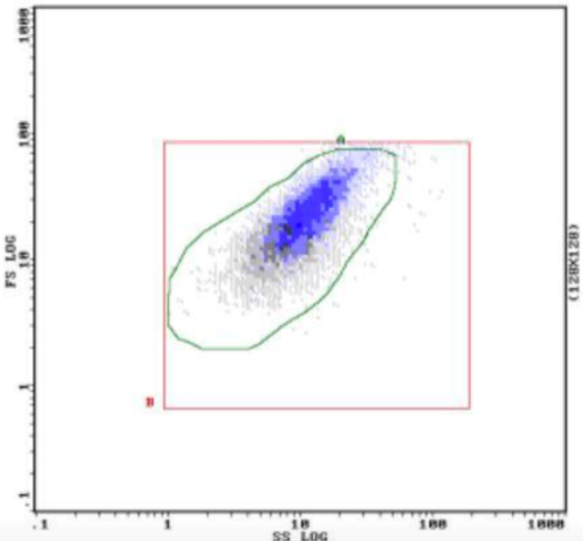


Figure 5.5.II



5.3.5. Leucocyte-Platelet Complexes (LPCs)

Principle

The expression of CD62P on the surface of an activated platelet allows the attachment of different leucocytes, including neutrophils, monocytes and lymphocytes (de Bruijne-Admiraal, Modderman et al. 1992). They attach predominantly via the P-Selectin Glycoprotein Ligand-1 (PSGL-1) receptor, and the percentage of leucocytes bound to platelets can be measured by flow cytometry (Li, Goodall et al. 1997; Furman, Benoit et al. 1998). As CD62P has been shown to be rapidly shed from the surface of degranulating platelets, elevated CD62P expression may not be found in patients with platelet activation unless the blood sample is drawn immediately distal to the site of platelet activation, the sample is taken within 5 minutes of the activating stimulus, or there is an ongoing stimulus to platelet activation (Michelson 1996; Michelson 2019). Previous studies on patients with acute myocardial infarction and those undergoing percutaneous coronary intervention (PCI) suggest that an increase in the percentage of circulating monocyte-platelet aggregates may be a more sensitive indicator of *in vivo* platelet activation than an increase in CD62P expression on platelets (Michelson 1996; Michelson, Barnard et al. 2001; Michelson 2019). Previous studies by our group have also shown elevated levels of specific leucocyte-platelet complexes in recently symptomatic compared with asymptomatic carotid stenosis (McCabe, Harrison et al. 2005; Kinsella, Tobin et al. 2013; Murphy, Lim et al. 2019). For these reasons, flow cytometric quantification of the percentage of leucocyte-platelet aggregates was performed as an additional sensitive marker of platelet activation status.

Reagents

- Distilled H_2O
- HBS
- Hanks Balanced Salt Solution (HBSS) without calcium, magnesium or phenol red (Gibco BRL, Life Technologies, Paisley, UK)
- 10% formaldehyde solution – 0.25 ml of 40% formaldehyde solution, diluted 1:4 with 0.75ml of distilled H_2O
- Diluent fixative – made up of 0.5 ml of 10% formaldehyde solution, 0.6 ml

of 10 x HBSS and 0.9 ml of distilled H_2O

- Anti-IgG1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25 ug/ml)
- Anti-CD42b-PE mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 1.56 ug/ml)
- Anti-CD45-RPE-Cy5 (Dako, Glostrup, Denmark. Concentration: 200 ug/ml)

Method

In order to equalise the concentration of the anti-IgG1-PE isotype control antibody (6.25 ug/ml) with the concentration of the anti-CD42b-PE monoclonal antibody (1.56 ug/ml), 5 µl of the anti-IgG1-PE control antibody was diluted 1:4 with 15 µl of HBS before use in the assay. The method used was based on one established by Joseph et al (Joseph, Harrison et al. 2001). 5µl of diluted anti-IgG1-PE control antibody was aliquoted into the isotype control sample tube, and 5 µl of anti-CD42b-PE antibody into the test sample tube. Afterwards, 5µl of anti-CD45-RPE-Cy5 antibody (a pan-leucocyte marker), followed by 65 µl of HBS were then added to both tubes, the samples were gently mixed, and covered until use. Within five minutes of venepuncture, 25 µl of whole blood was aliquoted into both the control and test tubes, and the samples were incubated at room temperature for 10 minutes. After incubation, the samples were fixed with 84 µl of diluent fixative. Following a further 10 minute incubation period, 840 µl of distilled water was added to each tube to lyse the erythrocytes. The samples were then analysed on the flow cytometer within three hours of venepuncture. A low flow rate was used to minimise the possibility of detecting 'dual events' i.e. the simultaneous passage of a single leucocyte and a single, unattached platelet through the flow chamber. A protocol was set up in which only CD45-positive events (leucocytes) were detected i.e. a scatterplot of log fluorescence of anti- CD45-RPE-Cy5-positive cells was plotted against side scatter, and a list-mode gate employed to exclude red cell debris from the analysis. A scatterplot of SS versus FS was then drawn to further analyse events in the gated region, and to separate three distinct subpopulations of leucocytes (neutrophils, monocytes, and lymphocytes); these were separated by manually drawing a gate around each of these subpopulations. Those cells which were dual

stained with anti-CD42b-PE and anti-CD45-RPE-Cy5 within these three separate gates were identified as platelets complexed to leucocytes, and the percentages of neutrophil-platelet, monocyte-platelet and lymphocyte-platelet complexes were calculated. The assay was terminated once 1000 “monocyte events” were detected.

Figure 5. 6 Scatterplot from a Leucocyte-Platelet Complexes Test Sample

(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

Figure 5.6.I: Only anti-CD45-RPE-Cy5-positive cells (leucocytes) are identified in region 'B' with RPE-Cy5 fluorescence detected in FL4

Figure 5.6.II: Three distinct leucocyte subpopulations can be identified by their forward and side light scatter profiles, and gates drawn around each subpopulation: C = Lymphocytes =, D = Monocytes, E = Neutrophils.

Figure 5.6.I

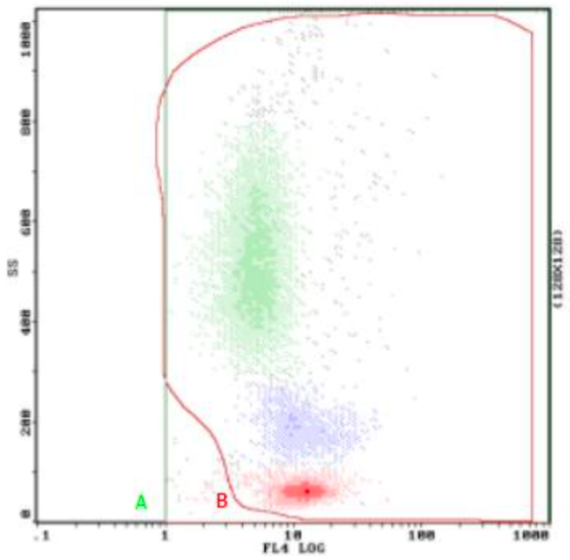
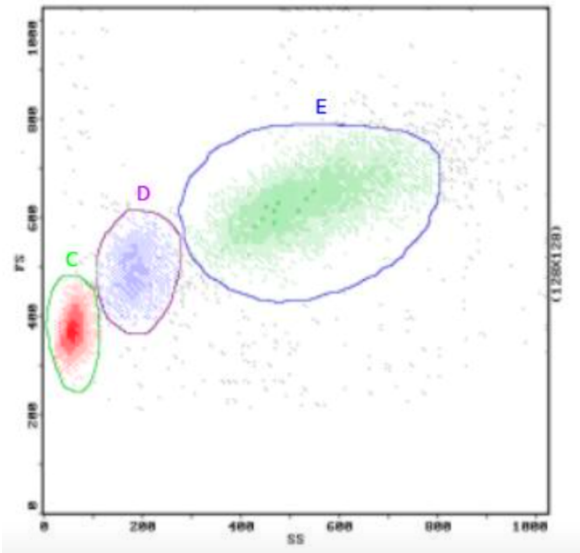


Figure 5.6.II



5.4. Platelet function testing

5.4.1. Background

As stated before, platelet function testing offers the potential to conduct reproducible, point-of-care analysis of platelet function/reactivity and assessment of the degree of inhibition of platelet function on antiplatelet therapy. Historically, the *in vivo* bleeding time was the initial most widely- available global screening test of platelet function. However, the test is invasive, crude, time consuming, poorly reproducible and insensitive (Harrison, Briggs et al. 2001; Harrison, Robinson et al. 2002) and cannot be used to serially monitor the response to therapy (Kerenyi, Schlammadinger et al. 1999). Platelet aggregometry has been considered the 'gold standard test' of platelet function over the past 6 decades. This method is based on the principle that platelets aggregate in response to exogenous agonists added to the system (Born and Cross 1963; Born and Cross 1963; Harrison 2000). Aggregometry has its own limitations and is often performed in platelet rich plasma (PRP) instead of in the physiological milieu of whole blood, with fewer data available from studies employing the well-established technique of impedance aggregometry in whole blood in patients with ischaemic cerebrovascular disease (Blann, Farrell et al. 2000). Aggregometry also requires a considerable amount of sample preparation, is labour intensive and expensive. Over the last 2-3 decades, several user-friendly devices have become available to assess platelet function in whole blood to overcome some of the problems associated with traditional aggregometry, and the advantages and disadvantages of these assays have been previously reviewed (Blann, Farrell et al. 2000). Three of the most easily-accessible, user-friendly tests of platelet function in whole blood which were available at the time we designed this study design were used in this thesis (Figure 5.7).

Figure 5. 7 Illustration of the 3 platelet function testing platforms used in this thesis.

Of note, the PFA-100 was originally supllied by Siemens and is now supplied by Sysmex, UK; the VerifyNow® was originally supplied by Accumetrics for this particular study, but is now supplied by Accriva Diagnostics (Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)

PFA-100®
(Siemens)

Multiplate®
(Roche)

Verify Now®
(Accumetrics)

5.5. PFA-100®

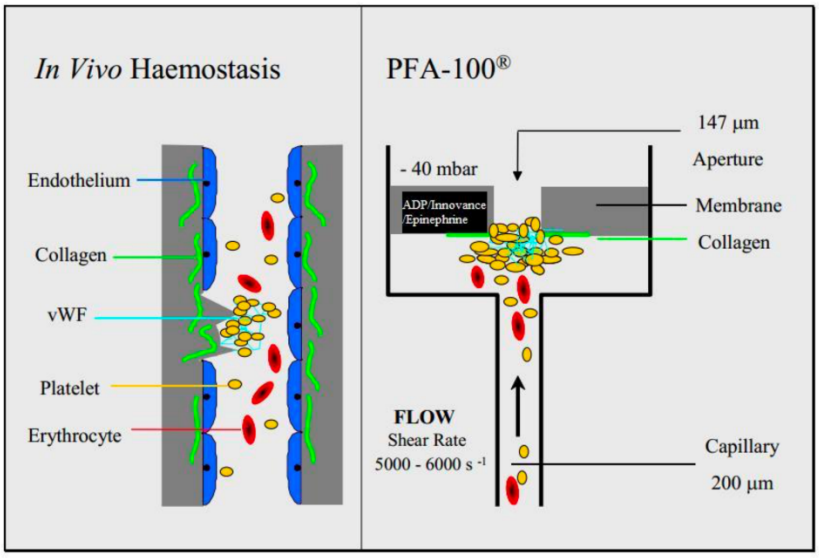
5.5.1. General Principles

The PFA-100® (Siemens, Germany) was developed to test platelet function in whole blood by simulating the *in vivo* haemostatic process at moderately high sheer stress rates (Kundu, Heilmann et al. 1995). To perform the test, 800µl of citrated anticoagulated whole blood is typically aliquoted into two disposable test cartridges that are placed on the carousel, but more recently a 3rd cartridge has been developed (see below). The carousel rotates and places the cartridges under the vacuum chuck inside the instrument, and heats the samples to 37°C prior to analysis. The blood sample is aspirated at a moderately high shear rate (5000 to 6000 s^{-1}) through a 200 µm capillary to a nitrocellulose membrane with a central 147 µm aperture (Figure 5.8). The shear rate to which platelets are exposed is consistent with that seen in a moderately stenosed artery (Kroll, Hellums et al. 1996). The membrane is coated with collagen (2 µg) in combination with either 50 µg ADP (C-ADP cartridge), 10 µg epinephrine bitartrate (C-EPI cartridge), or 20 µg ADP / 5 ng Prostaglandin E_1 / 459µg CaCl (INNOVANCE® PFA P2Y cartridge). To initialise the test, a predetermined volume of saline trigger solution is dispensed onto the membrane to solubilise the agonists. The combination of the high shear stress and biochemical stimulation activates the platelets, which adhere to the membrane and aggregate to one other forming a platelet plug that eventually occludes the aperture. The time from saline injection to complete occlusion of the aperture is called the 'closure time', and this provides a measure of platelet function in the sample. The machine is capable of recording test times up to 300 seconds, is fully automated and produces a printed result. Assays that lead to closure times greater than 300 seconds are recorded as 'test time exceeded'. We arbitrarily defined these closure times as (n+1) seconds, where n is the maximum closure time reported, and this was 300s in the majority of cases. The cartridges are disposable and are discarded at the end of each test. Because the high concentration of ADP in the C-ADP cartridge provides both a stronger stimulus to platelet activation than the epinephrine cartridge (Heilmann, Kundu et al. 1997) and can activate platelet aggregation independent of the arachidonic acid pathway, the C-EPI cartridge is much more sensitive at identifying

aspirin-induced platelet dysfunction than the C-ADP cartridge. The C-ADP cartridge has been shown to be insensitive at detecting the antiplatelet effects of clopidogrel in case-control studies (Grau, Reiners et al. 2003; Kinsella, Tobin et al. 2013; Tobin, Kinsella et al. 2013), so the INNOVANCE® PFA P2Y cartridge was specifically designed to detect the antiplatelet effects of $P2Y_{12}$ antagonists, such as clopidogrel (Kundu, Heilmann et al. 1995; Gum, Kottke-Marchant et al. 2001; Edwards, Jakubowski et al. 2012; Tsantes, Ikonomidis et al. 2012). The PFA-100 test results have been shown to be influenced by the levels of von Willebrand factor (VWF) in the circulation (Kundu, Heilmann et al. 1995; Fressinaud, Veyradier et al. 1998; Harrison, Robinson et al. 1999; McCabe, Harrison et al. 2005; McCabe, Murphy et al. 2015), as well as by the platelet count and haematocrit (Harrison, Robinson et al. 1999).

Figure 5. 8 Diagram of the in vivo haemostatic process and the PFA-100®

(*Figure adapted from Murphy SJX, PhD Thesis, TCD 2017*)

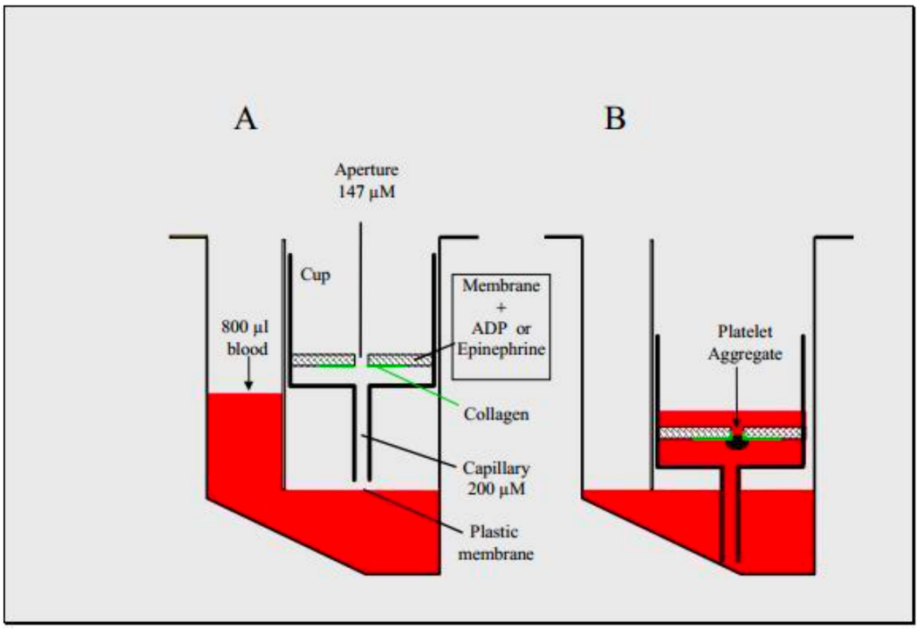


Legend for Figure 5.8: *In vivo* haemostasis: A defect in the lining of the endothelial surface of a blood vessel, such as that caused by the rupture of an atherosclerotic plaque, will lead to turbulent blood flow distal to the plaque rupture, thus increasing the shear stress platelets to which platelets are exposed. This in turn activates the platelets, and if sub-endothelial collagen is exposed, platelets will adhere to the plaque and then aggregate to each other. Ultimately, a platelet-rich thrombus forms that helps stabilise the plaque, but leads to a risk of distal platelet thromboembolism. (Figure redrawn from slide kindly donated by Dade-Behring, Germany, and adapted by Prof. D McCabe).

PFA-100®: Diagrammatic representation of a PFA-100® cup-capillary system within a test cartridge showing a platelet plug occluding the central aperture of the biologically active membrane

Figure 5. 9 PFA-100® Cup-capillary system

A:) The 800 µl blood sample is pipetted into the sample loading port at the start of the test and remains separated from the cup-capillary system by a plastic membrane; B:) During the test, the capillary tube is forced down through the membrane into the sample reservoir, and blood is aspirated through the capillary until a platelet plug forms at the aperture (redrawn from slides kindly donated by Siemens, Germany) (Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)



5.5.2 Quality Control:

The PFA-100® utilises a highly integrated microcontroller chip to manage and monitor the functions of the instrument (Kundu, Heilmann et al. 1995). Before using the instrument each day, an automated self-diagnostic test was run to ensure consistent function and integrity of the main components and sub-systems. If any faults registered, they were displayed on the LCD screen and an error code was printed. Sample cartridge testing could not proceed until all faults were resolved, and a repeat self-diagnostic performed to verify that the instrument was functioning normally.

In our laboratory, the co-efficient of variation (CV) for the C-ADP cartridge was

7%, the C-EPI cartridge was 7.5%, and the INNOVANCE® PFA P2Y cartridge was 7.8%.

Methodological Issues:

Previous studies had shown prolonged closure times with 0.129 M compared with 0.105 M or 0.106 M buffered sodium citrate blood collection systems (Mammen, Alshameeri et al. 1995; Heilmann, Kundu et al. 1997; von Pape, Dzijan-Horn et al. 2007). For this reason, only 0.105 M (3.2%) buffered sodium citrate Vacutainer® tubes were used in our laboratory for this thesis.

The INNOVANCE® PFA P2Y cartridge has been shown to have the highest correlation with other tests of platelet function if performed between 30 – 120 minutes after venepuncture (Tsantes, Ikonomidis et al. 2012). Because programming requirements prohibited running the INNOVANCE® PFA P2Y assay and the C-ADP or C-EPI assays side by side and consecutively on the device, we analysed platelet function with the INNOVANCE® PFA P2Y cartridge first between 100-120 minutes after venepuncture. To standardise our test methodology with previous studies in our laboratory (McCabe, Harrison et al. 2005; Tobin, Kinsella et al. 2011; Kinsella, Tobin et al. 2013), PFA-100® C-ADP and C-EPI testing was performed in all cases between 120 and 150 minutes after venepuncture.

The PFA-100 has been shown to be reliable and reproducible in various studies (Favaloro 2001) (Jilma 2001) (Lippi, Manzato et al. 2001) (Favaloro and Bonar 2018). The orginal normal ranges, as listed in the product inserts, are 71-118s for the C-ADP assay and 85-165s for the C-EPI assay. The normal ranges have also been established by other research groups. Franchini et al assessed 112 healthy controls and found the range for C-ADP was 70-110s and for C-EPI was 80-160s (Franchini et al. 2005). The slight variation in values between studies may be

attributed to local selection of healthy controls and other varaibles (Franchini et al. 2005)

Bock *et al*. studied the effects of smoking, oral contraceptive use and gender on platelet function in 309 healthy controls (Bock, De Haan et al. 1999). They identified slightly prolonged closure times on the C-EPI assay in smokers *vs*. non smokers, but the differences were not statistically significant. Sestito *et al*. evaluated 62 healthy controls aged 35 years to 75 years and found no correlation between age and closure times (Sestito, Sciahbasi et al. 1999). However, other studies have shown potential effects of age on PFA-100 closure times, in particular in neonates who were found to have shorter closure times compared with adults (Rand et al. 1998, Carcao et al. 1998, Lippi et al. 2001). Other factors which may be relevant include the patients' ABO blood grouping which may affect C-EPI closure times (Jilma-Stohlawetz et al. 2000, Lippi et al. 2001) and the concentration sodium citrate used to anticoagulate the blood samples (Heilmann et al. 1997, Reiter et al. 2003, Von pape et al. 2000). Low platelet counts, haematocrit or leucocyte counts may also affect PFA-100 closure times (Favalora et al. 2001, Escolar et al. 1999).

Existing 'cross-sectional/case-control' definitions of 'high on treatment platelet reactivity (HTPR)' on the PFA-100: Aspirin-HTPR has defined as failure to prolong C-EPI closure times (McCabe, Harrison et al. 2005; Kinsella, Tobin et al. 2013; Lim, Coughlan et al. 2015) and Clopidogrel-HTPR as failure to prolong INNOVANCE® PFA P2Y closure times beyond the normal range of controls, as per established manufacturer's definitions (Tsantes, Ikonomidis et al. 2012) (Tsantes et al. 2012).

5.6. VerifyNow®

5.6.1. General Principles

The VerifyNow® (Accriva Diagnostics®, USA; formerly Accumetrics® at the beginning of this thesis) is a user-friendly, *ex vivo* whole blood platelet function assay with disposable, single-use cartridges. In contrast to the PFA-100®, a combination of biochemical reagents, agonists and fibrinogen coated beads are used in this assay (van Werkum, Harmsze et al. 2008) which employs a modified light transmission aggregometry paradigm to assess inhibition of platelet function at low shear stress in response to stimulation with different platelet agonists, depending on the cartridge employed. During the test, a 2ml 3.2% sodium citrate-anticoagulated whole blood sample tube is inserted into the cartridge. The whole blood is mixed with the platelet agonists and the fibrinogen-coated beads by the movement of an electromagnetically-driven steel ball. The platelets become activated by the specific agonist in the cartridge to a degree that depends on the level of inhibition by the anti-platelet agent that the patient is receiving. Activated platelets will then bind to the fibrinogen-coated beads, cause agglutination and will fall out of the solution. Within the instrument itself, the light absorbance through the solution is measured 16 times per second. Both the rate and extent of platelet-induced agglutination/aggregation over a fixed period of time are measured and combined with a proprietary algorithm to report the values in 'reaction units' (van Werkum, Harmsze et al. 2008).

The agonists employed are arachidonic acid in the 'Aspirin cartridge' [Accriva Diagnostics VerifyNow-ASA (aspirin) assay package insert. San Diego, CA. 2012]; adenosine diphosphate (ADP), iso-thrombin receptor activating peptide (iso-TRAP), and PAR-4 activating peptide in the '$P2Y_{12}$ cartridge' [Accriva Diagnostics VerifyNow-P2Y12 (P2Y12) assay package insert; San Diego, CA. 2012]; and iso-TRAP alone which activates the platelet PAR–1 receptor in the 'GpIIb/IIIa assay' [Accriva Diagnostics VerifyNow-GP IIb/IIIa (GP IIb/IIIa assay) package insert. San Diego, CA. 2006, (Pamphlet)]. The GpIIb/IIIa assay was not studied in this thesis

and will not be discussed further.

5.6.2. Laboratory methods

Before each use of the instrument, an automated self-test was run to ensure the machine was free from errors and to promote consistency of output. Intra-assay coefficients of variation (CV) were measured in our laboratory and found to be 0.1% for the 'Aspirin cartridge' and 5.5% for the '$P2Y_{12}$ cartridge'. As described previously, two 2ml 3.2% sodium citrate-anticoagulated whole blood samples were added to the Aspirin and $P2Y_{12}$ cartridges, respectively. The 'Aspirin assays' were performed between 60-100 minutes, and the $P2Y_{12}$ assays were performed between 80-100 minutes after venepuncture. The test result was printed, with 'Aspirin Reaction units (ARU)' recorded for the Aspirin cartridge, and '$P2Y_{12}$ reaction units (PRU)' and levels of inhibition recorded for the $P2Y_{12}$ cartridge.

The VerifyNow was found to be reliable and reproducible in numerous studies (Coleman et al 2004, Malinin et al 2004, Aleil et al 2006, Dichiara et al 2007, Harrison et al 2005, Paniccia et al 2007 and Lordkipanidze 2007). This cross-sectional definition of aspirin-HTPR outlined below (ARU ≥ 550) is based on data from 65 patients on chronic aspirin therapy at a dose of 81 mg daily and 71 patients on 325 mg aspirin daily who were tested before and after ingestion of their medication (Accumetrics package insert 2004, Vanderkum et al 2008).

Existing cross-sectional definitions of HTPR on the VerifyNow

Based on the manufacture's 'cross-sectional/case-control' definitions of HTPR on antiplatelet therapy on the VerifyNow, Aspirin-HTPR has been defined as ARU ≥ 550 on the Aspirin cartridge (Accriva Diagnostics Verify Now Aspirin cartridge product insert, 2013), and Clopidogrel-HTPR as PRU ≥ 194 on the $P2Y_{12}$ cartridge (Accriva Diagnostics Verify Now $P2Y_{12}$ cartridge product insert, 2013).

Figure 5. 10 Diagrammatic representation of VerifyNow light absorbance system

(figure adapted from Murphy SJX, PhD Thesis, TCD 2017 which in turn had been adapted from the Accriva product demonstration manual)

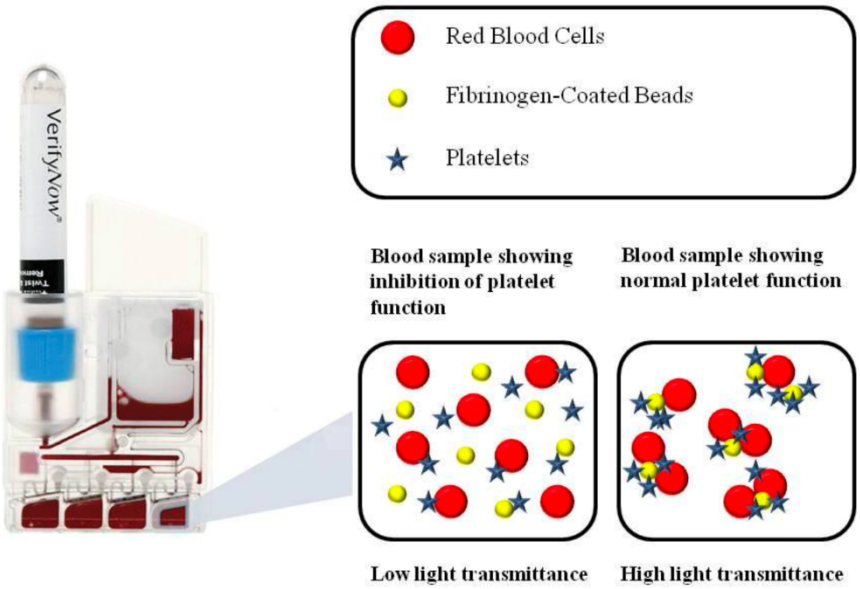
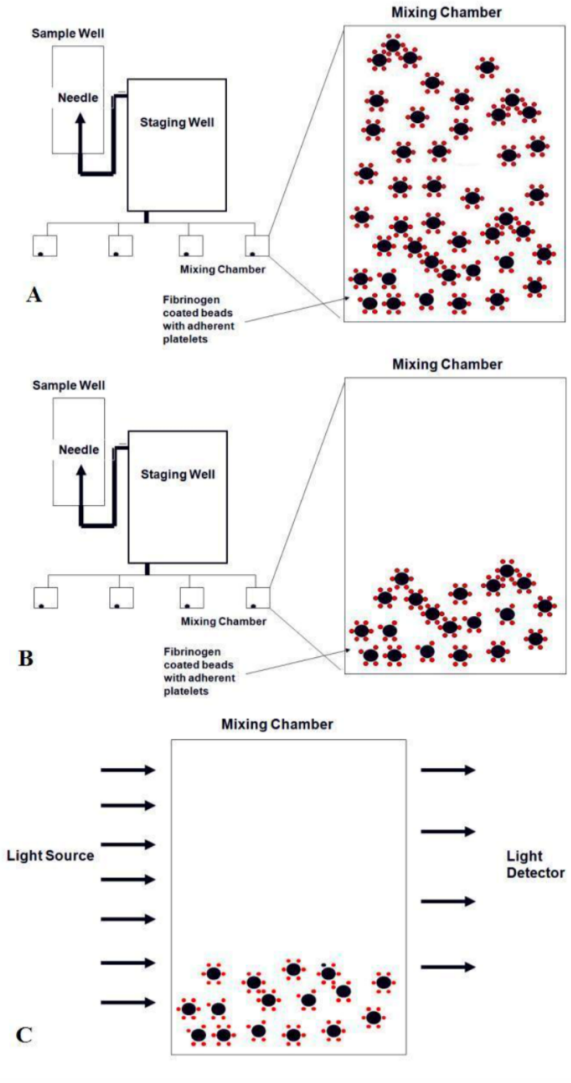


Figure 5. 11 Illustration of the VerifyNow Test principle

(Figure adapted from Murphy SJX, PhD Thesis, TCD 2017)



Legend for Figure 5.11: A: A 2 ml citrate-anticoagulated blood sample is loaded into a sample well at the start of the test and blood is aspirated into a staging well and distributed to mixing chambers containing fibrinogen coated beads and a metal ball to mix the sample.

B: During the test, the platelets adhere to the fibrinogen-coated beads depending on the degree of platelet function inhibition and fall out of solution.

C: Light from a source then passes through the sample, and the amount of light detected is dependent on the amount of platelet-fibrinogen bead complexes falling out of solution, and is converted to Aspirin Reaction Units in the Aspirin cartridge, and $P2Y_{12}$ Reaction Units in the $P2Y_{12}$ cartridge.

5.7. Multiplate® Assay (manufactured by Verum Diagnostica; owned by Roche)

5.7.1. General Principles

The Multiplate® whole blood platelet aggregation assay is performed on a multichannel, whole blood aggregometer that measures impedance to conduction of an electrical current at low shear stress as platelets adhere to 2 adjacent electrodes and aggregate to one another within a cuvette following exposure to platelet agonists.

5.7.2. Laboratory methods

A 'self-diagnostic test' was run each day before samples were analysed to ensure all assay components were functioning normally. The most consistent results in our lab were obtained when tests were performed between 100 to 120 minutes after venepuncture, so all further measurements were carried out simultaneously during this time interval.

The disposable test cuvettes contain two sets of independent impedance sensors. 300µl of hirudin-anticoagulated whole blood is added to 2 test cuvettes, diluted 1:2 with 300µl of 0.9% NaCl, heated to 37°C, and mixed with a magnetic stirrer for 3 minutes (Siller-Matula, Gouya et al. 2009). Platelets are then stimulated by the addition of either 20µL of 15mM arachidonic acid (Aspirin test; final concentration 0.5mM; Roche Multiplate ASP assay package insert 2014) or 20µL of 0.2mM ADP (ADP test; final concentration 6.5µM; Roche Multiplate ADP assay package insert 2014) to the individual cuvettes *in vitro* to measure the antiplatelet effects of aspirin or clopidogrel, respectively. Stimulated platelets adhere to the 2 adjacent electrodes and aggregate to one another within the cuvettes, thus increasing impedance at a rate proportional to the platelet reactivity in the sample. The increase in electrical impedance is recorded continuously for 6 minutes after the addition of the agonists,

and the mean values of the two independent determinations are expressed in units (U = one tenth of the Area Under the Curve), indicative of the extent of platelet adhesion and aggregation in the sample in patients on aspirin or clopidogrel, respectively.

The Multiplate assays have been shown to be reproducible in several centres, with similar intra- assay CVs for the Aspirin (8-8.6%) and ADP tests (9.5-10%) (Lee, Verheyden et al. 2012; Rubak, Villadsen et al. 2012). In our laboratory, the intra-assay CV for the Aspirin test was 7.3%, and for the ADP test was 7.8% (N = 8 assays).

The Multiplate assays were found to be reliable and reproducible in a study of 29 healthy volunteers and 81 stroke patients. (Mannu *et al.* 2015). Sabra *et al*. also assessed 72 healthy controls and 70 stroke patients and found that the mean AUC for the Aspirin Test was 97.6 +/- 27.3 and the mean AUC for the ADP test was 81.3 +/- 24.3 in the healthy controls (Sabra, Stanford et al. 2016)

Existing 'cross-sectional/case-control' definitions of HTPR on the Multiplate®:

The manufacture's recommended cross-sectional definition of Aspirin-HTPR is > 40 U for the Aspirin test [Roche Inc, Multiplate ASP assay package insert, Berlin; 2015], and of Clopidogrel-HTPR is > 46 U on the ADP test [Roche Inc, Multiplate ADP assay package insert, Berlin; 2015].

Figure 5. 12 Mutiplate test

Figure 5.12a: Multiplate ADP test output in a patient on no anti-platelet therapy

(*Figure adapted from Murphy SJX, PhD Thesis, TCD 2017*)

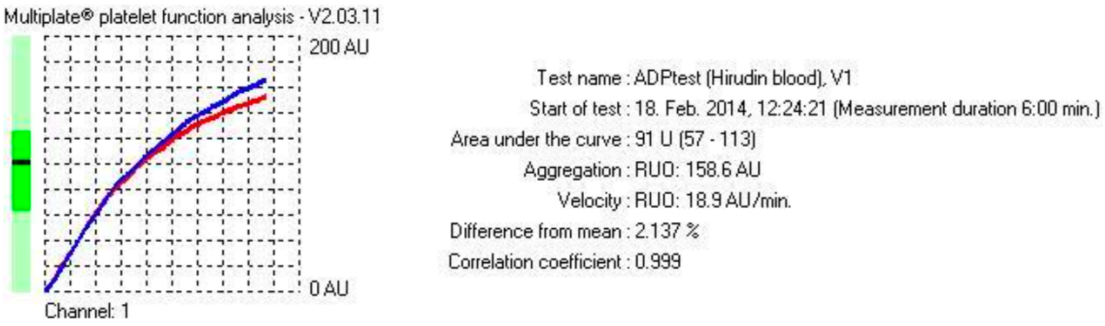


Figure 5.12a legend: AUC (Area under the curve) of 91 indicates HTPR for this assay for $P2Y_{12}$ antagonists. However, this result was expected because the patient was no on anti-platelet therapy at the time of the assay

Figure 5.12b: Multiplate Aspirin test output in a patient on Aspirin

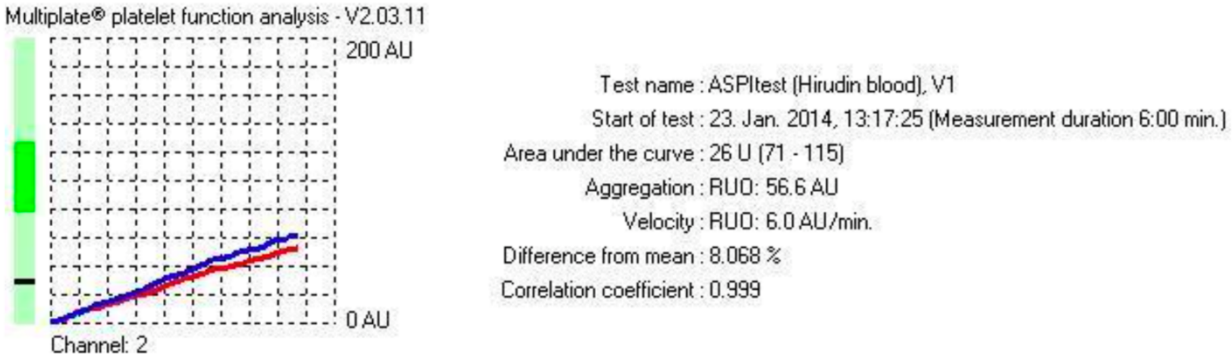


Figure 5.12b legend: AUC of 26 U indicates that this patient did not have Aspirin-HTPR on this assay on a dose of 75mg once daily for the preceding 3 months

5.8. Routine Haematological Investigations

5.8.1. Full Blood Count (FBC)

A full blood count (FBC) was performed on all study participants using a Sysmex XE-2100 haematology analyser (Sysmex U.K. Ltd., Milton Keynes, U.K.). As described above, 6ml of blood was drawn into two 3 ml sterile Vacutainer® tubes containing freeze-dried K_2 EDTA, and a further 3 ml sample was collected into a sterile Vacutainer® tube containing 0.105 M (3.2%) buffered sodium citrate. EDTA has been shown to promote more platelet swelling than citrate; however, the sodium citrate concentration dilutes the sample resulting in a lower platelet count than EDTA (P. M. Bath 1993). In view of this, total platelet count and mean platelet volume (MPV) were measured with both anticoagulants, and as stated above, all FBC measurements were performed between 2 and 4 hours after venepuncture.

6. Assessment of platelet activation status and on-treatment platelet reactivity in the early and late phases following TIA and ischaemic stroke in patients commencing aspirin monotherapy

6.1. Introduction

Aspirin is the most commonly prescribed antiplatelet drug for secondary prevention after TIA or stroke, but has traditionally been reported to reduce the relative risk of subsequent vascular events by only 13-18% (Algra and van Gijn 1996; Diener, Cunha et al. 1996). A more recent meta-analysis of 11 trials including 9635 patients has shown that the beneficial effect of aspirin may have been under-estimated in the early phase after TIA or minor ischaemic stroke, and that it may reduce the relative risk of recurrent ischaemic stroke by 59% within 6 weeks of symptom onset (HR 0.41; 95% CI: 0.30 - 0.56; $P < 0.001$) (Rothwell, Algra et al. 2016). However, the magnitude of the protective effects of aspirin following TIA or ischaemic stroke decreases over time (Rothwell, Algra et al. 2016).

Prior studies which assessed the impact of starting aspirin monotherapy on platelet activation status have been described in Chapters 3 and 4. A recent, pilot longitudinal study from our group did not show any change in platelet activation status (assessed by quantifying platelet surface CD62P and CD63 expression, and leucocyte-platelet complex formation) after commencing aspirin monotherapy or

changing from aspirin to clopidogrel monotherapy at 14 days or 90 days (Tobin, Kinsella et al. 2013).

As stated in chapter 3 and 4, the three most commonly-available commercial devices used to measure platelet function/reactivity at the time of this thesis were the PFA-100®, VerifyNow® and Multiplate®.

One can assess the *ex vivo* antiplatelet effects of aspirin in CVD patients by measuring the ability of aspirin to prolong C-EPI closure times at moderately high shear stress rates on the PFA-100® (Serebruany, Malinin et al. 2004; McCabe, Harrison et al. 2005; von Lewinski, Riggert et al. 2009). As outlined in Chapters 3 and 4, the prevalence of Aspirin-high on treatment platelet reactivity (Aspirin-HTPR) on the PFA-100 C-EPI assay varies between 15 - 60% in the early (Grundmann, Jaschonek et al. 2003; Alberts, Bergman et al. 2004; McCabe, Harrison et al. 2005; Godeneche, Sorel et al. 2009; Lai, Chen et al. 2012; Tobin, Kinsella et al. 2013) and 15 - 43% in the late phase (Grau, Reiners et al. 2003; Harrison, Segal et al. 2005; McCabe, Harrison et al. 2005; Boncoraglio, Bodini et al. 2009; Tobin, Kinsella et al. 2013) after TIA or ischaemic stroke. Quantification of inhibition of platelet function at low shear stress may also be clinically informative, especially in patients with TIA or stroke secondary to occlusion of a small intracranial artery where high shear stress-induced platelet function/reactivity might be less important. Therefore, simultaneous assessment of inhibition of platelet function at both high and low shear stress

rates is warranted in a heterogenous cerebrovascular disease patient population. Overall, using a cross-sectional case-control definition, Aspirin-HTPR on the VerifyNow Aspirin assay ranges from 6 - 33% on aspirin monotherapy during the subacute phase (Bennett, Yan et al. 2008; Seok, Joo et al. 2008; Ozben, Ozben et al. 2011; Sternberg, Ching et al. 2013; Agayeva, Gungor et al. 2014; Dharmasaroja and Sae-Lim 2014), and 8% in the late phase after a TIA or ischaemic stroke in patients taking aspirin and dipyridamole combination therapy (Kinsella, Tobin et al. 2013). As outlined in Chapter 4, several small studies have also shown variable results regarding the ability of the PFA-100 and VerifyNow to predict outcomes after TIA or ischaemic stroke. A small number of cross-sectional studies in CVD patients have also shown that the prevalence of Aspirin-HTPR on the low shear stress Multiplate Aspirin assay varies between 6.3 - 42.8% in the early phase (Yoo NT 2012; Azmin S 2013; Jastrzebska, Chelstowski et al. 2013; Łabuz-Roszak B 2015; Mannu, Macartney et al. 2015) and 12 - 13% in the late phase (Łabuz-Roszak B 2015) after symptom onset.

Monitoring the *ex vivo* antiplatelet effects of aspirin therapy with reliable laboratory tests of platelet function/reactivity has the potential to predict outcomes after TIA or stroke and further enhance our ability to facilitate personalised treatment of CVD patients.

As stated before, most prior studies have been relatively small and have used a ‘cross-sectional definition’ of Aspirin-HTPR. This definition typically categorises patients as

having antiplatelet-HTPR if their test results remain within a 'normal, healthy control range' whilst on antiplatelet treatment. There are several limitations to such a definition. Firstly, each individual is biologically different and may respond differently to an antiplatelet regimen, and an arbitrary cross-sectional definition does not take these intra-individual variables into account during platelet function/reactivity testing. Secondly, patients may not necessarily require complete inhibition of a particular platelet function pathway to gain adequate protection against a further ischaemic event as a certain degree of inhibition might be sufficient. Prospective 'longitudinal studies' in CVD patients, in which the same patients were studied before and after commencement or modification of antiplatelet therapy are relatively few (Grau, Reiners et al. 2003; Raman and Jilma 2004; Serebruany, Malinin et al. 2004; Serebruany, Malinin et al. 2005; Serebruany, Steinhubl et al. 2005; Serebruany, Malinin et al. 2008; Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013). As stated before, a novel 'longitudinal definition' of antiplatelet-HTPR has been proposed and explored by our research group as failure to alter the degree of inhibition of platelet function compared with a patient's own 'baseline value' before he/she started or changed antiplatelet therapy by more than twice the coefficient of variation of the assay (Tobin, Kinsella et al. 2013). Such 'longitudinal definitions' of antiplatelet-HTPR in CVD patients have the potential to provide more clinically meaningful information than traditional cross-sectional definitions of HTPR (Tobin, Kinsella et al. 2013). Nevertheless, cross-sectional data may also be very clinically informative (Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013), but studies simultaneously collecting both sets of data have rarely been performed.

6.2. Aims and Hypothesis

The **aims** of this aspect of the longitudinal, observational OATS study were:

1. To assess the ability of aspirin to influence platelet activation status using established, sensitive and specific 'unstimulated whole blood flow cytometry' assays;

2. To simultaneously assess and compare the ability of 3 different commercially-available laboratory tests of platelet function/reactivity to identify patients with recent TIA or ischaemic stroke who have Aspirin-HTPR *ex vivo* according to established 'cross-sectional' and 'novel longitudinal definitions' of Aspirin-HTPR;

3. To formally assess inter-device agreement in the definition of Aspirin-HTPR status using established 'cross-sectional' and 'novel longitudinal definitions' of Aspirin-HTPR;

Hypotheses:

1. Based on prior pilot data from our group and others, commencement of aspirin would not significantly influence platelet activation status using 'unstimulated whole blood flow cytometry' assays in patients with TIA or ischaemic stroke;

2. There would be a higher prevalence of Aspirin-HTPR *ex vivo* during simultaneous testing with certain platelet function assays than with others, and Aspirin-HTPR would be more commonly observed in CVD patients with a

moderately high shear stress test of platelet adhesion/aggregation than with low shear stress tests of platelet aggregation

3. Estimates of the prevalence of Aspirin-HTPR would be lower with novel longitudinal definitions than with established cross-sectional definitions of HTPR;

6.3. Methods

6.3.1. Recruitment

Patients who met the inclusion criteria were recruited during the period between 26/10/2011 and 13/01/2016 at AMNCH-TUH.

Inclusion criteria

Patients were eligible for inclusion if they were ≥ 18 years of age, within 4 weeks of onset of a TIA or ischaemic stroke, 'antiplatelet naive' and their treating physician decided to commence them on aspirin monotherapy.

Exclusion criteria

Patients were excluded if they had myocardial infarction, DVT, PE or surgery within the preceding three months; ongoing unstable angina or unstable symptomatic peripheral vascular disease; platelet count < 100 x 10^9/L; known bleeding or clotting diathesis, including known platelet-related bleeding disorders;

active proven vasculitis; active neoplasia; non-steroidal anti-inflammatory drug (NSAID) intake, other than aspirin, in the preceding 11 days; were unlikely to be able to attend for clinical follow-up and repeat testing at 14 +/- 7 days.

6.3.2. Controls

Nineteen healthy controls of similar age and sex, with no prior history of cerebrovascular disease, were recruited from the local population and from amongst relatives and acquaintances of participating subjects to determine the 'normal range' of the C-ADP and C-EPI PFA-100 assays in our laboratory (mean age 54 years; 64% male; Kinsella JA, personal communication, 2016]). The normal ranges for the PFA INNOVANCE P2Y, VerifyNow and Multiplate assays were determined from data published by the manufacturers on larger numbers of healthy controls. In addition, 10 healthy controls of similar sex and age, with no prior history of cerebrovascular disease were also recruited from among hospital staff to establish the 'local normal range' of the PFA INNOVANCE P2Y, VerifyNow and Multiplate assays in our own laboratory to ensure our control values fell within these expected published ranges. All controls were studied once and underwent colour Doppler ultrasound of carotid and vertebral arteries (CDUS) to exclude asymptomatic ≥50% carotid or vertebral stenosis prior to inclusion. Subjects were also excluded from the control group if they were on antiplatelet therapy or NSAIDs within the preceding 14 days, or had any other exclusion criteria which applied to the patients, as outlined before.

Ethical approval:

Written informed consent, or 'proxy consent' where appropriate, was obtained from all subjects. This study was approved by the St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2011/35/03).

6.3.3. Clinical Assessment, Timing and Follow-up

Patients underwent detailed clinical assessment and were classified as having had a TIA or ischaemic stroke when this was confirmed by their Consultant Neurologist or Stroke Physician after clinical investigation according to ESO recommendations (European Stroke Organisation Executive and Committee 2008). The diagnosis was also confirmed in all cases by me as a clinically experienced Research SpR in Vascular Neurology under the supervision of Prof McCabe, or by Prof McCabe himself. The underlying mechanism responsible for TIA or Stroke was categorised according to the TOAST classification (Adams, Bendixen et al. 1993) and ASCOD classification (Amarenco, Bogousslavsky et al. 2013)

Patients underwent detailed clinical and laboratory assessment with venepuncture before (**baseline**), 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after starting aspirin. In addition, clinical follow-up to assess longer-term risk of recurrent vascular events and outcome measures was performed using a validated in-person or telephone questionnaire at ≥ 1 year after symptom onset.

6.3.4. Laboratory Assessments

A detailed description of the laboratory testing performed has been previously described in Chapter 5 (General Methods). In summary, careful, 'atraumatic' venepuncture was performed in a standardised manner, as outlined previously (McCabe, Harrison et al. 2004) (Kinsella, Tobin et al. 2013). 2 ml sterile 3.2% buffered sodium citrate-anticoagulated samples were taken for analysis of platelet function with the VerifyNow® system (Accriva Diagnostics). Six further samples were taken in sterile 3 ml Vacutainers® containing 3.2% buffered sodium citrate. The first and second of these citrate-anticoagulated samples were used for whole blood flow cytometric analysis and for measurement of platelet function with the platelet function analyser (PFA-100®, Dade- Behring, Germany). Subsequently, one 3 ml double-walled Vacutainer® tube containing recombinant 'hirudin anticoagulant' was taken for analysis of platelet function on the Multiplate® system. Three 3ml sterile Vacutainer® tubes containing K_2EDTA were obtained. This first sample was used to measure the full blood count (FBC), including measurement of the mean platelet volume (MPV) and platelet distribution width (PDW).

Whole Blood Flow Cytometry: Platelets were distinguished from red and white blood cells, as described previously (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013). The expression of certain proteins that are upregulated to the platelet surface during platelet activation were quantified, including CD62P and CD63 (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013). The percentages of circulating leucocyte-platelet complexes were also quantified as additional, more sensitive markers of platelet activation using previously described methods (chapter 5).

PFA-100® Platelet function analyser: The degree of inhibition of platelet function in whole blood at **moderately high shear stress** was assessed before and after starting aspirin in response to biochemical stimulation with collagen and epinephrine (C-EPI cartridge), collagen-ADP (C-ADP cartridge), or ADP/Prostaglandin E1 (INNOVANCE P2Y cartridge, as previously described (McCabe, Harrison et al. 2005). As stated before, this device mimics the *in vivo* haemostatic process that one may see in a moderately stenosed artery. The time taken for activated platelets to occlude the aperture in the cartridge is called the closure time. The maximum closure time recorded by the device is 300s, and we arbitrarily defined closure times above 300s as 301s for statistical analyses.

Cross-sectional definitions of Aspirin-HTPR on the PFA-100*:* Aspirin-HTPR was defined as failure to prolong C-EPI closure times beyond the mean + 2 standard deviations of our control range (176 seconds) in patients on aspirin monotherapy (Tobin, Kinsella et al. 2011; Kinsella, Tobin et al. 2013).

Novel longitudinal definition of Aspirin-HTPR on PFA-100*:* The intra-assay CV for C-EPI cartridge assay in our lab was 7.5%. Based on recent pilot data from Prof McCabe's lab, 'Aspirin-HTPR' was also defined as failure to prolong C-EPI closure times compared with the patient's baseline value on no antiplatelet treatment by more than twice the intra-assay CV of the assay i.e. failure to prolong C-EPI closure times by >15% of the patient's baseline C-EPI closure time (Kinsella, Tobin et al. 2013; Tobin, Kinsella et al. 2013).

VerifyNow® Platelet Function Analyser: The VerifyNow® is a cartridge-based analyser, containing fibrinogen coated beads which assesses *ex vivo* platelet reactivity at **low shear stress** in response to stimulation with fixed doses of

different platelet agonists, depending on the cartridge employed (van Werkum, Harmsze et al. 2008) (see chapter 5 for a detailed description of this device and the constituents of the Aspirin and P2Y12 cartridges). Both the rate and extent of platelet-induced aggregation over a fixed period of time are measured and combined with a proprietary algorithm to report the values in 'reaction units' (van Werkum, Harmsze et al. 2008).

Current cross-sectional definition of Aspirin-HTPR on the VerifyNow: Aspirin-HTPR in patients on aspirin monotherapy was defined according to the manufacturer's definition as Aspirin Reaction Units (ARU) ≥550 on the Aspirin cartridge (Kinsella, Tobin et al. 2013).

Novel longitudinal definition of Aspirin-HTPR on VerifyNow®: Intra-assay coefficients of variation (CV) were measured in our laboratory and found to be 0.1% for the Aspirin cartridge. As stated before, Aspirin-HTPR was defined as failure to shorten the ARU on aspirin compared with the patient's baseline on no antiplatelet treatment by more than twice the CV of the Aspirin assay i.e. failure to shorten the ARU by ≥ 0.2% of patient's baseline ARU.

Multiplate® assay*:* As outlined previously, this whole blood platelet aggregation assay is based on measurement of impedance at **low shear stress** as platelets adhere to 2 adjacent electrodes and aggregate to one another within a cuvette (Chapter 5). The extent of platelet adhesion and aggregation is recorded as the Area Under the Curve (AUC) up to 6 minutes after the addition of either arachidonic acid or ADP to measure the antiplatelet effects of aspirin or clopidogrel, respectively.

Cross-sectional definition of HTPR on Multiplate*:* The manufacturer's recommended cross-sectional definition of Aspirin-HTPR is > 40U for the Aspirin

test [Roche Inc, Multiplate ADP assay package insert, Berlin; 2015].

Novel longitudinal definition of Aspirin-HTPR on Multiplate: In our laboratory, the intra-assay CV for the Aspirin test was 7.3% (N = 8 assays). Aspirin-HTPR was defined as failure to reduce aggregation on the Multiplate Aspirin test compared with the patient's baseline value on no antiplatelet treatment by more than twice the intra-assay CV of the assay i.e. failure to decrease the AUC by > 14.6% of the patient's baseline AUC.

6.3.5. Statistical Methods

All statistical analysis was performed using SPSS (Version 23). The Wilcoxon signed rank test was used for comparison of median C-EPI, C-ADP, Innovance P2Y, VerifyNow Aspirin, VerifyNow P2Y12, Multiplate Aspirin and Multiplate ADP results at 14d and 90d *vs*. baseline. McNemar's test was used to compare the cross-sectional and longitudinal HTPR data on the PFA-100, VerifyNow and Multiplate devices. Agreement between the different testing devices was assessed with Cohen's unweighted kappa statistics; 95% confidence intervals (CIs) were calculated. Kappa values of < 0 were taken to indicate 'poor agreement'; 0 - 0.2 indicate 'slight agreement'; 0.21 - 0.40 indicate 'fair agreement'; 0.41 - 0.60 indicate 'moderate agreement'; 0.61 - 0.80 indicate 'substantial agreement', and 0.81 - 1.00 indicate 'almost perfect agreement', 1.0 represents 'perfect agreement' (Kinsella, Tobin et al. 2011). Logistic regression analysis was used to assess the relationship between HTPR status and the risk of recurrent TIA, stroke or vascular events in this patient subgroup. $P < 0.05$ was considered to represent statistical

significance.

6.4. Results

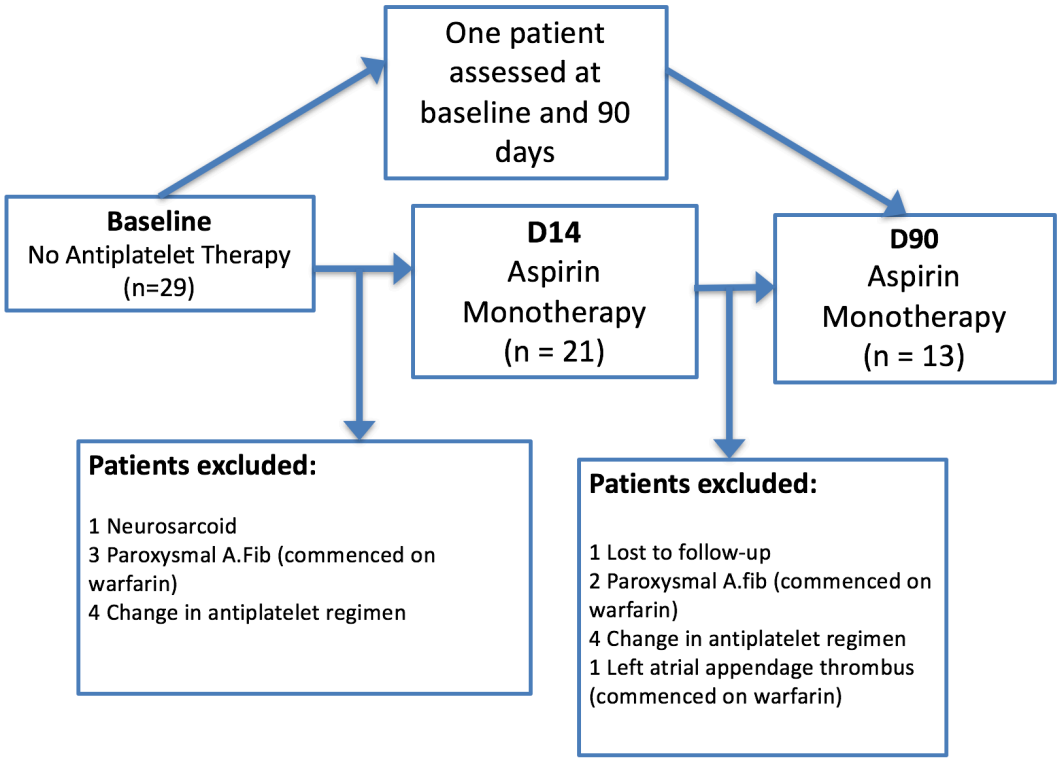
Subject recruitment in the No Medication to Aspirin Group

Twenty-nine patients were assessed at baseline on no medication. Twenty-one of these patients were retested at 14d on aspirin monotherapy, and thirteen patients were subsequently followed up at 90d. All thirteen patients were followed up for at least one year to assess clinical outcomes, including the risk of any further ischaemic events either in person or via telephone.

Some patients were excluded from the study for the following reasons: one patient was lost to follow up and was not contactable; one was subsequently diagnosed as having neurosarcoidosis; five patients were found to have paroxysmal atrial fibrillation and were subsequently commenced on warfarin; one patient had a left atrial appendage thrombus and was also commenced on warfarin. One patient was assessed at baseline and 90 days but had missed the 14d follow-up due to intercurrent illness. The remaining patients did not have 90d follow-up data on aspirin monotherapy because dipyridamole was added to their anti-platelet treatment regimen by their treating physician. Data were also unavailable from some of the whole blood flow cytometry and platelet function assays during follow up because the devices required servicing intermittently when appointments were due following recruitment.

The median daily dose of Aspirin was 300 mg at 14d and 75 mg at 90d. None of the patients in this subgroup on ‘no medication to aspirin’ had a recurrent vascular event by the 1 year follow-up visit.

Figure 6. 1 Flowchart of patients who were studied on no antiplatelet therapy and subsequently at 14d and 90d on aspirin monotherapy



The clinical details of the study subjects (table 6.1), and their TIA/stroke subtypes (tables 6.2 and 6.3) are outlined below.

Table 6. 1 Demographic and vascular risk factor profiles at enrolment in patients who changed from no medication to aspirin
TIA = Transient Ischaemic Attack; IHD = Ischaemic Heart Disease; DVT = Deep Venous Thrombosis; PE = Pulmonary Embolism

Characteristic	**Nil to Aspirin (n = 29) Number (%)**
Mean Age (years)	56 (SD 10.9)
Gender (M/F)	14/13
Ischaemic Stroke at Presentation	1 (3.4%)
TIA at Presentation	28 (96.6%)
Thrombolysed	0
Prior Stroke/TIA	3 (10.3%)
IHD	3 (10.3%)
Hypertension	9 (31%)
Diabetes Mellitus	3 (10.3%)
Atrial Fibrillation at Enrolment	0
Hyperlipidaemia	10 (34%)
Family History of Stroke	9 (31%)
Prior DVT/PE	0
Peripheral Vascular Disease	0
Migraine with and without aura	6 (20.7%)
Current Smoker	9 (31%)
Ex-Smoker	8 (27.6%)
Never Smoker	12 (41.4%)
Statin Therapy	7 (24.1%)

Table 6. 2 TOAST classification at enrolment of patients who changed from no medication to aspirin

	Nil to Aspirin (Total N = 29) % (N)
Large artery atherosclerotic	3.4% (1)
Small vessel disease	13.8% (4)
Cardioembolic	20.7% (6)
Other determined	3.4% (1)
Undetermined aetiology	58.6% (17)

Table 6. 3 ASCOD Classification at enrolment of patients who changed from no medication to aspirin

ASCOD Phenotype	**Disease present (ASCO 1,2,3)**	**Disease absent (ASCO 0)**	**Insufficient investigation (ASCO 9)**
A % (N)	31.0% (9)	69.0% (20)	0
S % (N)	55.2% (16)	44.8% (13)	0
C % (N)	58.6% (17)	41.4% (12)	0
O % (N)	31.0% (9)	69.0% (20)	0
D % (N)	0	100% (29)	0

A: atherosclerosis, S: small vessel disease, C: cardiac source, O: other cause; D: Dissection
1: 'definitely a potential cause of the index stroke', 2: 'causality uncertain', 3: 'unlikely a direct cause of the index stroke (but disease is present)' 0: 'disease absent'

6.4.1. Impact of commencing aspirin on platelet activation status

Data were available for analysis on 22 patients at baseline, 21 patients at 14d and 13 patients at 90d. There were no statistically significant changes in platelet surface CD62P or CD63 expression, or the percentages of any circulating leucocyte-platelet complexes between the baseline assessment and 14d or 90d after commencing aspirin monotherapy (Table 6.4; Figures 6.3a, 6.3b, 6.4 A-C).

Table 6. 4 Comparison of platelet activation markers between baseline and 14d and 90d in patients commencing aspirin.

Values are medians (range: min-max*)*

	Baseline (N = 22)	**14 days (N = 21)**	**90 days (N = 13)**
Platelet surface markers			
% CD62P	3.09 (0.42 – 8.40)	3.54 (0.30 – 10.19)	2.19 (1.10 – 9.08)
P value		0.79	0.79
% CD63	14.75 (0.25 – 30.20)	16.20 (0.21 – 30.80)	17.10 (1.66 – 27.90)
P value		0.74	0.72
Leucocyte-Platelet Complexes			
% Neutrophil-Platelets	2.73 (0.53 – 4.59)	2.87 (1.41 – 4.83)	2.66 (0.49 – 5.76)
P value		0.89	0.39
% Monocyte – Platelets	6.45 (0.53 – 13.60)	5.46 (1.90 – 12.80)	4.93 (0.60 – 9.70)
P value		0.81	0.72
% Lymphocyte-Platelets	2.29 (0.39 – 3.77)	2.18 (1.21 – 6.82)	2.06 (0.55 - 5.22)
P value		0.76	0.72

Figure 6. 2 % CD62P expression after starting aspirin

Median: 3.09 (0.42 – 8.4) Median: 3.54 (0.3 – 10.19) Median: 2.19 (1.1 – 9.08)

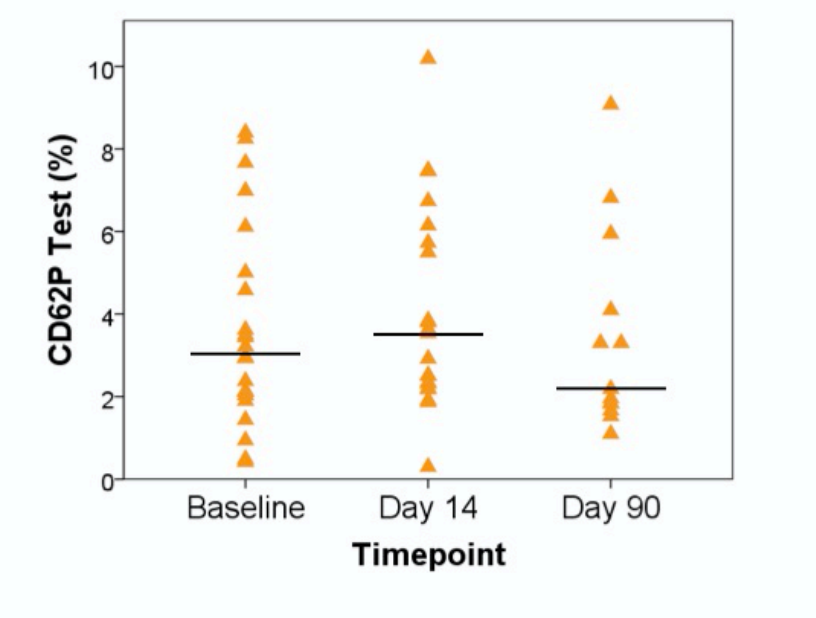


Figure 6. 3 %CD63 expression after starting aspirin

Median: 14.75 (0.25 – 30.2) Median: 16.2 (0.21 – 30.8) Median: 17.1 (1.66 – 27.9)

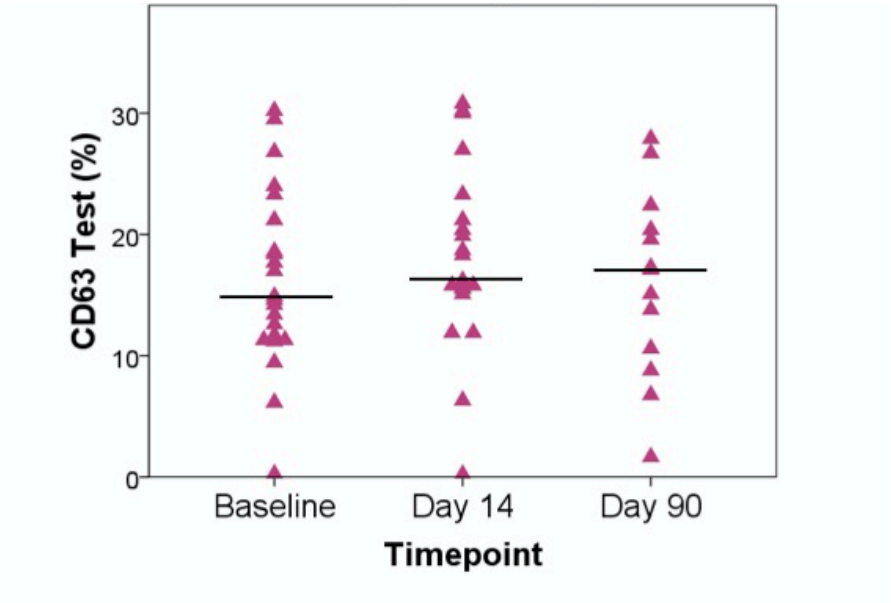
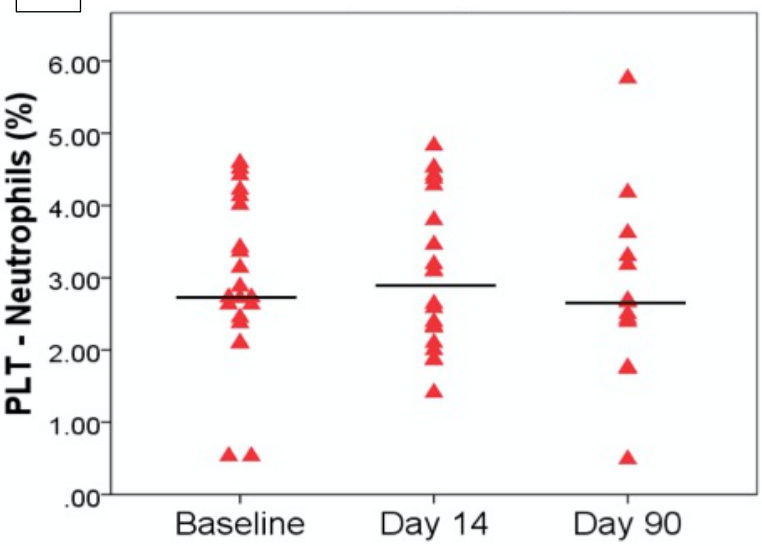


Figure 6. 4 % Circulating leucocyte-platelet complexes after starting aspirin

Median: 2.73 (0.53 – 4.59)	Median: 2.87 (1.41 – 4.83)	Median: 2.66 (0.49 – 5.76)

A



B

Median: 6.45 (0.53 – 13.6)

Median: 5.46 (1.9 – 12.8)

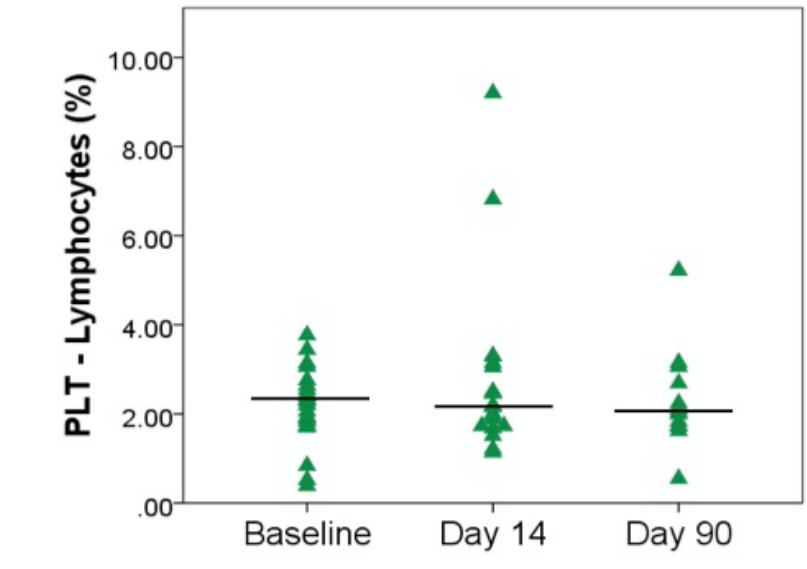
Median: 4.93 (0.6 – 9.7)

Median: 2.29 (0.39 – 3.77)

Median: 2.18 (1.21 – 6.82)

Median: 2.06 (0.55 – 5.22)

C



6.4.2. Impact of commencing aspirin on platelet function / reactivity

Twenty-nine patients were assessed at baseline on no medication. Data were available in 21 of those patients at 14d and 13 patients at 90d after commencing aspirin monotherapy.

PFA-100® data

C-EPI cartridge

As expected after commencing aspirin monotherapy (Tobin, Kinsella et al. 2013; Lim, Coughlan et al. 2015), median C-EPI closure times were significantly prolonged at both 14d [301s (range: 114 – 301s) *vs*. 115.5s (range: 80-196s), $p < 0.001$] and 90d [301s (range: 155 – 301s) *vs*. 114s (range: 80-196s), $p = 0.002$) (figure 6.5a).

Using a cross-sectional, case-control definition, 5/21 patients (23.8%) had Aspirin-HTPR at 14d; four of these five patients did not have follow-up data at 90d, but one patient no longer exhibited Aspirin-HTPR when retested at 90d. One of 13 patients (7.6%) had Aspirin-HTPR at 90d but had not had Aspirin-HTPR at 14d. The other

12 patients retained the same Aspirin-HTPR status during follow-up between 14d and 90d. Therefore, amongst patients who had data at both 14d and 90d after commencing aspirin, 12/13 (92.3%) retained the same cross-sectional/case-control Aspirin-HTPR status at 14d and 90d.

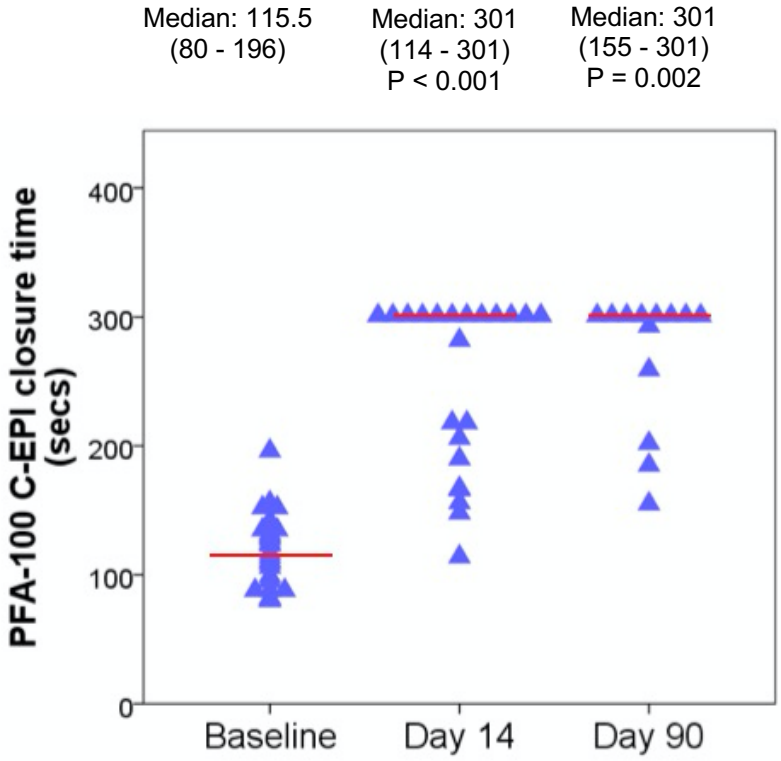
Using our novel longitudinal definition, only 1/21 patients (4.8%) had Aspirin-HTPR at 14d but this patient did not have follow-up data at 90d. One of 13 patients (7.6%) had Aspirin-HTPR at 90d, but did not have longitudinal data on Aspirin-HTPR status at 14d. Amongst patients who had data at both 14d and 90d after commencing aspirin, 12/13 (92.3%) retained the same Aspirin-HTPR status at 14d and 90d.

When we compared the classification of Aspirin-HTPR status according to the 2 definitions, four patients had Aspirin-HTPR using a cross-sectional definition at 14d, but none of these four had Aspirin-HTPR using the longitudinal definition at this time point. Only 1/21 patients (4.7%) had Aspirin-HTPR by both cross-sectional and longitudinal definitions at 14d, but this patient did not have data available at 90d (table 6.6).

Figure 6. 5 PFA-100® data in patients commencing aspirin

Figure 6.5a: Scatterplot of PFA-100® C-EPI closure times at baseline, 14d and 90d in patients changing from no medication to aspirin

Values are medians (Range: min-max) in the following scatterplots

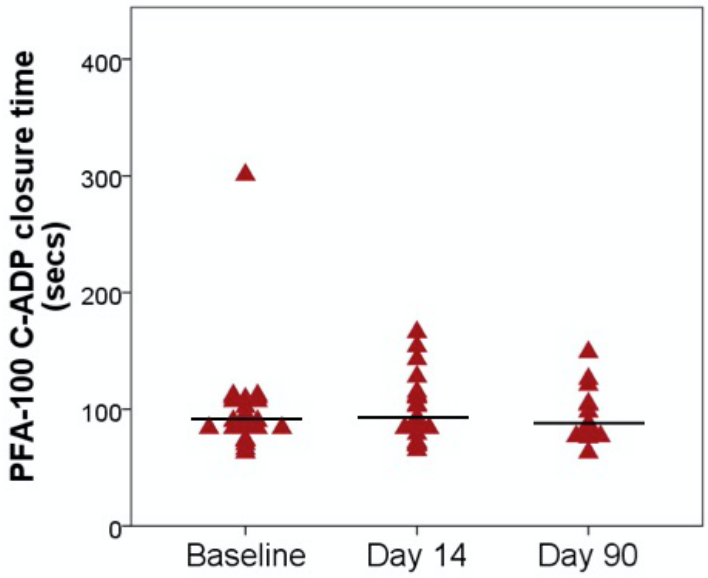


C-ADP cartridge

C-ADP data were available in 26 patients at baseline, 21 patients at 14d and 13 patients at 90d. One patient had a baseline C-ADP closure time of > 300s, but was not known to have any clinical history of a platelet-related bleeding disorder and was not on any antiplatelet treatment or NSAIDs at that time. This patient's baseline C-EPI closure time of 135s was

within the 'normal control range'. Median C-ADP closure times did not significantly change after commencing aspirin monotherapy at 14d [93s (range: 65–166s) *vs*. 88s (range: 66 -301s), p = 0.19] or 90d [88s (range 63 – 149) *vs*. 88s (range: 70 – 301s), p = 0.6] (figure 6.5b).

Figure 6.5b: PFA-100® C-ADP closure times at baseline, 14d and 90d in the no medication to aspirin group

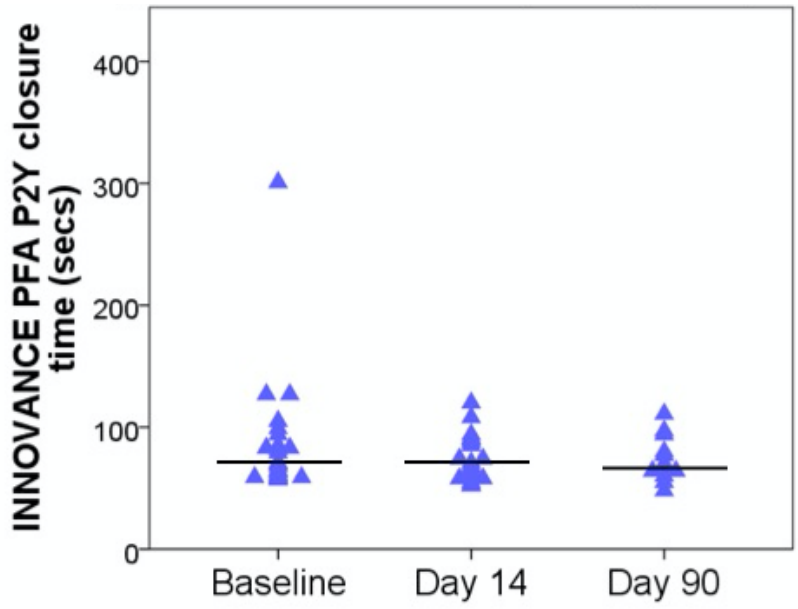


Innovance P2Y Cartridge

Innovance P2Y data were available on 24 patients at baseline, 18 patients at 14d and 11 patients at 90d. One patient also had a closure time of 301s at baseline, but was not known to have any platelet-related bleeding disorder and was not on any antiplatelet treatment or NSAIDs. This was a different patient to the patient who had a C-ADP closure time of >300s who did not have any known platelet-related bleeding disorder either.

There were no significant changes in Innovance P2Y closure times after commencing aspirin monotherapy at 14d [72.5s (range: 52– 120s) *vs*. 69s (range: 57 -301s), p = 0.99; N = 18] or 90d [68s (range: 48–111s) *vs*. 70s (range: 58 – 301s), p = 0.62; N = 11) (figure 6.5c).

Figure 6.5c: PFA-100® Innovance P2Y closure times at baseline, 14d and 90d in the no medication to aspirin group



VerifyNow® data

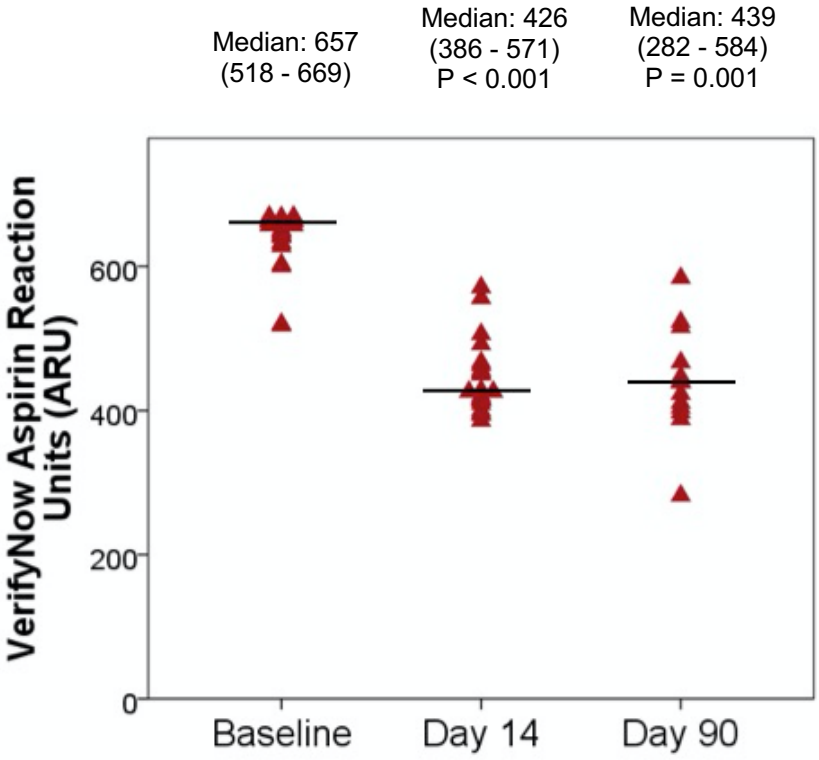
Aspirin Cartridge

VerifyNow Aspirin data were available in 29 patients at baseline, 21 patients at 14d and 13 patients at 90d. After commencing aspirin monotherapy, Aspirin Reaction Units (ARU) decreased significantly at 14d [426 (range: 386–571) *vs*. 657 (range 518 - 669); p <0.001] and 90d [439 (range: 282 – 584) *vs*. 662 (range: 603 - 669); p = 0.001] (figure 6.6a).

Using a cross-sectional definition, 2/21 patients (9.5%) had aspirin-HTPR at 14d. Of these two patients, one did not have persistent Aspirin-HTPR at 90d and the other patient did not have available data at 90d. 1/13 (7.7%) had aspirin-HTPR at 90d, but had not had Aspirin-HTPR at 14d. Amongst patients who had data at both 14d and 90d after commencing aspirin, 10/13 (76.9%) retained the same Aspirin-HTPR status at 14d and 90d. No patients had aspirin-HTPR at 14d or 90d using the novel longitudinal definition.

Figure 6. 6 (a) and (b) Data from VerifyNow ® in patients changing from no medication to Aspirin

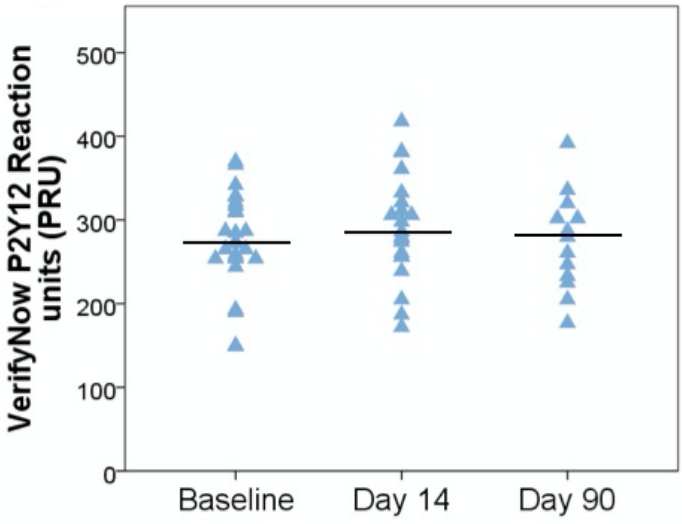
Figure 6.6a: VerifyNow® ARU at baseline, 14d and 90d in patients changing from no medication to aspirin



P2Y12 cartridge

VerifyNow P2Y12 data were available in 26 patients at baseline, 21 at 14d and 13 patients at 90d. There was a statistically significant increase in P2Y12 reaction units (PRU) at 14d compared with baseline [286s (range: 172-418s) *vs*. 265s (range: 149-370s); p = 0.045] (Figure 6.6b). However, this was not sustained during follow-up, with no significant change in median PRU values at 90d compared with baseline [280s (range: 177-392s) *vs*. 285s (range: 190-366s); p = 0.25] (Figure 6.6b).

Figure 6.6b: VerifyNow® PRU at baseline, 14d and 90d in the no medication to aspirin group



Multiplate® data

Multiplate Aspirin assay

Multiplate Aspirin data were available in 29 patients at baseline, 21 at 14d and 13 patients at 90d. After treatment with aspirin monotherapy, AUC significantly decreased at 14d [19 U (range: 4-78) *vs*. 107 U (range: 48 -179); $p < 0.001$, N=21] and at 90d [13.5 U (range: 3-101) *vs*. 106.5 U (range: 75 – 179); $p < 0.001$, N = 13] (figure 6.7a).

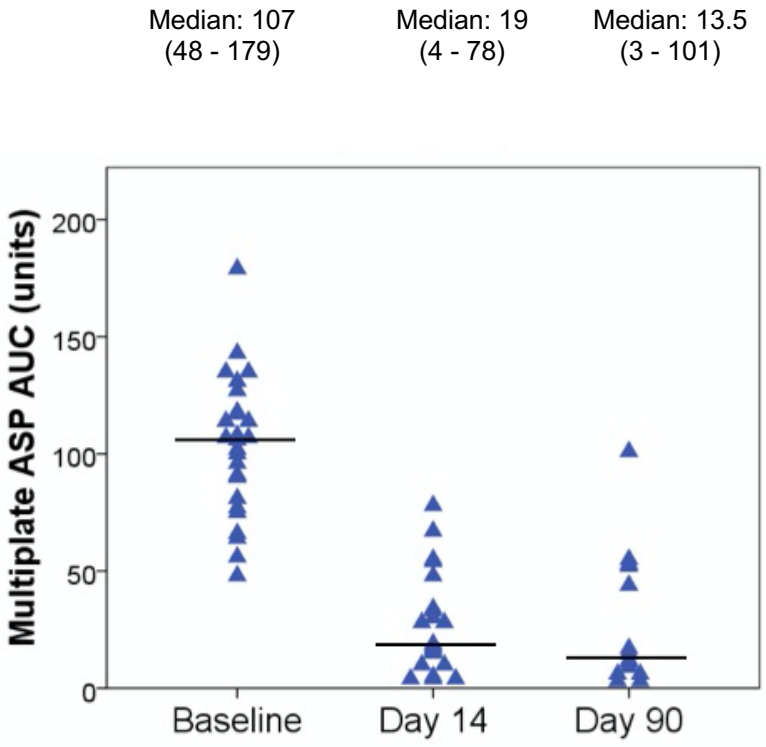
Using a cross-sectional, case-control definition, 5/21 (23.8 %) patients had Aspirin-HTPR at 14d. Of these five patients, three had persistent Aspirin-HTPR at 90d but two did not have available data at 90d. One patient did not have aspirin-HTPR at 14d but developed aspirin-HTPR at 90d. Therefore, a total of 4/13 (30%) patients had Aspirin-HTPR at 90 days. Amongst patients who had data at both 14d and 90d after commencing aspirin, 12/13 (92%) retained the same Aspirin-HTPR status at 14d and 90d.

Using the longitudinal definition, only 1/21 (4.8%) patients had Aspirin-HTPR at 14d.

This patient also had aspirin-HTPR at 14d using the cross-sectional definition, but there were no data available for this patient at 90d. In contrast to the cross-sectional definition, none of the patients had Aspirin-HTPR at 90d according to the longitudinal definition.

Figure 6. 7 (a) and (b) Data from Multiplate® in patients from no medications to Aspirin

Figure 6.7a: Multiplate® Aspirin assay AUC data at baseline, 14d and 90d in the no medication to aspirin group



Multiplate ADP assay

Multiplate ADP data were available in 25 patients at baseline, 20 patients at 14d and 13 patients at 90d. After treatment with aspirin monotherapy, there was no significant change in the ADP AUC at 14d [101.5 U (range: 65-140) *vs*. 88.5 U (range: 55 -155); p = 0.054, N = 20] or at 90d [100.5 U (range: 52-129) *vs*. 91 U (range: 55-155); p = 0.64, N = 13] (figure 6.7b).

Figure 6.7b: Multiplate® ADP assay AUC data at baseline, 14d and 90d in the no medication to aspirin group

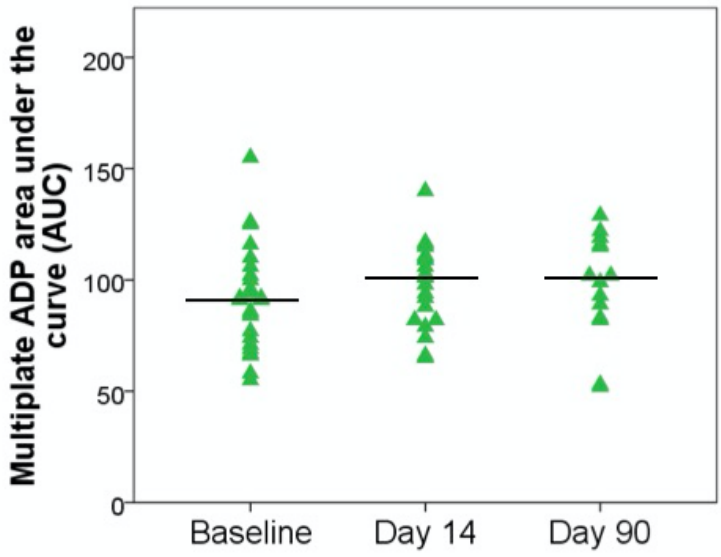


Table 6. 5 Summary of results of all platelet function/reactivity assays in response to commencing aspirin monotherapy in 'antiplatelet naïve' CVD patients.

Values represent medians (range: min-max). P values refer to comparisons between data at baseline *vs*. 14d and baseline vs. 90d. Significant P values highlighted in bold. Of note, median values at baseline on each assay represent values in the 'entire dataset' because comparisons between matched datasets varied between 14d and 90d; relevant values are described in the text above

	Baseline	**14d**	**90d**
Median Daily Aspirin Dose	0 mg	300 mg	75 mg
PFA-100			
C-EPI (S) **P value**	115.5 (80 – 196)	301 (114 – 301) **p < 0.001**	301 (155 – 301) p = **0.002**
C-ADP (S) **P value**	90 (63 – 301)	93 (65 – 166) p = 0.19	88 (63 – 149) p = 0.6
INNOVANCE P2Y (S) **P value**	70.5 (57 – 301)	72.5 (52 -120) p = 0.99	68 (48 – 111) p = 0.62
VerifyNow			
Aspirin (ARU) **P value**	654 (518 – 669)	426 (386 – 571) **P < 0.001**	439 (282 – 584) P = 0.001
P2Y12 (PRU) **P value**	273 (149 – 370)	286 (172 – 418) **p = 0.045**	280 (177 – 392) p = 0.25
Multiplate			
ASP (U) **P value**	106.5 (48 – 179)	19 (4-78) **p < 0.001**	13.5 (3- 101) **p < 0.001**
ADP (U) **P value**	91 (55 – 155)	101.5 (65-140) p = 0.054	100.5 (52-129) p = 0.64

6.4.3. Comparison of prevalence of Aspirin-HTPR according to cross-sectional *vs.* longitudinal definitions of HTPR on each platelet function testing platform in patients commencing aspirin

Although there were clear trends towards a lower prevalence of Aspirin-HTPR using longitudinal *vs*. cross-sectional/case-control definitions, these differences in prevalence did not reach statistical significance at 14d or 90d on any device in this group of patients (table 6.6)

Table 6. 6 Comparison of the prevalence of Aspirin-HTPR with cross-sectional vs. longitudinal definitions of HTPR on individual platelet function testing platforms. Values represent percentages (absolute numbers) of patients with Aspirin-HTPR at each time point

	14 days (Total N = 21)		90 days (Total N = 13)	
	Cross-sectional HTPR % (N)	**Longitudinal HTPR % (N)**	**Cross-sectional HTPR % (N)**	**Longitudinal HTPR % (N)**
PFA-100 C-EPI	23.8% (5)	4.8% (1)	7.7% (1)	7.7% (1)
P value		0.38		1.00
VerifyNow Aspirin	9.5% (2)	0%	7.7% (1)	0%
P value		0.50		1.00
Multiplate Aspirin	23.8% (5)	4.8% (1)	30% (4)	0%
P value		0.13		0.13

6.4.4. Inter-device agreement between different platelet function testing platforms

Using a cross-sectional definition of Aspirin-HTPR, there was a moderate agreement between the PFA-100 C-EPI and VerifyNow Aspirin assays (κ = 0.50, 95% CI: 0.05-0.96; P = 0.008). However, there was only 'fair agreement' between the PFA-100 C-EPI and Multiplate Aspirin assays (κ = 0.21, 95% CI: -0.25 to 0.68, P = 0.33), and 'slight agreement' between the VerifyNow Aspirin and Multiplate Aspirin assays (κ = 0.17, 95% CI: -0.28 to 0.63; P = 0.36). Using a longitudinal definition of Aspirin-HTPR, there was poor agreement between the PFA-100 C-EPI and Multiplate Aspirin assays (κ = -0.05, 95% CI: -0.11 to 0.02, P = 0.82). Because no patients had Aspirin-HTPR on the VerifyNow Aspirin assay according to our longitudinal definition, inter-device agreement with the VerifyNow and the other two devices could not be performed for our novel longitudinal definition.

6.4.5. Clinical outcomes and recurrent vascular events during follow-up.

Only one patient (3.4 %) had a stroke and all remaining patients had a TIA as their index event at the time of initial enrolment in this component of the OATS study. The single patient who presented with a minor ischaemic stroke had a modified Rankin scale score of 1.0 at baseline, but was subsequently excluded from the study because of a requirement for anti-coagulation prior to the 14d follow-up visit; therefore, there were no data available at 14d or 90d to comment on Aspirin-HTPR status in this patient's case. None of the patients in this group had a recurrent TIA, stroke or other vascular event during follow-up to at least 1 year in our study.

6.5. Discussion

To our knowledge, this pilot study is also the most comprehensive, simultaneous assessment of *ex vivo* platelet activation with whole blood flow cytometry and on-treatment platelet reactivity at high and low shear stress with these three devices after commencing aspirin in the early and late phase after TIA and ischaemic stroke.

In keeping with the findings of prior pilot studies, commencement of aspirin did not affect platelet surface CD62P or CD63 expression (Meiklejohn, Vickers et al. 2001; Grau, Reiners et al. 2003; Tobin, Kinsella et al. 2013), or the percentage of circulating leucocyte-platelet complexes at 14d or 90d (Tobin, Kinsella et al. 2013). This study confirms that measurement of levels of these markers on 'unstimulated whole blood flow cytometry' is not a sensitive method of detecting the effects of antiplatelet treatment *ex vivo* in CVD, in contrast to preliminary data from flow cytometry studies of 'stimulated CD62P expression' (Bath, May et al. 2017). Bath *et al*. found that 4.7% of patients in the early phase after TIA or ischaemic stroke had aspirin-HTPR with that stimulated CD62P flow cytometry assay (Bath, May et al. 2017).

The PFA-100 C-EPI, VerifyNow Aspirin and Multiplate Aspirin assays were found to be sensitive at detecting the antiplatelet effects of aspirin *ex vivo* in this study, as expected from prior studies on the PFA-100 (Grau, Reiners et al. 2003; Grundmann, Jaschonek et al. 2003; Alberts, Bergman et al. 2004; Harrison, Segal et al. 2005; McCabe, Harrison et al. 2005; Harrison, Segal et al. 2008; Zytkiewicz, Gielwanowska et al. 2008; Boncoraglio, Bodini et al. 2009; von Lewinski, Riggert

et al. 2009; Tobin, Kinsella et al. 2013), VerifyNow (Bennett, Yan et al. 2008; Seok, Joo et al. 2008; Ozben, Ozben et al. 2011; Sternberg, Ching et al. 2013; Agayeva, Gungor et al. 2014; Dharmasaroja and Sae-Lim 2014) and Multiplate® (Yoo NT 2012; Azmin S 2013; Jastrzebska, Chelstowski et al. 2013; Łabuz-Roszak B 2015; Mannu, Macartney et al. 2015), respectively. However, aspirin did not influence PFA-100 C-ADP or Innovance P2Y closure times, consistent with prior studies with the C-ADP cartridge (Grau, Reiners et al. 2003; Grundmann, Jaschonek et al. 2003; Serebruany, Malinin et al. 2004; McCabe, Harrison et al. 2005; Tobin, Kinsella et al. 2013), and did not significantly affect Multiplate ADP AUC values. There was a borderline statistically significant increase in VerifyNow P2Y12 PRU values at 14d in patients after commencing aspirin monotherapy, but this was not sustained at 90d. These VerifyNow P2Y12 findings are most likely to reflect a type I error because it is not anticipated that the commencement of aspirin would actually enhance platelet aggregation in response to ADP and the differences were not sustained during follow-up. Furthermore, as stated above, aspirin significantly inhibited platelet aggregation on the VerifyNow Aspirin cartridge in the same patient group. Therefore, allowing for our interpretation of these VerifyNow P2Y12 findings at 14d, we have confirmed that the 'non-aspirin specific' assays on these devices, which are essentially designed to detect inhibition with P2Y12 or other ADP receptor antagonists, are not significantly influenced by the commencement of aspirin monotherapy. These data are informative and may aid in the interpretation of relevant test results in patients on dual antiplatelet therapy e.g. with aspirin and clopidogrel in future. However, we must stress that this aspect of the OATS study was not designed assess the impact of the commencement of aspirin followed by the later addition of clopidogrel on platelet function/reactivity.

This needs to be formally addressed in future studies on this topic.

We simultaneously compared the prevalence of Aspirin-HTPR using cross-sectional and novel longitudinal definitions, which to our knowledge, has not previously been performed in CVD patients on all of these three devices. The prevalence of Aspirin-HTPR varied between 9.5-23.8% in early phase and 7.7-30% in late phase CVD patients with cross-sectional definitions, and 0-4.8% in early phase and 0-7.7% in late phase patients with novel longitudinal definitions in this study. These findings indicate that up to almost a quarter of early phase and almost one third of late phase CVD patients have 'cross-sectional' Aspirin-HTPR on certain platelet function assays despite the use of commonly prescribed doses of aspirin (median daily dose of 300 mg at 14 days and 75mg at 90d). Prior studies using cross-sectional definitions reported that the prevalence of Aspirin-HTPR on the PFA-100 C-EPI cartridge ranged from 15-60% during the early phase (within 4 weeks) (Grundmann, Jaschonek et al. 2003; Alberts, Bergman et al. 2004; McCabe, Harrison et al. 2005; Godeneche, Sorel et al. 2009; Lai, Chen et al. 2012; Tobin, Kinsella et al. 2013; Lim, Coughlan et al. 2015), and between 15-43% during the late phase (≥4 weeks) after a TIA or ischaemic stroke on 75mg-300mg of aspirin daily (Grau, Reiners et al. 2003; Harrison, Segal et al. 2005; McCabe, Harrison et al. 2005; Boncoraglio, Bodini et al. 2009; Tobin, Kinsella et al. 2013; Lim, Coughlan et al. 2015). Other studies identified Aspirin-HTPR in 6-33% of patients on aspirin monotherapy with the VerifyNow Aspirin cartridge (Bennett, Yan et al. 2008; Seok, Joo et al. 2008; Lee, Cha et al. 2010; Ozben, Ozben et al. 2011; Sternberg, Ching et al. 2013; Agayeva, Gungor et al. 2014; Dharmasaroja and Sae-Lim 2014; Lim, Coughlan et al. 2015), and in 6.3-42.8% of CVD patients in the early phase (Yoo NT 2012; Azmin S 2013; Jastrzebska, Chelstowski et al. 2013; Łabuz-Roszak B 2015;

Mannu, Macartney et al. 2015) and 12-13% in the late phase after symptom onset (Łabuz-Roszak B 2015) with the Multiplate Aspirin assay. Therefore, our findings fall within the ranges reported in these prior studies.

There are several factors which may influence Aspirin-HTPR status *ex vivo*, including the presence of specific single nucleotide polymorphisms (SNPs) (Postula, Kaplon-Cieslicka et al. 2011; Voora, Horton et al. 2011), medication adherence (Tantry, Bliden et al. 2005; Cuisset, Frere et al. 2009), use of enteric coated *vs*. soluble aspirin (Ridker, Hennekens et al. 1996; Cox, Maree et al. 2006; Peace, McCall et al. 2010; Grosser, Fries et al. 2013), possibly the concurrent use of proton pump inhibitors as reported in some studies in IHD patients (Wurtz, Grove et al. 2010; Charlot, Grove et al. 2011) and body weight (Rothwell, Cook et al. 2018).

There was a 'statistically non-significant' but 'numerically lower' prevalence of Aspirin-HTPR with our novel longitudinal compared with traditional cross-sectional definitions on the PFA-100 C-EPI and Multiplate Aspirin assays (4.8 *vs*. 23.8%) and on the VerifyNow Aspirin assay (0 *vs*. 9.5%) at 14d, and on the Multiplate Aspirin (0 *vs*. 30%) and VerifyNow Aspirin assays (0 *vs*. 7.7%) at 90d. These non-significant results may also reflect a type II error due to the small number of subjects included in this aspect of the OATS study, and further work is clearly required to reassess this issue.

There were no recurrent vascular events observed during comprehensive, prospective clinical and laboratory follow-up to at least 1 year after symptom onset in any of our patients who had available Aspirin-HTPR data. Therefore, we cannot reliably comment on the value of Aspirin-HTPR status at predicting the risk of recurrent vascular events over time from this pilot study. It is clear that large,

adequately-powered, multi-centre, international studies are needed to properly address this critically-important clinical question.

There was no statistically significant difference in the prevalence of Aspirin-HTPR with the PFA-100 C-EPI cartridge in this study compared with the data from a study with a similar longitudinal design which was conducted by Tobin *et al*. (4.8% *vs*. 24% at 14d, and 7.7% *vs*. 18% at 90d; P = NS) (Tobin, Kinsella et al. 2013). A significantly higher proportion of patients had a stroke (rather than a TIA) in that prior study by Tobin *et al*. compared with our current study [38.5% (10/26) *vs*. 3.45% (1/29); P = 0.0037]. Some studies have shown that aspirin-HTPR is associated with more severe strokes at baseline (P=0.005)(Englyst, Horsfield et al. 2008; Schwammenthal, Tsabari et al. 2008; Zheng, Churilov et al. 2013; Coignion, Poli et al. 2015; Oh, Yu et al. 2016), so this could partly explain the trend towards a numerically lower prevalence of Aspirin-HTPR in our study population which was essentially composed of patients who had experienced TIAs.

There is also a large variation in the reported prevalence of antiplatelet-HTPR in the published literature, partly due to a lack of a standardised definition of Aspirin-HTPR using case-control/cross-sectional definitions (Mansour, Taher et al. 2009). It has been suggested that antiplatelet-HTPR should not be viewed as an 'all or nothing phenomenon', but instead as an entity on a continuous spectrum (Hedegaard, Hvas et al. 2009; Alberts 2010; Topcuoglu, Arsava et al. 2011). The cross-sectional definitions used is this study were based on published cut-off values on the PFA-100 (Kinsella, Tobin et al. 2013; Tobin, Kinsella et al. 2013), and on the manufacturers' definition of Aspirin-HTPR on the various platelet

function/reactivity platforms. Other studies have also used different threshold values for cross-sectional definitions of Aspirin-HTPR e.g., at least seven different cross-sectional definitions of Aspirin-HTPR have been used in studies on the PFA-100 (Grundmann, Jaschonek et al. 2003; Mansour, Taher et al. 2009). In addition, a patient's Aspirin-HTPR status may fluctuate over time despite being on the same treatment (Helgason, Bolin et al. 1994; Tobin, Kinsella et al. 2013), and one may regain a certain degree of platelet aggregability despite the presence of continued inhibition of one particular platelet pathway from chronic antiplatelet use via activation of alternative intra-platelet pathways. For example, long-term aspirin use has been shown to increase the platelet response to thromboxane A2-independent stimuli, such as ADP, thrombin, epinephrine, and collagen over time (Hedegaard, Hvas et al. 2009). Nevertheless, in the absence of an international consensus regarding the optimal threshold to define Aspirin-HTPR, one has to rely on 'manufacturers' definitions' or locally established 'healthy control ranges' on each device if one is using cross-sectional/case-control definitions of Aspirin-HTPR in the interim.

We found moderate inter-device agreement between the PFA-100 C-EPI and VerifyNow Aspirin assays, fair agreement between the PFA-100 C-EPI and Multiplate Aspirin assays, but only slight agreement between the VerifyNow Aspirin and Multiplate Aspirin assays using cross-sectional definitions of Aspirin-HTPR. Using a longitudinal definition of Aspirin-HTPR, there was poor agreement between the PFA-100 C-EPI and Multiplate Aspirin assays, but the inter-device agreement between the VerifyNow and the other two devices could not be calculated. There was no clear reproducible pattern of a higher prevalence of Aspirin-HTPR on the high shear stress PFA-100 than on the low shear stress

VerifyNow or Multiplate, as we had hypothesised and anticipated (Harrison, Segal et al. 2008). Therefore, it is important to simultaneously assess platelet function/reactivity with more than one testing platform to assess the potential clinical predictive value of one or more of the relevant devices in CVD.

6.6. Conclusions

Measurement of unstimulated levels of platelet surface activation markers or leucocyte -platelet complexes on whole blood flow cytometry is not a sensitive method to detect the effects of aspirin treatment *ex vivo* in patients after a recent TIA or ischaemic stroke. The inhibitory effects of aspirin were reliably detected by the PFA-100 C-EPI cartridge, VerifyNow Aspirin cartridge and Multiplate Aspirin assay, but up to one quarter to one third of patients exhibit 'cross-sectional' Aspirin-HTPR on certain platelet function assays despite the use of commonly prescribed doses of aspirin. The prevalence of aspirin-HTPR was 'numerically' but not statistically-significantly lower when one used a longitudinal definition *vs*. a cross-sectional of HTPR, as shown previously in an earlier study by our group (Tobin, Kinsella et al. 2013). Larger, prospective studies are warranted to determine whether cross-sectional/case-control or relatively novel longitudinal definitions of Aspirin-HTPR may predict the risk of recurrent vascular events and outcomes in the clinical setting in individual CVD patients who are treated with aspirin.

7. Assessment of platelet activation status and on-treatment platelet reactivity in the early and late phases following TIA or ischaemic stroke in patients commencing clopidogrel monotherapy

7.1. Introduction:

The CAPRIE study revealed that clopidogrel monotherapy appears to be as good or slightly more effective than aspirin for the secondary prevention of vascular events after ischaemic stroke, but the 7.3% relative reduction in risk of recurrent vascular events in the ischaemic stroke subgroup alone did not reach statistical significance (P = 0.26) (CAPRIE Steering Committee 1996). The CAPRIE study was not actually powered to detect a benefit of clopidogrel over aspirin in the stroke subgroup, but *post-hoc* analysis showed that clopidogrel appeared superior to aspirin monotherapy at preventing recurrent vascular events in the subgroup of patients with ischaemic cerebrovascular disease (CVD) or peripheral vascular disease who had co-existing ischaemic heart disease (22.7% relative risk reduction; 95% CI: 4.9-37.2%; P = 0.043) (CAPRIE Steering Committee 1996). Therefore, the ideal regimen for individual patients who are not monitored with laboratory tests of platelet function/reactivity is yet to be fully determined (1997; 1997; Kinsella, Tobin et al. 2013).

The PFA-100 C-ADP assay has been shown to be insensitive at detecting the *ex*

vivo antiplatelet effects of clopidogrel in several prior studies employing a 'cross-sectional definition' of Clopidogrel-HTPR (Grau, Reiners et al. 2003; McCabe, Harrison et al. 2005; Tobin, Kinsella et al. 2011; Kinsella, Tobin et al. 2013; Tobin, Kinsella et al. 2013; Lim, Coughlan et al. 2015). However, the PFA-100® INNOVANCE P2Y cartridge has been reported to have overcome this issue (Edwards, Jakubowski et al. 2012; Tsantes, Ikonomidis et al. 2012), and the prevalence of Clopidogrel-HTPR using a cross-sectional definition in a pilot study in CVD patients was 39% during the early phase (day 7) and 56% during the late phase (day 90) after an ischaemic stroke on 75 mg of clopidogrel daily (Jover, Rodriguez et al. 2014). Clopidogrel-HTPR on the VerifyNow P2Y12 assay was identified in 26-44% of patients in the early phase (Kinsella, Tobin et al. 2013; Sternberg, Ching et al. 2013) and 29-61% of patients in the late phase (Maruyama, Takeda et al. 2011; Kinsella, Tobin et al. 2013; Jover, Rodriguez et al. 2014) following an ischaemic stroke. The Mutiplate ADP assay may also detect the *ex vivo* antiplatelet effects of clopidogrel at low shear stress and cross-sectional data from CVD patients indicate that the prevalence of Clopidogrel-HTPR on this assay varies between 18-57.1% in the early phase (within one week) after TIA or ischaemic stroke onset (Meves, Schroder et al. 2014; Coignion, Poli et al. 2015; Lundstrom, Wallen et al. 2015). Prior to the conduct of this study, no published studies had assessed the prevalence of Clopidogrel-HTPR in the late phase following an ischaemic stroke using the Multiplate ADP assay. As stated in our prior reviews (Chapters 3 and 4), no adequately-sized study has assessed the value of the Multiplate assays at predicting the risk of recurrent events or outcomes following TIA or ischaemic stroke (Lim, Coughlan et al. 2015).

7.2. Aims and Hypothesis

The **Aims** of this component of the OATS study were:

1) To simultaneously assess and compare the ability of established and novel laboratory tests of platelet activation and reactivity to identify patients with recent TIA or ischaemic stroke who have *ex vivo* Clopidogrel-HTPR according to established 'cross-sectional' and 'novel longitudinal definitions' of HTPR.

2) To improve our understanding of the underlying clinical, demographic and pharmacodynamic mechanisms influencing *ex vivo* Clopidogrel-HTPR on each of these devices in CVD patients.

3) To collect pilot data to contribute to the assessment of the comparative effectiveness and test characteristics of each assay, using both established 'cross-sectional' and 'novel longitudinal definitions' of Clopidogrel-HTPR, at predicting the risk of recurrent vascular events during long-term follow up in patients with recent TIA or ischaemic stroke on clopidogrel monotherapy.

Hypotheses:

1. There will be a higher prevalence of *ex vivo* Clopidogrel-HTPR during simultaneous testing with certain platelet function assays than with others in patients with TIA or ischaemic stroke, and prevalence estimates will be higher with established cross-sectional than with novel longitudinal definitions.

2. This study will improve our understanding of the clinical, demographic, and pharmacodynamic mechanisms influencing *ex vivo* Clopidogrel-HTPR in CVD patients.

3. Platelet reactivity testing will ultimately improve our ability to predict the risk of recurrent vascular events in CVD patients treated with clopidogrel monotherapy. Pilot data from this study will be utilised to progress to a definitive multi-centre study.

7.3. Methods

7.3.1. Recruitment

Patients who met the inclusion criteria were recruited during the period between 26/10/2011 and 13/01/2016 at AMNCH-TUH.

Inclusion criteria

Patients were eligible if they were ≥18 years of age, within 4 weeks of onset of a TIA or ischaemic stroke, were either on no antiplatelet therapy, aspirin monotherapy or aspirin & dipyridamole combination therapy and their treating physician decided to commence or change their antiplatelet treatment regimen to clopidogrel monotherapy.

Exclusion criteria

Patients were excluded if they had myocardial infarction, DVT or PE within the preceding three months; ongoing unstable angina or unstable symptomatic peripheral vascular disease; platelet count < 100 x 10^9/L; known bleeding or clotting diathesis, including known platelet-related bleeding disorders; active proven vasculitis; active neoplasia; non-steroidal anti-inflammatory drug (NSAID) intake other than aspirin or aspirin in combination with dipyridamole in the preceding 11 days; CVD patients unable to attend for clinical follow-up and repeat testing at 14 +/- 7 days; patients who had recent surgery or a recent intracranial haemorrhage in the past three months.

Written informed consent was obtained from all subjects, and this study was approved by the St. James's Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2011/35/03).

7.3.2. Controls

Inclusion and exclusion criteria for control subjects have been outlined in chapter 6, and will not be duplicated here.

7.3.3. Clinical assessment, Timing and Follow-up

Patients underwent detailed clinical assessment and were classified as having a TIA or ischaemic stroke if this was confirmed by their Consultant Neurologist or Stroke Physician after clinical investigation according to ESO recommendations (European Stroke Organisation Executive and Committee 2008). As outlined before, the diagnosis was confirmed in all cases by Dr Lim and/or Prof. McCabe. The

underlying mechanism responsible for TIA or Stroke was categorised according to the TOAST (Adams, Bendixen et al. 1993) and ASCOD classification systems (Amarenco, Bogousslavsky et al. 2009).

Patients underwent detailed clinical and laboratory assessment with blood sampling before (**baseline**), 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after commencing or changing to clopidogrel monotherapy. In addition, clinical follow-up to assess longer-term risk of recurrent vascular events and outcome measures was performed using a validated in-person or telephone questionnaire at ≥1 year after symptom onset.

7.3.4. Laboratory Assessments

Detailed descriptions of the venepuncture technique and laboratory testing have been outlined previously in Chapter 6 (General Methods). In summary, venepuncture was performed in a standardised manner, as described previously (McCabe, Harrison et al. 2004) (Kinsella, Tobin et al. 2013). Greiner Vacuette® tubes containing 3.2% sodium citrate are taken for **VerifyNow, PFA-100** and **whole blood flow cytometry** analysis, with an additional hirudin-anticoagulated tube taken for **Multiplate analysis.**

Whole blood flow cytometry: Platelets were distinguished from red and white blood cells, as described previously (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013). Platelet surface CD62P and CD63 expression (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013) and the percentages of circulating leucocyte-platelet complexes were quantified, using previously described and validated

methods (Chapter 6, (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013; Tobin, Kinsella et al. 2013)).

PFA-100® Platelet function analyser: The degree of inhibition of platelet function in whole blood at moderately high shear stress was assessed before and after commencing clopidogrel in response to biochemical stimulation with collagen and epinephrine (C-EPI cartridge), collagen-ADP (C-ADP cartridge), and ADP/Prostaglandin E_1 (INNOVANCE® PFA P2Y cartridge), as previously described (Chapter 6) (McCabe, Harrison et al. 2005; Jover, Rodriguez et al. 2014).

The maximum closure time recorded by the device is 300s, and we arbitrarily defined closure times above 300s as 301s for statistical analyses.

Cross-sectional definition of Clopidogrel-HTPR on the PFA-100*:* Clopidogrel-HTPR was defined as failure to prolong INNOVANCE® PFA P2Y closure times beyond the normal control range, as per the established manufacturer's definition i.e. failure to prolong INNOVANCE® PFA P2Y closure times > 106s in patients on clopidogrel, alone or in combination with aspirin [Siemens Inc. INNOVANCE P2Y assay, package insert, Erlangen, Germany, 2015].

Novel longitudinal definition of Clopidogrel-HTPR on the PFA-100*:*

In our laboratory, the intra-assay co-efficient of variation (CV) for the INNOVANCE® PFA P2Y cartridge was 7.8% (Chapter 6). Clopidogrel-HTPR was defined as failure to prolong INNOVANCE® PFA P2Y closure times on clopidogrel compared with baseline values before starting clopidogrel by more than twice the

CV of the assay i.e. failure to prolong INNOVANCE® PFA P2Y closure times by > 15.6% of the patient's baseline INNOVANCE® PFA P2Y closure time.

VerifyNow® Platelet Function Analyser: The VerifyNow® is a cartridge-based analyser, containing fibrinogen coated beads that assesses *ex-vivo* platelet reactivity at low shear stress in response to stimulation with fixed doses of different platelet agonists, depending on the cartridge employed (van Werkum, Harmsze et al. 2008). The reagents bound to the fibrinogen beads are arachidonic acid in the Aspirin cartridge and adenosine diphosphate (ADP), iso- thrombin receptor activating peptide (iso-TRAP), and PAR-4 activating peptide in the P2Y12 cartridge (see chapter 5). As explained in chapters 6 and 8, both the rate and extent of platelet-induced agglutination over a fixed period of time are measured and combined with a proprietary algorithm to report the values in 'reaction units'.(van Werkum, Harmsze et al. 2008)

Current cross-sectional definition of Clopidogrel-HTPR on the VerifyNow: Clopidogrel-HTPR in patients on clopidogrel, alone or in combination with aspirin, has been defined as P2Y12 reaction units (PRU) ≥ 194 on the P2Y12 cartridge [Accumetrics Inc. VerifyNow-P2Y12 (P2Y12 assay) package insert. San Diego, CA. 2006, (Pamphlet)].

Novel longitudinal definition of Clopidogrel-HTPR on VerifyNow®: The intra-assay CV for the P2Y12 cartridge in our laboratory was 5.5%. Clopidogrel-HTPR was defined as failure to shorten PRUs on clopidogrel compared with the patient's own baseline value by more than twice the CV of the P2Y12 assay i.e. failure to shorten the PRU by ≥ 11% of patient's baseline PRU.

Multiplate® assay*:* This whole blood platelet aggregation assay is based on measurement of impedance at low shear stress as platelets adhere to 2 adjacent electrodes and aggregate to one another within a cuvette. The extent of platelet adhesion and aggregation is recorded as the Area Under the Curve (AUC) up to 6 minutes after the addition of either arachidonic acid or ADP to measure the antiplatelet effects of aspirin or clopidogrel, respectively.

Cross-sectional definition of Clopidogrel-HTPR on the Multiplate*:* The manufacturer's recommended cross-sectional definition of clopidogrel-HTPR is > 46U on the ADP test [Roche Inc, Multiplate ADP assay package insert, Berlin; 2015].

Novel longitudinal definition of Clopidogrel-HTPR on the Multiplate: In our laboratory, the intra-assay CV for the ADP test was 7.8% (N = 8 assays). Clopidogrel-HTPR was defined as failure to reduce aggregation on the ADP test with clopidogrel compared with the patient's own baseline value by more than twice the CV of the assay, i.e. failure to decrease the AUC by > 15.6% of the patient's baseline AUC.

7.3.5. Statistical Methods

All statistical analysis was performed using SPSS (Version 23). The Wilcoxon signed rank test was used for comparison of C-EPI, C-ADP, Innovance P2Y, VerifyNow Aspirin, VerifyNow P2Y12, Multiplate Aspirin and Multiplate ADP

results at 14d and 90d relative to baseline. McNemar's test was used to compare proportions of patients with clopidogrel-HTPR based on cross-sectional *vs*. longitudinal definitions on the PFA-100, Multiplate and VerifyNow devices. Inter-device agreement in the diagnosis of clopidogrel-HTPR between the different testing platforms was determined by Cohen's unweighted kappa testing. 95% confidence intervals (CIs) were calculated. Kappa values of < 0 indicate 'no agreement', 0 to 0.2 indicate 'slight agreement', 0.21 to 0.40 indicate 'fair agreement', 0.41 to 0.60 indicate 'moderate agreement', 0.61 to 0.80 indicate 'substantial agreement' and 0.81 to 1.00 indicate 'almost perfect agreement' (Kinsella, Tobin et al. 2011; Bradley, Cronin et al. 2013). Logistic regression analysis was used to assess the relationship between clopidogrel-HTPR status and the risk of recurrent TIA, stroke or vascular events in this patient subgroup. The relationship between platelet activation status and clopidogrel-HTPR status was also assessed with logistic regression analysis. $P < 0.05$ was considered to represent statistical significance.

7.4. Results

Subgroups of patients commencing Clopidogrel

This aspect of the OATS study included three main subgroups of patients changing from (a) aspirin to clopidogrel monotherapy, (b) aspirin & dipyridamole combination therapy to clopidogrel monotherapy, and (c) no medication to clopidogrel monotherapy (figure 7.1). To simplify some data analysis, laboratory data were subsequently combined into one overall group of patients who were

'commencing clopidogrel'. Some patients did not have simultaneous data from all assays if e.g. specific devices were being serviced at the time of recruitment. The demographic, clinical and vascular risk factor profiles of the study subjects (table 7.1), and their TIA or ischaemic stroke subtypes (tables 7.2 and 7.3) are tabulated below.

Aspirin Monotherapy to Clopidogrel Monotherapy subgroup

Thirty-five patients had baseline data; 31 were reassessed at 14d and 22 patients were reassessed at 90d. The reasons for subsequent exclusion of 13 patients at 14d or 90d were as follows: One patient underwent aortic valve replacement and required warfarin; one had a recurrent cardio-embolic stroke attributed to unstable atheroma in the aortic arch and was commenced on warfarin by the treating physician; one had recurrent embolic pontine ischaemia from a vertebral artery dissection and was commenced on warfarin; two patients did not ultimately have their medications changed by the treating physician as initially planned; two were changed back to aspirin and clopidogrel was stopped by their treating cardiologist; two patients could not attend within the required time frame for their 14d follow up due to lack of transportation; one patient died from pneumonia, one developed an allergic reaction to clopidogrel; one had a further TIA on clopidogrel, resulting in alteration of the antiplatelet regimen to aspirin and dipyridamole; one patient dropped out due to busy family commitments.

The median dose of aspirin prior to the changing treatment was 75mg daily and the median dose of clopidogrel was 75mg at 14d and 90d. Three patients had recurrent TIAs during follow up. One patient had a recurrent TIA attributed to embolisation from aortic arch atheroma within a week of recruitment; one had recurrent pontine

TIAs from a vertebral artery dissection within a week of recruitment; and one patient had a left hemispheric TIA due to possible embolism from a stenosed internal carotid artery within three months of recruitment.

Aspirin & Dipyridamole to Clopidogrel group

Five patients had baseline data; 3 had available data at 14d, and one patient had data at 90d. Two patients were subsequently found to have paroxysmal atrial fibrillation and anticoagulated with warfarin; one was changed to a combination of aspirin and clopidogrel by the treating physician; one patient dropped out due to busy family commitments. The median daily dose of aspirin was 75 mg at baseline and the median daily dose of dipyridamole MR was 200mg bd. Median daily dose of clopidogrel was 75mg at 14d and 90d.

No Medication to Clopidogrel group

Data were available in 4 patients at baseline, 4 at 14d and 2 patients at 90d. One patient was excluded subsequently because clopidogrel was later changed to prasugrel by the treating cardiologist; another patient could not attend for follow-up due to multiple ‘non-cerebrovascular-related co-morbidities’, including arthritis and exertional dyspnoea. The median daily dose of clopidogrel was 75 mg at 14d and 90d.

Figure 7. 1 Flow Chart of Study Patients 'Commencing Clopidogrel'

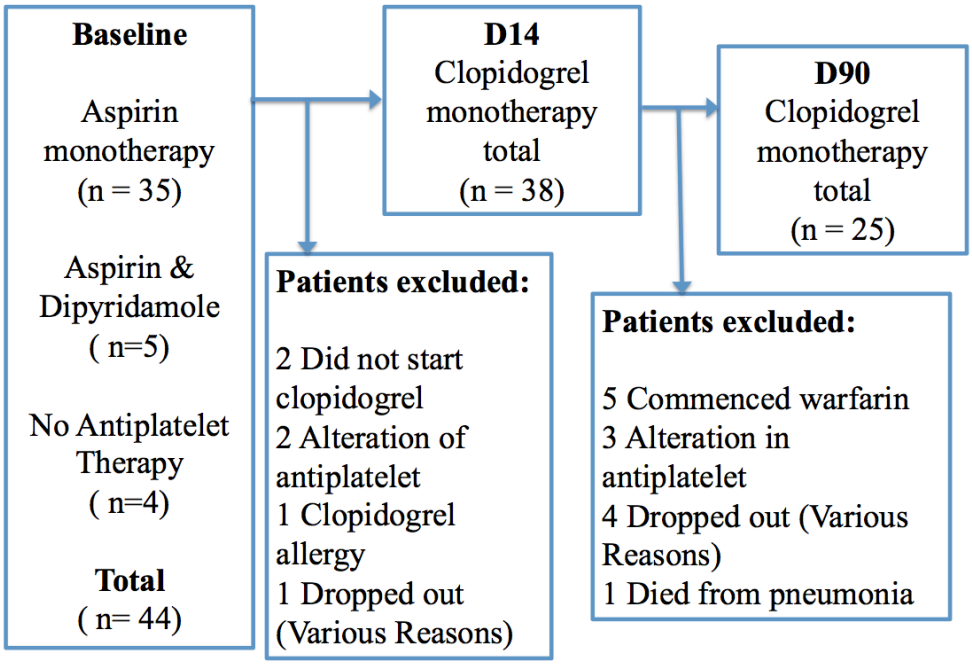


Table 7. 1 Demographic and Clinical Profiles of Patients at Enrolment

Parameter	Clopidogrel starters (N = 44)	Number (%)
Mean Age (years) [SD]	64 [SD 13]	
Gender (M/F)	31/13	
Prior Stroke/TIA	23 (52%)	
Index event = TIA	42 (95.5%)	
Index event = Stroke	2 (4.5%)	
Thrombolysed	2 (5%)	
Hyperlipidaemia	24 (54%)	
IHD	16 (36%)	
Hypertension	21 (48%)	
Diabetes Mellitus	9 (20.5%)	
Atrial Fibrillation at Enrolment	0	
Family History of Stroke	11 (25%)	
Prior DVT/PE	0	
Peripheral Vascular Disease	1 (2.2%)	
Migraine with Aura	3 (7 %)	
Migraine without Aura	2 (5%)	
Current Smoker	11 (25%)	
Ex-smoker	15 (34%)	
Never smoker	18 (44%)	
Statin Therapy	33 (75%)	
Index Event on Anti-platelet Therapy	40 (91%)	

Table 7. 2 TOAST Classification of patients who commenced Clopidogrel

	Patients Commencing Clopidogrel (N = 44) % (N)
Large Artery Atherosclerotic	6.8% (3)
Small Vessel Disease	11.4% (5)
Cardioembolic	6.8% (3)
Other Determined	0% (0)
Undetermined Aetiology	75% (33)

Table 7. 3 ASCOD Classification of patients commencing Clopidogrel

ASCOD Phenotype	**Disease Present (ASCOD 1,2,3)**	**Disease Absent (ASCOD 0)**	**Insufficient Investigations (ASCOD 9)**
A % (N)	61.4% (27)	38.6% (17)	0
S % (N)	70.5% (31)	29.5% (13)	0
C % (N)	36.4% (16)	63.6% (28)	0
O % (N)	25% (11)	75% (33)	0
D % (N)	2.3% (1)	97% (43)	0

A: Atherosclerosis; S: small vessel disease; C: cardiac source; O: other cause; D: dissection
1: 'definitely a potential cause of the index stroke',
2: 'causality uncertain',
3: 'unlikely a direct cause of the index stroke but disease is present'
0: 'disease absent'

7.4.1. Impact of commencing clopidogrel monotherapy on platelet activation status

In the combined group of patients, commencement of clopidogrel did not significantly affect platelet surface CD62P or CD63 expression, or the percentage of any circulating leucocyte-platelet complexes during follow-up at either 14d or 90d *vs*. baseline (table 7.4 and figures 7.2, 7.3 & 7.4 a-c).

Table 7. 4 Platelet activation markers in patients commencing clopidogrel. P values refer to comparisons between matched data at 14d and 90d vs. baseline. Values represents medians (range: min - max)

	Baseline N = 38	**14 days N = 34**	**90 days N = 23**
Platelet surface markers			
CD62P %	3.28 (0.62 – 21.6)	3.55 (0.76 – 10.3)	3.86 (1.07 – 9.94)
P value		0.08	0.39
CD63 %	16.89 (5.12 – 35.6)	16.15 (0.37 – 31.7)	11.5 (0.57 – 29.2)
P value		0.23	0.07
Leucocyte-Platelet Complexes:			
Neutrophil-Platelet (%)	2.59 (0.53 – 5.3)	2.85 (0.51 – 11.20)	2.7 (1.31 – 3.96)
P value		0.49	0.83
Monocyte-Platelet (%)	6.15 (0.57 – 17.1)	5.50 (0.51 – 13.2)	5.21 (0.60 – 10.5)
P value		0.31	0.051
Lymphocyte-Platelet (%)	2.23 (0.50 – 9.76)	1.91 (0.50 – 6.13)	1.89 (1.12 – 5.0)
P value		0.41	0.33

Figure 7. 2 CD62P% expression in patients commencing Clopidogrel. Values represents medians (range: min-max):

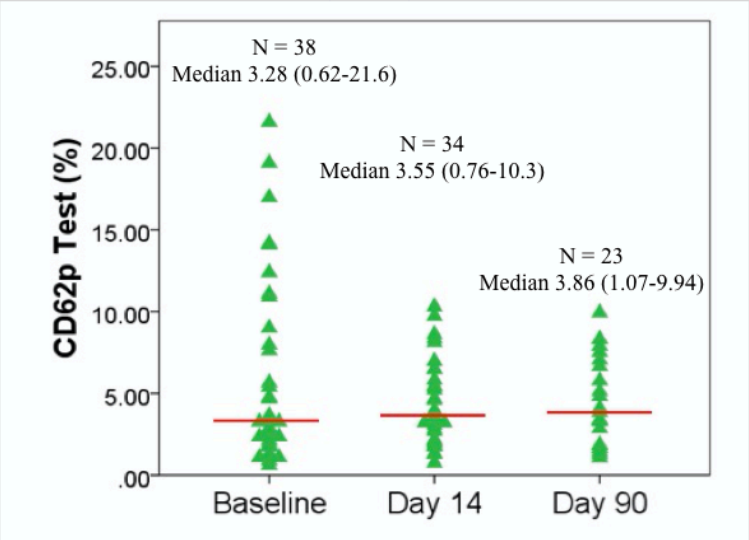


Figure 7. 3 CD63% expression in patients commencing clopidogrel. Values represents medians (range: min-max):

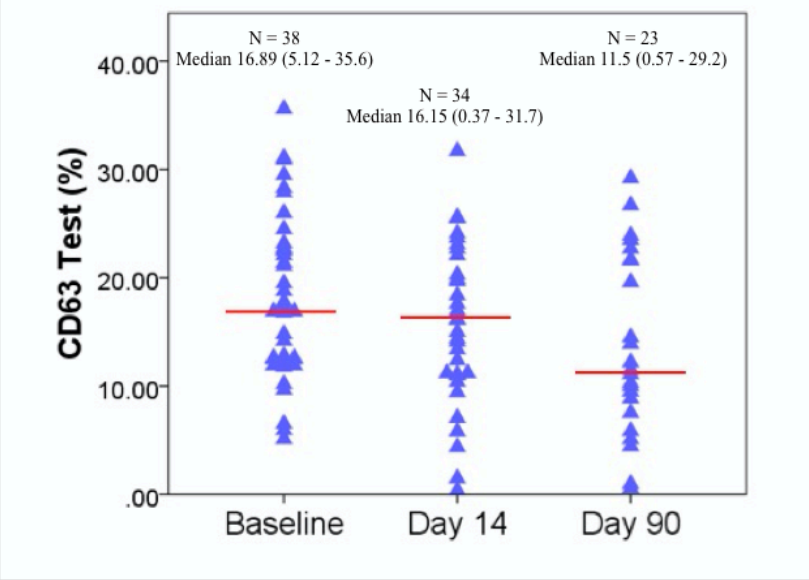


Figure 7. 4 Leucocyte platelet complexes in patients commencing clopidogrel

Figure 7.4a: Neutrophil-platelet complexes in patients commencing Clopidogrel. Values represents medians (range: min-max):

7.4a

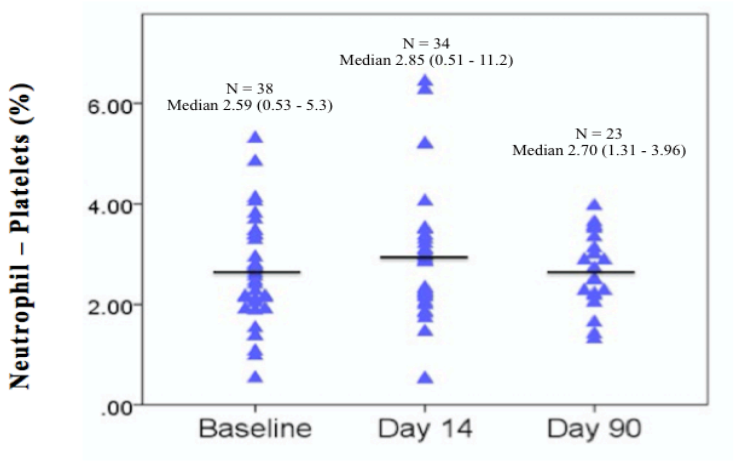


Figure 7.4b: Monocyte-platelet complexes in patients commencing Clopidogrel. Values represents medians (range: min-max):

7.4b

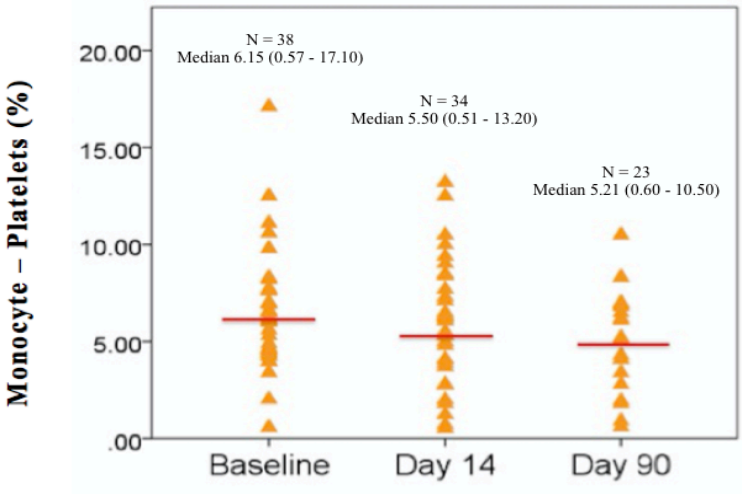
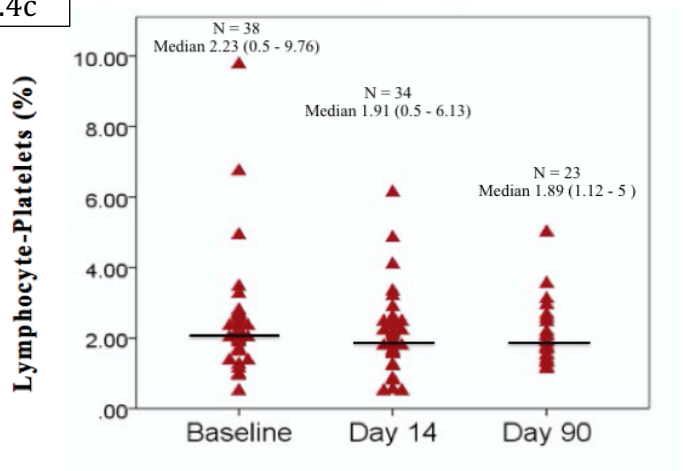


Figure 7.4c: Lymphocyte-platelet complexes in patients commencing Clopidogrel. Values represents medians (range: min-max):

7.4c



7.4.2. Platelet function / reactivity status after commencing Clopidogrel

PFA-100® analysis

Innovance P2Y cartridge

Data were available for analysis in 39 patients at baseline, 38 at 14d and 24 patients at 90d. Three patients did not have data at baseline because they were recruited prior to the final installation of the validated Innovance P2Y system in our lab, and two patients did not have baseline data because there was an 'error reading' from the cartridge at the time of analysis. One patient did not have data at 90d because the device was being serviced.

After commencing clopidogrel, median Innovance P2Y closure times significantly increased at 14d [170.5s (range: 58–301s) *vs*. 64.5s (range: 80-196s), $p < 0.001$; N = 34] and 90d [272.5s (range 61-301s) *vs*. 68s (range 45-131s), $p < 0.001$; N = 22] (table 7.5). At 14d, 31.6% (12/38) of patients had Clopidogrel-HTPR according to a cross-sectional definition, but only 16.7% (6/36) had Clopidogrel-HTPR according

to our novel longitudinal definition. Two patients who had cross-sectional Clopidogrel-HTPR at 14d did not have baseline data, so one could not comment on their longitudinal Clopidogrel-HTPR status at 14d. Four patients (10.5%) had Clopidogrel-HTPR according to a cross-sectional definition at both 14d and 90d, but only one had Clopidogrel-HTPR by both cross-sectional and longitudinal definitions at the 14d and 90d time points. 5/24 (20.8%) patients had Clopidogrel-HTPR using a cross-sectional definition, but only 1/22 (4.5%) had Clopidogrel-HTPR at 90d according to our longitudinal definition.

Overall, amongst patients who had data at both 14d and 90d after commencing clopidogrel, 12/22 (54.5%) retained the same cross-sectional/case-control Clopidogrel-HTPR status, and 14/20 (70%) retained the same novel longitudinal Clopidogrel-HTPR status at 14d and 90d.

Of the three patients who had recurrent TIAs, one patient had Clopidogrel-HTPR by the cross-sectional definition on the Innovance P2Y at 14d (table 7.6), but none of these three patients had 90d Innovance P2Y follow-up data.

C-ADP cartridge

Data were available on 42 patients at baseline, 38 patients at 14d and 24 patients at 90d. Compared with baseline values, there was no significant prolongation of median C-ADP closure times after commencing clopidogrel at 14d [89.5s (range: 53–301s) *vs*. 83s (range: 50-185s), p = 0.59; N = 38], or 90d [97.5s (range: 70-154s) *vs*. 85s (range: 62-185), p = 0.07; N = 24] (table 7.5)

C-EPI cartridge

Data were available on 41 patients at baseline, 37 patients at 14d, and 23 patients at 90d. After changing from either aspirin monotherapy, aspirin & dipyridamole combination therapy or no medication to clopidogrel monotherapy, there was a significant decrease in median C-EPI closure times compared with baseline at 14d [109s (range: 67–218) *vs*. 202s (range: 65-301), $p < 0.001$; N = 37] and 90d [114s (range: 85 – 211s) *vs*. 301s (range: 95 – 301s), $p < 0.001$; N = 23] (table 7.5)

VerifyNow® analysis in patients commencing Clopidogrel

VerifyNow P2Y12 cartridge

Data were available in 40 patients at baseline, 36 patients at 14d and 25 patients at 90d. Compared with baseline, PRU significantly decreased at 14d [211.5s (range 7 – 341) *vs*. 308.5 (range: 195 - 418); $p <0.001$, N = 36] and 90d [218 (range 32 – 284) *vs*. 319 (range: 221 - 418); $p < 0.001$, N = 25] after commencing clopidogrel (table 7.5). Using a cross-sectional definition, the prevalence of clopidogrel-HTPR was 55.3% (21/38) at 14d and 56% (14/25) at 90d. However, the prevalence of clopidogrel-HTPR was only 13.9% (5/36) at 14d and 1/25 (4%) at 90d using our novel longitudinal definition.

Amongst patients who had data at both 14d and 90d after commencing clopidogrel, 21/25 (84%) retained the same cross-sectional/case-control Clopidogrel-HTPR status, and 20/25 (80%) retained the same novel longitudinal Clopidogrel-HTPR status at 14d and 90d.

Of the three patients who had recurrent vascular events, one patient had clopidogrel-HTPR at 14d by the cross-sectional definition, but none had clopidogrel-HTPR according to the longitudinal definition (table 7.6).

VerifyNow Aspirin Cartridge

There was a significant increase in the Aspirin Reaction Units (ARU) after cessation of aspirin or aspirin and dipyridamole combination therapy and commencement of clopidogrel monotherapy at 14d [650.5 (range: 350 – 672) *vs*. 457 (range: 350 - 665); p <0.001, N = 36] and 90d [640 (range: 422 – 670) *vs*. 463 (range: 365 - 665); p < 0.001, N = 25] (table 7.5).

Multiplate® analysis in patients commencing clopidogrel

Multiplate® ADP assay

Data were available on 42 patients at baseline, 38 patients at 14d and 25 patients at 90d. After commencing clopidogrel monotherapy, Area Under the Curve (AUC) significantly decreased at 14d [54 U (range: 9 - 120) *vs*. 86 U (range: 43 -132); p < 0.001, N = 38] and 90d [50 U (range: 12 – 110) *vs*. 87 U (range: 45 – 128); p = 0.02, N = 25] (table 7.5). The prevalence of Clopidogrel-HTPR was 60.5% (23/38) at 14d, and 52% (13/25) at 90d using a cross-sectional definition. However, using a longitudinal definition, clopidogrel-HTPR was noted in only 21% (8/38) at 14d and 4% (1/25) at 90d.

Overall, amongst patients who had data at both 14d and 90d after commencing clopidogrel, 20/25 (80%) retained the same cross-sectional/case-control Clopidogrel-HTPR status, and 15/25 (60%) retained the same novel longitudinal

Clopidogrel-HTPR status at 14d and 90d.

Of the four patients who had recurrent events, two patients had Clopidogrel-HTPR according to the cross-sectional definition, but none had clopidogrel-HTPR according to our longitudinal definition at 14d (table 7.6).

Multiplate® ASPIRIN assay

Data were available on 42 patients at baseline, 38 patients at 14d and 25 patients at 90d. After changing from aspirin, aspirin & dipyridamole or no medication to clopidogrel monotherapy, median AUC significantly increased compared with baseline at 14d [93.5 (range: 28 - 158) *vs*. 24.5 (range: 5 -123); $p < 0.001$, N = 38] and 90d [96 (range: 21 – 134) *vs*. 26 (range: 5 – 123); $p < 0.001$, N = 25] (table 7.5).

Table 7. 5 Platelet function/reactivity data after commencement of clopidogrel monotherapy in patients previously on aspirin monotherapy, aspirin-dipyridamole combination therapy or no antiplatelet therapy

Results are presented as medians (range: min-max). Of note, median values at baseline on each assay represent values in the 'entire dataset' because comparisons between matched datasets varied between 14d and 90d; relevant values are described in the text above.

Timepoint	**Baseline**	**14d**	**90d**
Median Daily Dose of Clopidogrel	0 mg	75 mg	75 mg
PFA-100			
C-EPI (S) **P value**	193 (65 – 301)	109 (67-218) $p < 0.001$	114 (85 – 211) $p < 0.001$

C-ADP (S) **P value**	85.5 (50-185)	89.5 (53 – 301) p = 0.59	97.5 (70 – 154) p = 0.07
INNOVANCE P2Y (S) **P value**	65 (45-131)	170.5 (58 – 301) p < 0.001	272.5 (61 – 301) p < 0.001
VerifyNow			
ASPIRIN (ARU) **P value**	464.5 (350-665)	650.5 (350 – 672) p < 0.001	640 (422 – 670) p < 0.001
P2Y12 (PRU) **P value**	300.5 (195 -148)	211.5 (7 – 341) p < 0.001	218 (32 – 284) p < 0.001
Multiplate			
ASPIRIN (U) **P value**	25.5 (5-123)	93.5 (28 - 158) p < 0.001	96 (21 – 134) p < 0.001
ADP (U) **P value**	84 (40-132)	54 (9 - 120) p < 0.001	50 (12 – 110) p = 0.02

Table 7. 6 Clopidogrel-HTPR status at 14d in patients who had recurrent TIAs during follow up

	Aetiology of recurrent event	**PFA-100® Innovance P2Y**		**VerifyNow® P2Y12**		**Multiplate® ADP**	
		Cross-Sectional HTPR	Longitudinal HTPR	Cross-Sectional HTPR	Longitudinal HTPR	Cross-Sectional HTPR	Longitudinal HTPR
Patient 1	Embolism from aortic arch atheroma	No	No	Yes	No	No	No
Patient 2	Vertebral Artery Dissection	No	No	No	No	Yes	No
Patient 3	Embolism from stenosed ICA	Yes	No	No	N/A	Yes	No

7.4.3. Comparison of prevalence of clopidogrel-HTPR according to cross -sectional *vs*. longitudinal definitions of HTPR on each platelet function testing platform in patients commencing clopidogrel

There was a significantly higher prevalence of clopidogrel-HTPR with cross-sectional than longitudinal definitions on the VerifyNow P2Y12 and Multiplate ADP assays at both 14d and 90d. Although there was a trend towards a higher prevalence of clopidogrel-HTPR with cross-sectional than longitudinal definitions on the INNOVANCE® PFA P2Y assay, the differences were not statistically significant at either time point (Table 7.7).

Table 7. 7 Percentages reflect total % with Clopidogrel-HTPR at each time point.

P values refer to comparison of prevalence of Clopidogrel-HTPR between cross-sectional and longitudinal definitions at relevant timepoints. Significant P values in bold.

	14 days		**90 days**	
	Cross-sectional HTPR, % (N)	**Longitudinal HTPR, % (N)**	**Cross-sectional HTPR, % (N)**	**Longitudinal HTPR, % (N)**
PFA Innovance P2Y	31.6% (12/38)	15.8% (6/38)	20.8% (5/24)	4.5% (1/22)
P value		0.13		0.25
VerifyNow P2Y12	55.3% (21/38)	13.9% (5/36)	56% (14/25)	4% (1/25)
P value		**< 0.001**		**< 0.001**
Multiplate ADP	60.5% (23/38)	21% (8/38)	52% (13/25)	4% (1/25)
P value		**< 0.001**		**= 0.03**

7.4.4. Inter-device agreement between different platelet function testing platforms

Using a cross-sectional definition of HTPR, there was moderate agreement between the VerifyNow P2Y12 and Multiplate ADP assays (κ = 0.46), and fair agreement between the INNOVANCE PFA P2Y and both the Multiplate ADP (κ = 0.33) and VerifyNow P2Y12 assays (κ = 0.33) (Table 7.8). However, using longitudinal definitions, there was only slight agreement between the three devices, with kappa values ≤ 0.195 (Table 7.8).

Table 7. 8 Inter-device agreement between different platelet function testing platforms

Cross-sectional definition		
Device and Assay	**Inter-device agreement (κ)**	**Conclusion**
VerifyNow P2Y12 & Multiplate ADP	κ = 0.46 (95% CI: 0.18 to 0.75)	Moderate agreement
INNOVANCE PFA P2Y & Multiplate ADP	κ = 0.33 (95% CI: 0.097 to 0.6)	Fair agreement
INNOVANCE PFA P2Y & VerifyNow P2Y12	κ = 0.33 (95% CI: 0.07 to 0.59)	Fair agreement
Longitudinal definition		
Multiplate ADP & Verify Now P2Y12	κ = 0.006 (95% CI: -0.32 to 0.33)	Slight agreement
INNOVANCE® PFA P2Y & Multiplate ADP	κ = 0.195 (95% CI: -0.197 to 0.59)	Slight agreement
INNOVANCE® PFA P2Y & VerifyNow P2Y12	κ = 0.06 (95% CI: -0.32 to 0.43)	Slight agreement

7.4.5. Relationship between platelet activation status and Clopidogrel-HTPR status on each platelet function testing platform

To assess the relationship between simultaneously-recorded platelet activation markers on flow cytometry and HTPR status, we calculated the 'Exp(B)' for each device, which refers to the odds ratio of the independent variable influencing the dependent variable. There was a positive relationship between platelet activation status and cross-sectional clopidogrel-HTPR status at 14d on 2 of the 3 devices. Increasing % CD62P expression was associated with a greater likelihood of having clopidogrel-HTPR on the VerifyNow P2Y12 at 14d (Exp(B) = 1.71, 95% CI: 1.06 – 2.77; p = 0.029). In addition, increasing percentages of circulating lymphocyte-platelet complexes were associated with a greater likelihood of having clopidogrel-HTPR on the Multiplate ADP assay at 14d (Exp(B) = 13.81, 95%: CI 1.74 – 109.83; p = 0.013). However, there was no association between % CD62P expression or % lymphocyte-platelet complexes and clopidogrel-HTPR on the VerifyNow or Multiplate ADP assays at 90d, or on the INNOVANCE PFA P2Y assay at any timepoint. Furthermore, there was no association between % CD63 expression, % neutrophil-platelet complexes or % monocyte-platelet complexes and clopidogrel-HTPR status on any device at 14d or 90d using either cross-sectional or longitudinal definitions.

7.4.5. Clinical outcomes and recurrent vascular events during follow-up.

Patients were followed up prospectively up to 1 year. Only two patients (4.5%) in this subgroup had a qualifying stroke and the remaining patients had a TIA at the

time of presentation. One stroke patient had a modified Rankin scale score of 4 at baseline, 14d and 90d, and had clopidogrel-HTPR on the VerifyNow and Multiplate assays but had no INNOVANCE PFA P2Y data available. The other stroke patient had a modified Rankin scale score of 1 at baseline and 14d, and did not have clopidogrel-HTPR on any device; he did not have follow-up laboratory data at 90d because he had a recurrent TIA and required anticoagulation with warfarin prior to the scheduled 90d follow-up visit.

Three patients had a recurrent TIA during follow up, as outlined above. No other patients had recurrent stroke, MI or vascular death, and none had venous thrombo-embolism during follow-up. All patients with recurrent TIAs had clopidogrel-HTPR according to one of the cross-sectional definitions on at least one device (table 7.6).

7.5. Discussion

To our knowledge, this innovative pilot study is one of the most comprehensive, simultaneous assessments of *ex vivo* platelet activation and on-treatment platelet reactivity at high and low shear stress, using established cross-sectional and novel longitudinal definitions of clopidogrel-HTPR, in both the early and late phases after TIA and ischaemic stroke in patients commencing clopidogrel.

In agreement with prior pilot studies, the % platelet surface CD62P or CD63 expression (Meiklejohn, Vickers et al. 2001; Grau, Reiners et al. 2003; Tobin, Kinsella et al. 2013) and the % circulating leucocyte-platelet complexes (Tobin, Kinsella et al. 2013) were not significantly influenced by changing from aspirin, aspirin-dipyridamole or no medication to clopidogrel monotherapy at 14d or 90d.

These pilot studies indicate that quantifying alterations in 'unstimulated' platelet activation status with whole blood flow cytometry alone will not inform clinicians whether patients are 'responsive' to clopidogrel monotherapy *ex vivo*. However, assessment of platelet activation status may still provide very important insights into the relationship between platelet activation status and platelet function-reactivity *ex vivo* in patients who commence clopidogrel following TIA or ischaemic stroke. Furthermore, whole blood flow cytometry studies following agonist stimulation *in vitro* have been found to be feasible and informative following TIA or stroke (Bath, May et al. 2018). Using cross-sectional definitions, higher % CD62P expression was associated with a higher probability of having clopidogrel-HTPR on the VerifyNow P2Y12 assay at 14d, and higher % lymphocyte-platelet complexes increased the likelihood of having clopidogrel-HTPR on the Multiplate ADP assay at 14d. These associations were not observed at 90d on either of these low shear stress devices, but this might reflect a type II error due to smaller numbers of patients with data available at 90d. Some of these analyses might also have been partly influenced by the effects of resolution of the acute phase response over time.

The INNOVANCE® PFA P2Y assay did detect the antiplatelet effects of clopidogrel monotherapy in the majority of patients at both 14d and 90d in this study. The cross-sectional prevalence was not significantly higher than the longitudinal prevalence of clopidogrel-HTPR on the INNOVANCE® PFA P2Y at 14d or 90d, respectively (table 7.7). However, there was a clear trend towards a lower prevalence of clopidogrel-HTPR with our novel longitudinal than with the traditional cross-sectional definition, so this may also reflect a type II error. A prior, smaller pilot study in 18 patients treated with aspirin (100-300mg daily) and clopidogrel (75 mg daily) combination therapy or clopidogrel monotherapy (75 mg

daily) found a similar cross-sectional prevalence of clopidogrel-HTPR on the INNOVANCE® PFA P2Y of 39% during the early phase (day 7), but an apparently higher prevalence (possibly 61%) on day 90 after TIA or stroke on either regimen (Jover, Rodriguez et al. 2014). The authors of that study indicated that they also phoned patients to promote adherence with antiplatelet therapy for at least 10 days before their 3 month follow-up visit. However, only 7 patients in early phase and 12 patients in the late phase after stroke or TIA who were included in that study were on clopidogrel monotherapy; these subgroup data were not presented separately in the text, so any apparent difference in prevalence of clopidogrel-HTPR between that study and ours could simply be due to chance.

As previously reported, the C-ADP cartridge was not found to be sensitive at detecting the antiplatelet effects of clopidogrel *ex vivo* (Edwards, Jakubowski et al. 2012; Tsantes, Ikonomidis et al. 2012; Tobin, Kinsella et al. 2013). This may be attributed to the concentration of ADP within the C-ADP cartridge which could cause excessive, 'non-physiological' stimulation of platelets, thus circumventing P2Y12 receptor blockade in the laboratory setting. Furthermore, both the $P2Y_1$ and $P2Y_{12}$ ADP platelet receptors in blood samples from patients on clopidogrel may be activated by the ADP in the C-ADP cartridge, thus contributing to a falsely high cross-sectional prevalence of clopidogrel HTPR on this cartridge (Gachet 2006).

There was a numerically lower cross-sectional prevalence of clopidogrel-HTPR on the INNOVANCE® PFA P2Y assay than on the VerifyNow P2Y12 or Multiplate ADP assays, with only fair agreement between the INNOVANCE® PFA P2Y and the other assays. However, one must note that the PFA-100 assesses both platelet

adhesion and aggregation under moderately high shear stress conditions which are heavily influenced by ADP (Harrison, Robinson et al. 1999; Harrison, Robinson et al. 2002; Paniccia, Priora et al. 2015), whereas the VerifyNow and Multiplate assays essentially assess platelet aggregation under low shear stress conditions which are less ADP-dependent.

The cross-sectional prevalence of Clopidogrel-HTPR on the VerifyNow P2Y12 assay was 55.3% at 14d and 56% at 90d. Our findings at 14d are higher than the reported prevalence of clopidogrel-HTPR of 26-44% in the 'early phase' after ischaemic stroke (Kinsella, Tobin et al. 2013; Sternberg, Ching et al. 2013), but in keeping with the prevalence range reported in the late phase after ischaemic stroke (Maruyama, Takeda et al. 2011; Kinsella, Tobin et al. 2013; Jover, Rodriguez et al. 2014). This higher prevalence at 14 +/7 days after TIA/stroke onset in our study in patients on 75 mg of clopidogrel daily could be explained by the fact that 300 mg loading doses of clopidogrel were given and Clopidogrel-HTPR status measured at 26 and 64 hours following medication intake in patients studied by Stenberg et al (Sternberg, Ching et al. 2013).

The cross-sectional prevalence of Clopidogrel-HTPR on the Multiplate ADP assay in our study was 60.5% at 14d and 52% at 90d. The prevalence in the early phase is also slightly higher than the range of 18-57.1% reported previously within one week after TIA or ischaemic stroke onset (Meves, Schroder et al. 2014; Coignion, Poli et al. 2015; Lundstrom, Wallen et al. 2015). However, to our knowledge, no prior studies have longitudinally followed up the same CVD patients from the early to late phase after TIA or stroke onset with clinical assessment and Multiplate assays

at each timepoint. Our data confirm that over half of patients also have Clopidogrel-HTPR on the Multiplate at least 90d after symptom onset.

The prevalence of Clopidogrel-HTPR was significantly lower at both 14d and 90d using our novel longitudinal compared with traditional cross-sectional definitions on both the VerifyNow P2Y12 and Multiplate ADP assays. These novel longitudinal data reflect the dynamic change in HTPR status over time after starting clopidogrel, and longitudinal definitions have the potential to be more clinically informative than cross-sectional definitions of Clopidogrel-HTPR in individual patients. However, larger follow-up studies are needed to determine whether longitudinal assessment of Clopidogrel-HTPR status is more or less effective than cross-sectional assessment of HTPR status at predicting outcomes and/or recurrent events over time (Kinsella, Tobin et al. 2013; Mannu, Macartney et al. 2015).

The median PFA-100 C-EPI closure time decreased, and the median ARU on the VerifyNow Aspirin assay and the median AUC on the Multiplate Aspirin assay increased in patients after commencing clopidogrel, driven by the cessation of aspirin or aspirin & dipyridamole combination therapy. This confirms that clopidogrel does not affect aspirin-HTPR status on any of these 3 assays, as noted previously for the PFA-100 C-EPI assay (Tobin, Kinsella et al. 2013).

Clopidogrel-HTPR status is a potentially very important phenomenon, which may affect the efficacy of the drug *in vivo.* On-treatment platelet reactivity could be influenced by a number of factors including e.g. medication dosage (Angiolillo, Fernandez-Ortiz et al. 2004) non-adherence, intestinal absorption, bioavailability, interactions with other medications (Gurbel, Bliden et al. 2003; O'Donoghue and

Wiviott 2006; Angiolillo, Fernandez-Ortiz et al. 2007; Armero, Camoin Jau et al. 2010), vascular risk factor profiles, activation of non-ADP mediated intracellular platelet signaling pathways and loss-of function CYP2C19 genetic polymorphisms involved in clopidogrel metabolism from its pro-drug to the active drug (Mega, Close et al. 2009; Wang, Zhao et al. 2016; Pan, Chen et al. 2017).

This study had a number of limitations. The relatively small sample size may have contributed to a type II error during some calculations, as clearly alluded to above. There was no clear relationship between cross-sectional or longitudinal Clopidogrel-HTPR status and the risk of recurrent vascular events using logistic regression, after adjusting for age, gender and smoking status. However, only 3 patients had recurrent TIAs during follow-up, so this study was not powered to reliably assess the relationship between either definition of Clopidogrel-HTPR and the risk of recurrent TIAs or vascular events overall. However, these data can be used to contribute to ongoing, pre-planned studies in our Department and future informative meta-analyses on this topic (Chapter 4). Because only 2 patients had stroke as their qualifying event, we could not reliably comment on the relationship between Clopidogrel-HTPR status and prospectively-collected MRS scores during follow-up following stroke.

7.6. Conclusion

The cross-sectional prevalence of Clopidogrel-HTPR is 31.6-60.5% in the early phase and 20.8-56% in the late phase after TIA or ischaemic stroke based on simultaneous assessment with all 3 platelet function/reactivity testing platforms. Comparing data from all 3 assays shows that the cross-sectional prevalence of

Clopidogrel-HTPR is higher on low shear stress than on moderately high shear stress platforms. Clopidogrel-HTPR prevalence is also higher with cross-sectional than with novel longitudinal definitions of HTPR, but the clinical predictive value of these findings is unclear from this study alone.

At present, one cannot recommend altering antiplatelet therapy based on the results of Clopidogrel-HTPR testing. However, our systematic literature review and meta-analysis indicate that such data have the potential to identify patients who should benefit from a modified, individualised antiplatelet regimen to optimise secondary prevention (see Chapter 4). Larger, adequately-powered, prospective, multi-centre studies are required to assess the value of testing for cross-sectional and longitudinal Clopidogrel-HTPR status with both high and low shear stress assays to enhance our ability to predict the risk of recurrent vascular events and outcomes following TIA and ischaemic stroke in diverse geographical populations.

8. Assessment of on-treatment platelet reactivity at high and low shear stress and platelet activation status after the addition of dipyridamole to aspirin in the early and late phases after TIA and ischaemic stroke

8.1. Introduction

In previous chapters, our findings on aspirin-HTPR, clopidogrel-HTPR and platelet activation status in patients with TIA and ischaemic stroke on these regimens were discussed. Relatively few studies have investigated the phenomenon of 'dipyridamole-HTPR' in ischaemic cerebrovascular disease (CVD) (Tobin, Kinsella et al. 2011).

Dipyridamole exerts its antithrombotic effects in several ways, including by inhibiting adenosine uptake by erythrocytes, which may in turn facilitate vasodilation and platelet inhibition (Kim and Liao 2008). Erythrocytes and endothelial cells remove free adenosine from circulating blood via a specific adenosine carrier called Equilibrative Nucleoside Transporter 1 (ENT1), and dipyridamole inhibits adenosine reuptake by erythrocytes in a dose-dependent manner (Klabunde 1983). Furthermore, dipyridamole inhibits phosphodiesterase 3 (PDE3) and phosphodiesterase 5 (PDE5) in platelets, thus increasing intra-platelet levels of cAMP, a potent inhibitor of 'platelet activation'. Dipyridamole can also

exert a vasodilatory effect on vascular smooth muscle by inhibiting PDE3- and PDE5-mediated cAMP/cGMP degradation. It may also promote prostaglandin I_2 (PGI_2) production by vascular smooth muscle cells which, in turn, also stimulates cAMP production, resulting in further vasodilatory and anti-platelet effects (Neri Serneri, Masotti et al. 1981; Gresele, Zoja et al. 1983). It has been demonstrated that dipyridamole may inhibit 'platelet aggregation' by inhibition of PDE5 (Sakuma, Akaishi et al. 1990; Aktas, Utz et al. 2003). Furthermore, it has been proposed that dipyridamole mediates the antiplatelet effects of a combination of NO and PGI_2 by increasing intracellular levels of cAMP/cGMP (Sakuma, Akaishi et al. 1990; Bult, Fret et al. 1991; Bult, Fret et al. 1991).

Dipyridamole may exert 'anti-inflammatory effects' by inhibiting secretion of monocyte chemotactic protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) from monocytes, resulting in reduced nuclear translocation of NF-kB and inhibition of transcription and generation of further MCP-1 (Weyrich, Denis et al. 2005). Additionally, dipyridamole may downregulate Macrophage-1 Antigen (MAC-1), leading to inhibition of neutrophil adhesion to cultured endothelial cells obtained from ischaemic stroke patients (Hallevi, Hazan-Halevy et al. 2007). Other anti-inflammatory effects of dipyridamole include reduction of lymphocyte recruitment, and reduced activation and secretion of other pro-inflammatory mediators (Coeugniet, Bendtzen et al. 1976; Dong, Osmanova et al. 2006). Monocytes/macrophages and microglial cells play a pivotal role in driving inflammation in evolving ischaemic stroke lesions (Chiba and Umegaki 2013). Dipyridamole has been shown to exert anti-inflammatory effects on human monocytes, which may contribute to its secondary preventive effects following ischaemic stroke (Massaro, Scoditti et al. 2013). Dipyridamole may also inhibit

ICAM-1 and MMP-9 expression in human brain endothelial cells exposed to TNFα, and may reduce levels of MMP-9 and apoptosis in 'oxygen-glucose starved' cerebral endothelial cells (Guo, Stins et al. 2010). More recently, dipyridamole has also been shown to reduce circulating VWF:Ag levels (Zhao, Fletcher et al. 2005; Tobin, Kinsella et al. 2014) and may have indirect 'anticoagulant effects' by reducing peak and total thrombin generation *ex-vivo* following TIA or ischaemic stroke (Tobin, Kinsella et al. 2013).

An early study by Heptinstall *et al*. revealed that there was inadequate inhibition of ADP-induced aggregation in whole blood after ingestion of either 600mg of aspirin alone or in combination with 200 mg of dipyridamole in healthy volunteers using a platelet counting method (Ultra-Flo 100®). However, inhibition of platelet function occurred with aspirin when Platelet Activating Factor (PAF) or low concentrations of Arachadonic Acid (AA) were used as the agonists; this inhibitory effect of aspirin was enhanced in the presence of adenosine when 200mg of dipyridamole was added to aspirin *in vitro* (Heptinstall, Fox et al. 1986).

A pilot, randomised clinical study showed that 30 days treatment with aspirin-dipyridamole combination therapy could lead to enhanced inhibition of platelet function compared with aspirin alone in Japanese patients with ischaemic CVD (Serebruany, Malinin et al. 2004). A further longitudinal, randomised study thoroughly assessed platelet function and activation in type II diabetic patients in the late phase after TIA who were assigned to receive either clopidogrel monotherapy, aspirin-dipyridamole combination therapy, or aspirin-clopidogrel combination therapy (Serebruany, Malinin et al. 2008). There were no significant

differences in platelet function on the PFA-100 between these 3 treatment groups. Another pilot longitudinal, observational study showed that 59% of CVD patients at approximately 14 days and 56% at ≥90 days following symptom onset did not have further inhibition of platelet function on the C-ADP cartridge when 200 mg of dipyridamole MR BD was added to aspirin (Tobin, Kinsella et al. 2011). Using a novel longitudinal definition of HTPR discussed in the previous chapter (Tobin, Kinsella et al. 2011), these patients were deemed to have 'dipyridamole-HTPR' on the PFA-100 C-ADP, but they did not have simultaneous platelet function/reactivity testing at low shear stress during that study (Tobin, Kinsella et al. 2011). It was of interest that monocyte-platelet complexes increased in patients with dipyridamole-HTPR, but not in those without dipyridamole-HTPR over time, suggesting that monocyte-platelet complexes may have influenced dipyridamole-HTPR status on the PFA-100 C-ADP assay in that pilot study. Overall, that study revealed that carefully-designed, prospective longitudinal studies may facilitate detection of additional inhibition of platelet adhesion/aggregation with the PFA-100 C-ADP cartridge in a proportion of patients when dipyridamole is added to aspirin, a finding which would not have been identified using cross-sectional definitions of HTPR.

In clinical trials, aspirin-dipyridamole combination therapy has been shown to be superior to aspirin alone at preventing recurrent stroke (23% relative risk reduction) (Diener, Cunha et al. 1996) or recurrent ischaemic vascular events overall (22% relative risk reduction) in patients with ischaemic cerebrovascular disease (Group, Halkes et al. 2006). The use of aspirin-dipyridamole combination therapy within 24 hours of TIA or ischaemic stroke onset has also been shown to be as safe and as effective as deferring the introduction of dipyridamole for 7 days after symptom onset (Dengler, Diener et al. 2010). However, the majority of patients are still not

protected from further vascular events by adding dipyridamole to aspirin, and this may partly relate to the phenomenon of dipyridamole-HTPR.

To further investigate this topic following a prior, novel pilot study by our group, we performed an independent, longitudinal, observational case-crossover study in TIA and ischaemic stroke patients whose treating physician opted to add dipyridamole to aspirin monotherapy.

8.2. Aims and Hypotheses

The **aims** of this aspect of the prospective, longitudinal, observational-analytical OATS study were:

1. To simultaneously assess and compare the ability of established and novel laboratory tests of platelet reactivity to identify patients with recent TIA or ischaemic stroke who have dipyridamole-HTPR *ex vivo* after the addition of dipyridamole to aspirin using 'novel longitudinal definitions' of HTPR.

2. To improve our understanding of the underlying platelet activation pathways and clinical, demographic and pharmacodynamic mechanisms influencing *ex vivo* on-treatment platelet reactivity in CVD patients when dipyridamole is added to aspirin.

3. To collect pilot data to assess the potential ability of this 'novel longitudinal definition of dipyridamole-HTPR', especially on the PFA-100 C-ADP cartridge, to predict the risk of recurrent vascular events during long-term follow-up.

Hypotheses:

1a. Inhibition of platelet function/reactivity would be enhanced in an important minority of patients on aspirin-dipyridamole combination therapy compared with aspirin alone, and the PFA-100 C-ADP cartridge would be confirmed to be able to detect such inhibition;

1b. The Multiplate ADP assay might be able to detect additional inhibition of platelet function/reactivity after the addition of dipyridamole to aspirin.

2. This study would provide further insights into the relationship between platelet activation and dipyridamole-HTPR status, and improve our understanding of the mechanisms influencing *ex vivo* on-treatment platelet reactivity on dipyridamole in CVD patients overall.

3. Platelet reactivity testing may improve our ability to predict the risk of recurrent vascular events in CVD patients treated with aspirin-dipyridamole combination therapy. Pilot data from this study will be utilised to design a definitive multi-centre study.

8.3. Methods

8.3.1. Recruitment

Patients who met the inclusion criteria were recruited during the period between 26/10/2011 and 13/01/2016 at AMNCH-TUH.

Inclusion criteria:

Patients were eligible if they were:

- ≥18 years of age;

- Within 4 weeks of onset of a TIA or ischaemic stroke;
- On aspirin monotherapy and their treating physician decided to change their antiplatelet treatment regimen to aspirin-dipyridamole combination therapy.

Exclusion criteria:

Patients were excluded if they had a myocardial infarction, DVT, PE or recent surgery within the preceding three months; ongoing unstable angina or unstable symptomatic peripheral vascular disease; platelet count < 100 x 10^9/L; known bleeding or clotting diathesis, including known platelet-related bleeding disorders; active proven vasculitis; active neoplasia; recent or prior history of intracranial haemorrhage, active infection and non-steroidal anti-inflammatory drug (NSAID) intake other than aspirin in the preceding 11 days; were unlikely to be able to attend for clinical follow-up and repeat testing at 14 +/- 7 days.

Written informed consent, or 'proxy consent' where appropriate, was obtained from all participants, and this study was approved by the St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2011/35/03).

8.3.2. Clinical assessment and outcome events

Clinical Assessment:

All patients underwent detailed clinical assessment and were given a clinical diagnosis of a TIA or ischaemic stroke by their attending Consultant Neurologist or Stroke Physician after detailed investigations according to ESO recommendations (European Stroke Organisation Executive and Committee 2008). The diagnosis was also confirmed in all cases by me as a clinically-experienced Vascular Neurology Research SpR (STL) or by the supervising Vascular Neurologist (DJHM). The underlying mechanism responsible for TIA or ischaemic stroke was categorised according to both the TOAST classification (Adams, Bendixen et al. 1993) and

ASCOD classification systems (Amarenco, Bogousslavsky et al. 2013).

Patients underwent detailed clinical and laboratory assessment with venesection at baseline before changing treatment (**baseline**), at 14 +/- 7 days after (**14d**), and at least 90 days (**90d**) after their treating physician added dipyridamole to aspirin monotherapy. In patients with recently symptomatic ≥50% carotid artery stenosis, the 90d follow-up was performed at least 3 months following carotid surgery or endovascular treatment, unless intervention had been delayed for at least 3 months after symptom onset. Adherence to prescribed antiplatelet therapy in inpatients was confirmed by assessing their inpatient drug chart. Adherence in all outpatients was checked by history taking alone, but all patients were phoned to emphasise the importance of medication-adherence in the week prior to reassessment. Reassessment was deferred for 14 days in any patients deemed possibly non-adherent to their antiplatelet treatment, and any issues potentially affecting adherence were addressed. In addition, clinical follow-up to assess the longer-term risk of recurrent vascular events and outcome measures was performed using a validated in-person or telephone questionnaire at ≥1 year after symptom onset.

Clinical outcome events:

Detailed information regarding primary and secondary clinical outcome events was collected in person at each clinical and/or laboratory follow-up visit to assess the incidence of recurrent events, and in particular between 14d and the date of last follow up. Clinical follow-up assessments were performed at ≥365 days (1 year) in all cases in person by Dr Lim. If 1 year clinic attendance was not possible, a validated and standardised telephone questionnaire was completed. If any patients reported recurrent vascular events, they were invited to re-attend for clinical

reassessment to confirm the outcome, and undergo repeat platelet function and activation testing, where appropriate. We prospectively planned to confirm any outcome events determined by telephone interview alone by contacting the GP, reviewing relevant hospital consultant's letters or notes, and if necessary, by reviewing death certificates if the patient had unfortunately died before the 1-year follow-up visit.

***Primary clinical endpoint*:** Composite endpoint of non-fatal ischaemic stroke, non-fatal MI or vascular death during follow-up.

***Secondary clinical endpoints*: 1**. Recurrent ischaemic stroke; **2.** Recurrent TIA or stroke; **3.** Symptomatic haemorrhagic infarct transformation; **4**. Primary intracerebral haemorrhage; **5.** Myocardial infarction or unstable angina requiring coronary intervention; **6.** Severe symptomatic peripheral arterial disease requiring lower limb endovascular or surgical revascularisation or major amputation; **7.** Major extracranial haemorrhage; **8.** Minor extracranial haemorrhage, as per definitions of haemorrhage in PRoFESS (Sacco, Diener et al. 2008).

8.3.3. Laboratory Assessments

Detailed descriptions of the protocols for 'careful, atraumatic venepuncture' and laboratory tests have been described previously in Chapter 5. In summary, venepuncture was performed in a standardised manner, as previously described (McCabe, Harrison et al. 2004) (Kinsella, Tobin et al. 2013). 2 ml sterile 3.2% buffered sodium citrate-anticoagulated samples were taken for analysis of platelet function with the VerifyNow® system (Accriva Diagnostics) Six further samples were taken in

sterile 3 ml Vacutainers® containing 3.2% buffered sodium citrate. The first and second of these citrate-anticoagulated samples were used for whole blood flow cytometric analysis and for measurement of platelet function with the platelet function analyser (PFA-100®, Dade-Behring, Germany). Subsequently, one 3 ml double-walled Vacutainer® tube containing recombinant 'hirudin anticoagulant' was taken for analysis of platelet function on the Multiplate® system. Three 3ml sterile Vacutainer® tubes containing K_2EDTA were obtained. This first sample was used to measure the full blood count (FBC), including measurement of the mean platelet volume (MPV) and platelet distribution width (PDW) (see chapter 5).

Whole Blood Flow Cytometry: Platelets were distinguished from red and white blood cells, as described previously (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013). The expression of certain 'activation-dependent markers' that were upregulated to the platelet surface during platelet activation were quantified, including CD62P and CD63 (McCabe, Harrison et al. 2004; Kinsella, Tobin et al. 2013). The percentages of circulating leucocyte-platelet complexes (neutrophil-platelet, monocyte-platelet and lymphocyte-platelet complexes) were also quantified as additional, more sensitive markers of platelet activation using previously described methods (Tobin, Kinsella et al. 2011).

PFA-100® Platelet Function Analyser: The degree of inhibition of platelet function in whole blood was assessed before and after adding dipyridamole to aspirin therapy at moderately high shear stress following biochemical stimulation with collagen and epinephrine (C-EPI cartridge), collagen-ADP (C-ADP cartridge), and ADP/Prostaglandin E_1/CaCl (INNOVANCE PFA P2Y™ cartridge), as

previously described (McCabe, Harrison et al. 2005). The membrane is coated with collagen (2 µg) in combination with either 50 µg ADP (C-ADP cartridge), 10 µg epinephrine bitartrate (C-EPI cartridge), or 20µg ADP / 5ng Prostaglandin E_1 / 459µg CaCl (INNOVANCE® PFA P2Y cartridge). This device mimics the *in vivo* haemostatic process that one may see in a moderately stenosed artery. The time taken for activated platelets to occlude an aperture in the cartridge is called the closure time. The maximum closure time recorded by the device is 300s; we arbitrarily defined closure times > 300s as 301s for statistical analyses using non-parametric tests because one could not assume that the data were normally distributed.

Recently-defined novel longitudinal definition of Dipyridamole-HTPR on the PFA-100*:* In our laboratory, the intra-assay co-efficient of variation (CV) for the C-ADP assay was 7%, for the C-EPI cartridge was 7.5%, and for the INNOVANCE® PFA P2Y cartridge was 7.8%. 'Dipyridamole HTPR' was defined as failure to prolong C-ADP closure times compared with the patient's baseline on aspirin monotherapy by more than twice the CV of the assay when dipyridamole was added to aspirin therapy i.e. failure to prolong C-ADP closure times by >14% of the patient's baseline C-ADP closure time (Tobin, Kinsella et al. 2011).

VerifyNow® Platelet Function Analyser: The VerifyNow® is a cartridge-based analyser which assesses *ex-vivo* platelet reactivity at low shear stress in response to stimulation with fixed doses of different platelet agonists in single-use cartridges containing fibrinogen coated beads (van Werkum, Harmsze et al. 2008). The reagents bound to the fibrinogen beads are arachidonic acid in the Aspirin cartridge, and adenosine diphosphate (ADP), iso- thrombin receptor activating peptide (iso-

TRAP), and PAR-4 activating peptide in the $P2Y_{12}$ cartridge. During the test, a 2ml 3.2% sodium citrate-anticoagulated whole blood sample tube is inserted into the cartridge. The whole blood is mixed with the platelet agonists and the fibrinogen-coated beads by the movement of an electromagnetically-driven steel ball. The platelets become activated by the specific agonist in the cartridge to a degree that is dependent on the level of inhibition by the anti-platelet agent which the patient is taking. Activated platelets will then bind to the fibrinogen-coated beads, cause agglutination and will fall out of the solution. Within the instrument, the light absorbance through the solution is measured 16 times per second. Both the rate and extent of platelet-induced agglutination over a fixed period of time are measured and combined with a proprietary algorithm to report the values in reaction units (van Werkum, Harmsze et al. 2008). Intra-assay CVs were measured in our laboratory and found to be 0.1% for the 'Aspirin cartridge' and 5.5% for the '$P2Y_{12}$ cartridge'.

Multiplate® Assay*:* This whole blood platelet aggregation assay is based on measurement of impedance at **low shear stress** as platelets adhere to 2 adjacent electrodes and aggregate to one another within a cuvette. The extent of platelet adhesion and aggregation is recorded as the Area Under the Curve (AUC) up to 6 minutes after the addition of either arachidonic acid (**Aspirin test**) or ADP (**ADP test**) to measure the antiplatelet effects of aspirin or clopidogrel, respectively. Based on our experience with the PFA-100 C-ADP assay, we assessed the potential ability of the Multiplate ADP assay to detect additional inhibition of platelet function *ex-vivo* following stimulation with ADP when dipyridamole was added to aspirin. In our laboratory, the intra-assay CV for the Aspirin test was 7.3% and for the ADP test was 7.8% (N = 8 assays).

Exploratory novel longitudinal definition of Dipyridamole-HTPR on the Multiplate ADP assay*:* 'Dipyridamole HTPR' was provisionally defined as failure to decrease the AUC of the Multiplate ADP test compared with the patient's baseline AUC on aspirin monotherapy by more than twice the CV of the assay when dipyridamole was added to aspirin therapy i.e. failure to decrease the AUC by > 15.6% of the patient's AUC at baseline.

8.3.4. Statistical Methods

All statistical analyses were performed using SPSS (Version 23). Descriptive statistical calculations were performed to describe demographic and vascular risk factors, and TIA/stroke subtyping in our patient population. PFA-100® closure times are reported by the device as a continuous scale in seconds up to 300s. All results above 300s are reported as >300s, and we arbitrarily classified these as 301s for the purpose of our analyses. The Wilcoxon signed rank test was used for comparison of C-EPI, C-ADP and Innovance P2Y closure times, VerifyNow Aspirin and VerifyNow P2Y12 reaction units, and Multiplate-Aspirin and Multiplate-ADP AUCs at 14d and 90d relative to baseline. Spearman rank-order correlation analysis assessed the relationship between platelet activation markers, leucocyte-platelet complexes and PFA-100 C-ADP closure times & Multiplate ADP AUC values.

8.4. Results

Sixty patients were recruited at baseline; 39 of these patients had analysable data at 14d and 31 had analysable data at 90d (figure 8.1). Fourteen patients discontinued treatment due to side effects with dipyridamole, most commonly headaches, nausea,

vomiting or dizziness. Three patients underwent urgent carotid endarterectomy and could not be reassessed at 14d, as per our study protocol; one of these patients was only assessed at baseline, and two of these patients had baseline data and follow up data at 90d. Six patients were subsequently found to have paroxysmal atrial fibrillation and required anticoagulation with cessation of antiplatelet therapy during early or delayed follow-up (figure 8.1). One patient was subsequently excluded due to an interval diagnosis of metastatic liver cancer. One patient was excluded during follow-up due to non-adherence with treatment at 14d. One patient could not attend for follow up due to severe arthritis limiting mobility, and one patient who was uncontactable was lost to follow-up. Four patients could not attend for follow up due to difficult personal circumstances. Furthermore, every laboratory assay could not be performed at each time point if e.g. certain devices were being serviced when patients re-attended for follow-up, this limiting some of our follow up laboratory data.

Of the patients recruited, four had a cerebral ischaemic stroke, one had a thoracic spinal cord infarct, and the remainder had TIAs at enrolment. The median daily dose of aspirin was 150mg at baseline, and 75mg at 14d and 90d.

Figure 8. 1 Flowchart of patients in whom dipyridamole was added to aspirin monotherapy.

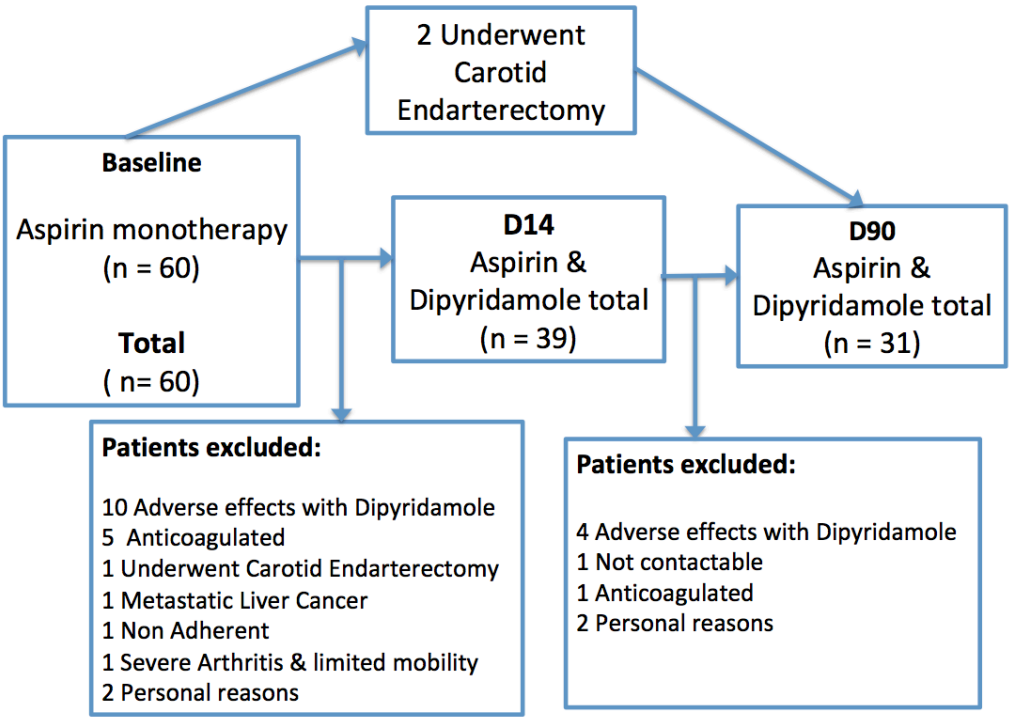


Table 8. 1 Demographic and vascular risk factor profiles in patients at enrolment

TIA = Transient Ischaemic Attack; IHD = Ischaemic Heart Disease; DVT = Deep Venous Thrombosis; PE = Pulmonary Embolism

Characteristic	Numbers	(%)
Mean Age (years)	60.2 (SD 12.7)	
Gender (M/F)	32/28	53.3%
Ischaemic Stroke at presentation	Cerebral (4); Spinal Cord (1)	8.3%
TIA at presentation	55	91.7%
Thrombolysed	2	3.3%
Prior Stroke/TIA	4	6.7%
IHD	3	5%
Hypertension	18	30%
Diabetes Mellitus	2	3.3%
Atrial Fibrillation at Enrolment	0	0
Family History of Stroke	13	21.7 %
Prior DVT/PE	0	0
Peripheral Vascular Disease	2	3.3%
Hyperlipidaemia	14	23%
Migraine (with or without aura)	11	18.3%
Current Smoker	12	20%
Ex-smoker	20	33.3%
Never smoker	26	43.3 %
Statin Therapy	31	51.7%
Index Event on Antiplatelet Therapy	9	15%

Table 8. 2 TOAST classification in patients at enrolment

(Total N = 60)

Stroke / TIA Subtype	Numbers (%)
Large Artery Atherosclerotic	4 (6.7 %)
Small Vessel Disease	5 (8.3 %)
Cardioembolic	6 (10 %)
Other Determined	0
Undetermined Aetiology	45 (75%)

Table 8. 3 ASCOD classification in patients at enrolment

(Total N = 60)

ASCOD Phenotype	Disease present (ASCOD 1,2,3)	Disease absent (ASCOD 0)	Insufficient investigation (ASCOD 9)
A % (N)	28 (46.7%)	32 (53.3%)	0
S % (N)	36 (60%)	24 (40%)	0
C % (N)	30 (50%)	30 (50%)	0
O % (N)	17 (28%)	43 (72%)	0
D % (N)	0 (0%)	60 (100%)	0

A: Atherosclerosis; S: small vessel disease; C: cardiac source; O: other cause; D: dissection
1: 'definitely a potential cause of the index stroke',
2: 'causality uncertain',
3: 'unlikely a direct cause of the index stroke but disease is present',
0: 'disease absent'

8.4.1. Impact of the addition of dipyridamole to aspirin on platelet activation and platelet function.

8.4.1.1. Platelet activation status in patients in whom dipyridamole was added to aspirin

There were no statistically significant changes in platelet surface CD62P or CD63 expression at 14d or 90d *vs*. baseline after commencing dipyridamole overall. Compared with baseline values, the % circulating neutrophil-platelet complexes did not change at 14 days, but significantly increased at 90d (P = 0.01). Monocyte-platelet (%) complexes initially increased at 14d (P = 0.02), but this increase did not reach statistical significance at 90d after commencing dipyridamole (P = 0.06).

Table 8. 4 Platelet Activation Markers after adding Dipyridamole to Aspirin.

Values are medians (range: min-max). Significant P values in bold.

	Baseline N = 47	**14 days N = 30**	**90 days N = 23**
Platelet surface markers:			
CD62P %	4.0 (0.67 – 13)	3.14 (0.59 – 18.3)	3.3 (0.51 – 13.4)
P value		0.37	0.18
CD63 %	13.5 (3.39 – 34.6)	12.9 (2.49 – 27.7)	12.8 (4.07 – 29.60)
P value		0.18	0.32
Leucocyte-Platelet Complexes:			
Neutrophil-Platelet (%)	2.63 (0.88 – 7.14)	2.69 (0.8 – 8)	3.04 (0.3 – 5.31)
P value		0.59	**0.01**
Monocyte – Platelet (%)	6.0 (0.4 – 14.1)	6.1 (1.1 – 12.8)	6.45 (2.2-9.8)
P value		**0.02**	0.06
Lymphocyte-Platelet (%)	2.0 (0.65 – 8.0)	2.2 (1.11 – 8.77)	2.42 (1.00 – 8.33)
P value		0.25	0.18

Figure 8. 2 Scatterplot of CD62% in patients commencing dipyridamole

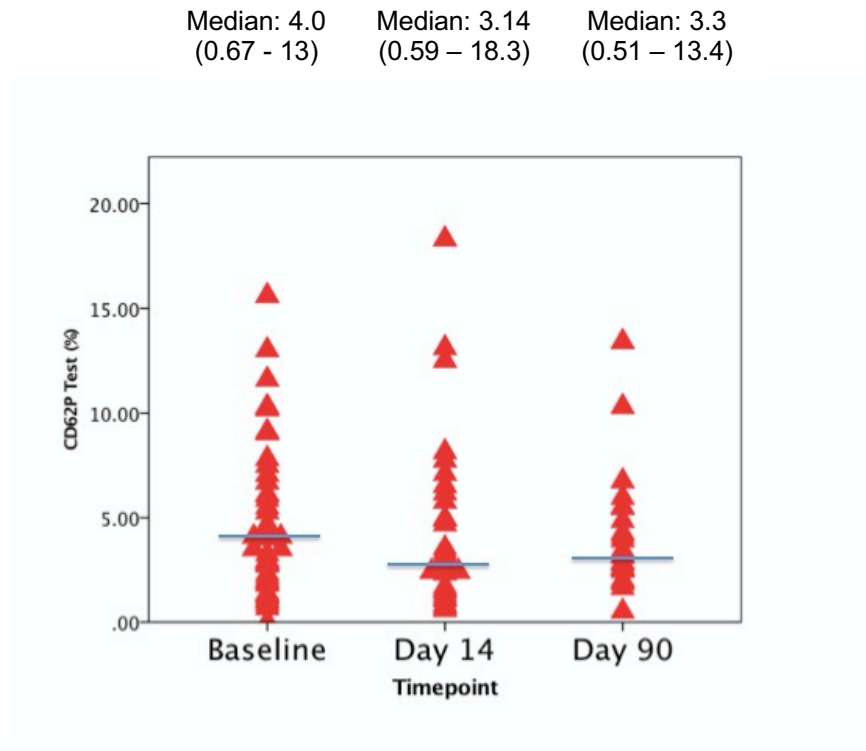


Figure 8. 3 Scatterplot of CD63% in patients commencing dipyridamole

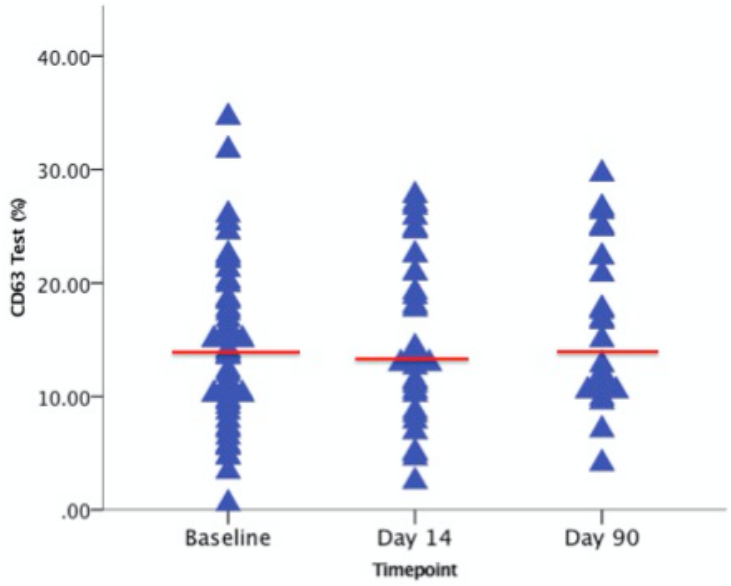


Figure 8. 4(a), (b) and (C) Leucocytes-Platelet Complexes in Patients Commencing Dipyridamole

Figure 8.4 (a): % Neutrophil-Platelets in patients commencing dipyridamole

Median: 2.63 (0.88 – 7.14) | Median: 2.69 (0.8 – 8) | Median: 3.04 (0.3 – 5.31) **P = 0.01**

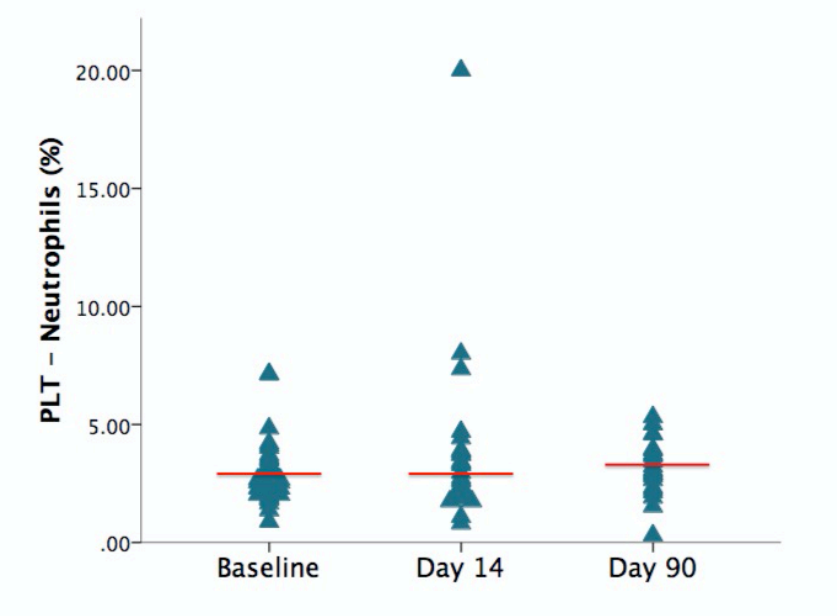


Figure 8.4 (b): % Monocyte-Platelets in patients commencing dipyridamole

Median: 6.0 (0.4 – 14.1) | Median: 6.1 (1.1 – 12.8) **P = 0.02** | Median: 6.45 (2.2-9.8)

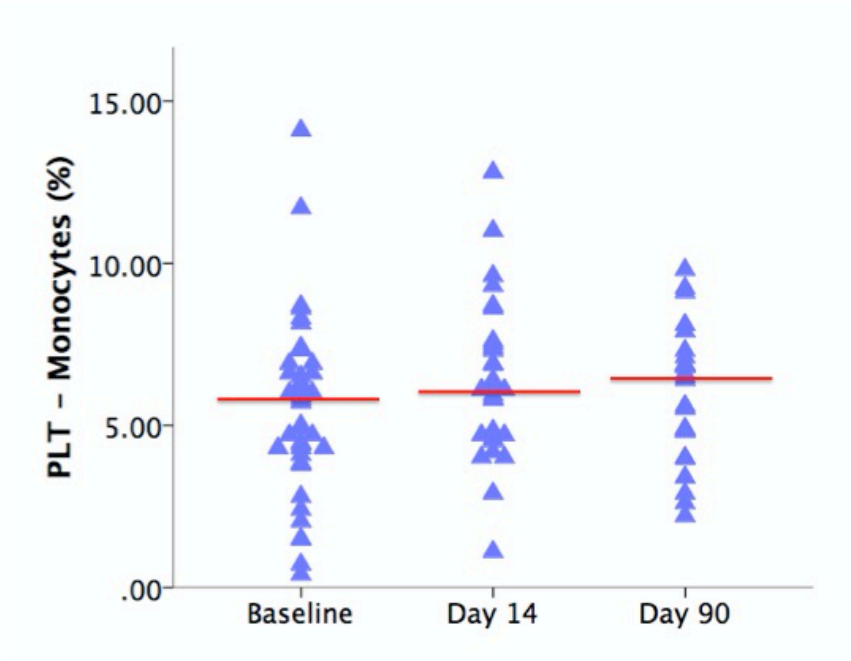
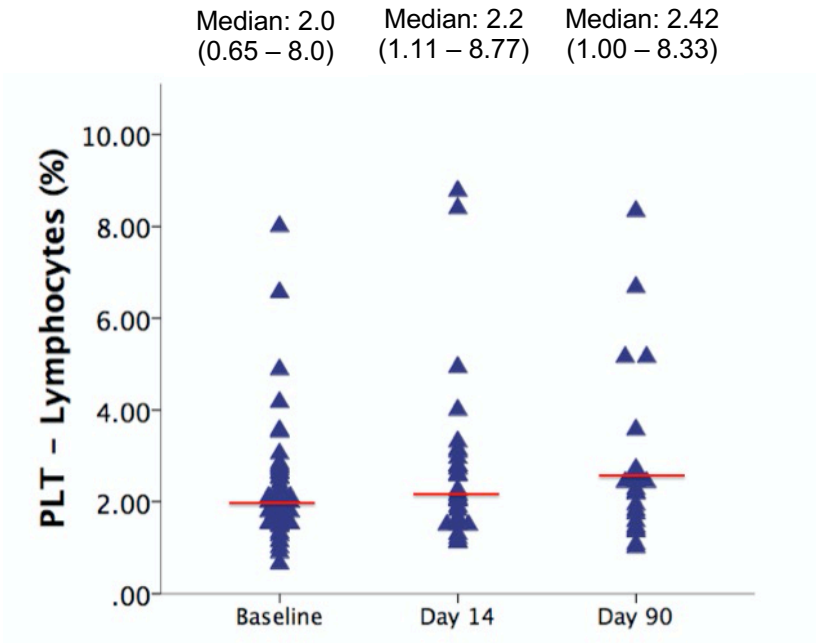


Figure 8.4 (c): % Lymphocytes-Platelets in patients commencing dipyridamole



8.4.1.2. Platelet function/reactivity after the addition of dipyridamole to aspirin

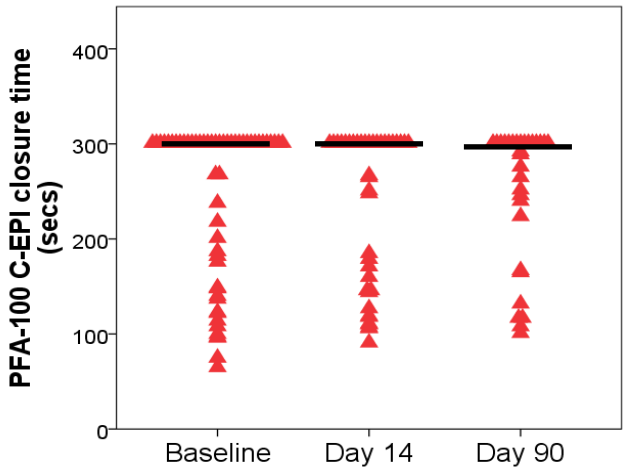
PFA-100® analysis in patients commencing dipyridamole

In the following sections, follow-up biomarker data are presented first and baseline data are presented second for comparative purposes in the text, unless otherwise specified.

C-EPI assay

Compared with baseline, there was no significant change in median C-EPI closure times after adding dipyridamole to aspirin monotherapy at either 14d [301s (range: 91 - 301s) *vs*. 301s (range: 75 - 301s); p = 0.36; N = 35] or 90d [292s (range: 101 – 301s) *vs*. 301s (range: 96 – 301s); p = 0.1; N = 27) (table 8.5 and figure 8.5).

Figure 8. 5 Scatterplot of PFA-100® C-EPI closure times at baseline, 14d and 90d in patients commencing dipyridamole.
Symbols represent individual patient's data and black bars represent median values in all the scatterplots below.

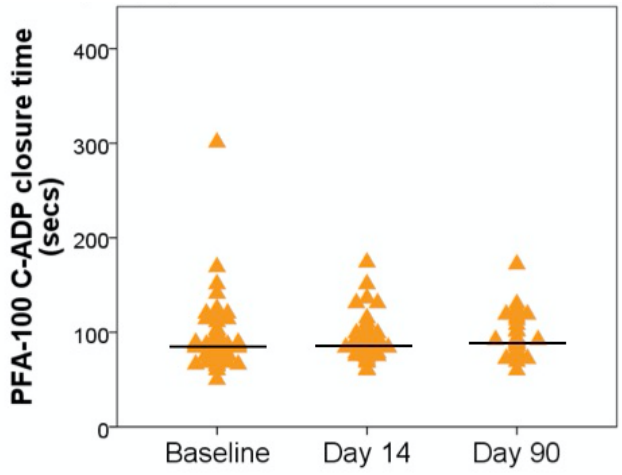


C-ADP assay

There was no statistically significant increase in median C-ADP closure times after adding dipyridamole to aspirin monotherapy at 14d [87.5s (range: 60 - 174s) *vs*. 88.5s (range: 64 - 169), p = 0.2; N = 35] or 90d [92s (range: 60 - 172s) *vs*. 90.5s (range: 68 - 169s), p = 0.6; N = 27] (table 8.5 and figure 8.6).

However, using our longitudinal definition, the prevalence of dipyridamole-HTPR on the PFA-100 C-ADP assay was 75% (27/36) at 14d and 71.4% (20/28) at 90d. Six patients who had dipyridamole-HTPR at 14d did not have persistent dipyridamole-HTPR at 90d. Four patients who had dipyridamole-HTPR at 14d had no data available for analysis at 90d because the patients either dropped out of the study or the device was not functioning at the 90d follow-up visit. Two patients with dipyridamole-HTPR at 90d had not had dipyridamole-HTPR at 14d, and a further two patients with dipyridamole-HTPR at 90d had no 14d data available. Overall, amongst patients who had matched data at both 14d and 90d (N = 27), 18/27 (66.7%) retained the same dipyridamole-HTPR status during follow-up.

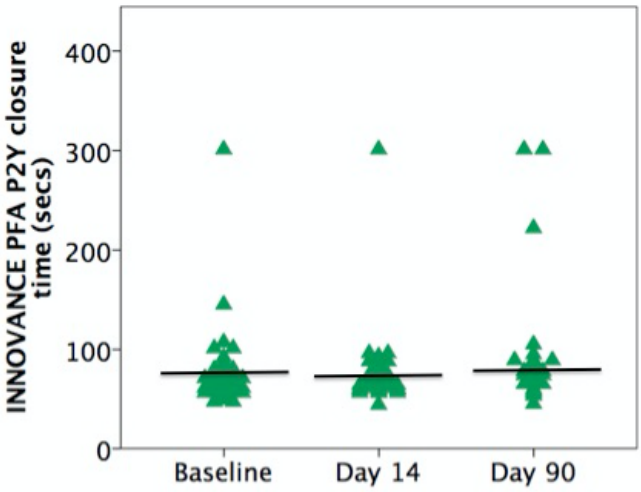
Figure 8. 6 Scatterplot of PFA-100® C-ADP closure times at baseline, 14d and 90d in patients commencing dipyridamole



Innovance P2Y Assay

There was no significant change in Innovance P2Y closure times after commencing dipyridamole in patients on aspirin monotherapy at either 14d [67s (range: 44 – 301s) *vs*. 70s (range: 49 – 301s), p = 0.25; N = 36] or 90d [74.5s (range: 45 – 301s) *vs*. 72s (range: 50 – 301s); p = 0.1; N = 26). (table 8.5 and figure 8.7).

Figure 8. 7 Scatterplot of PFA-100® Innovance P2Y closure times at baseline and 14d and 90d in patients commencing dipyridamole

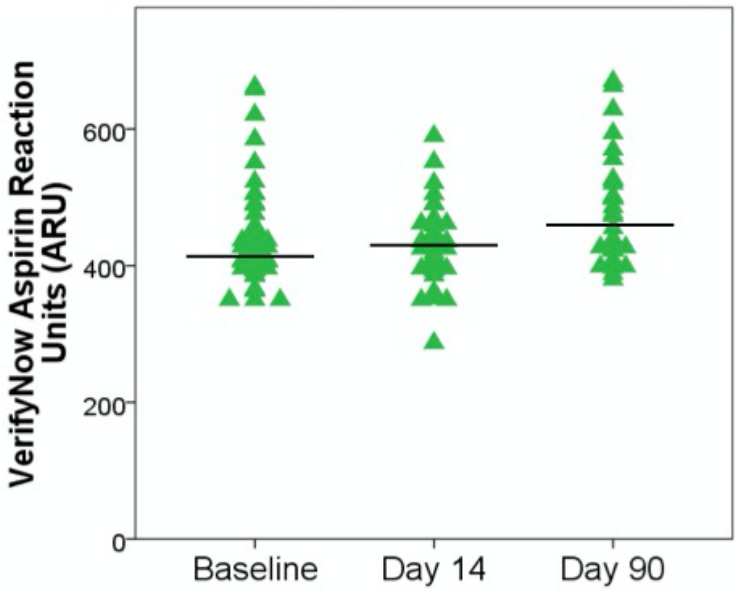


VerifyNow® analysis in patients commencing Dipyridamole

Aspirin Assay

There was no significant change in Aspirin Reaction Units (ARU) after adding dipyridamole to aspirin monotherapy at 14d [431.5 (range 350 – 590) *vs*. 412 (range 350 - 662), p = 0.5; N = 38]. However, the median ARU was significantly higher at 90d *vs*. baseline [455 (range: 380 – 670) *vs*. 409 (range: 350 – 662), p = 0.008; N = 32] (table 8.5 and figure 8.8).

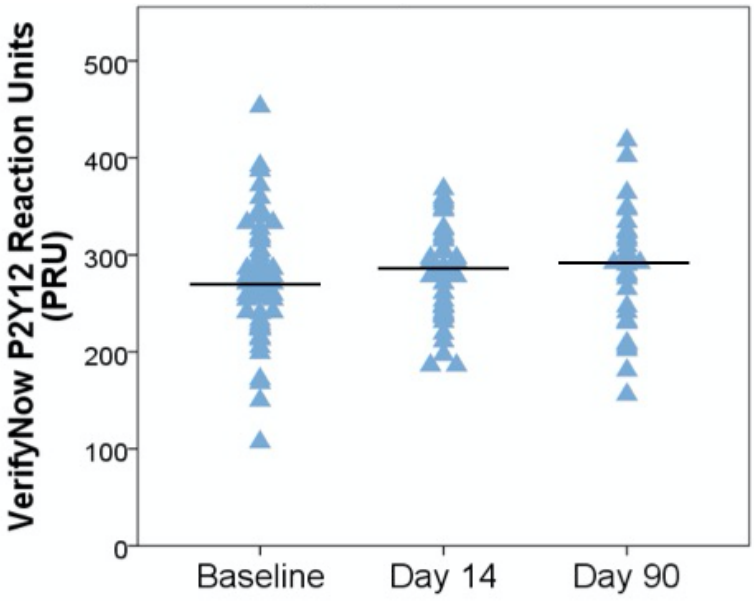
Figure 8. 8 Scatterplot of VerifyNow® Aspirin ARU at baseline, 14d and 90d in patients commencing dipyridamole



P2Y12 Assay

There was no significant change in median P2Y12 Reaction Units (PRU) after adding dipyridamole to aspirin monotherapy at 14d [284 (range: 186 - 368) *vs*. 278.5 (range: 150 - 392), p = 1.0; N = 36] or 90d [292.5 (range: 156 – 418) *vs*. 278.5 (range: 150 - 387), p = 0.13; N = 30) (table 8.5 and figure 8.9).

Figure 8. 9 Scatterplot of VerifyNow® P2Y12 PRU at baseline, 14d and 90d in patients commencing dipyridamole

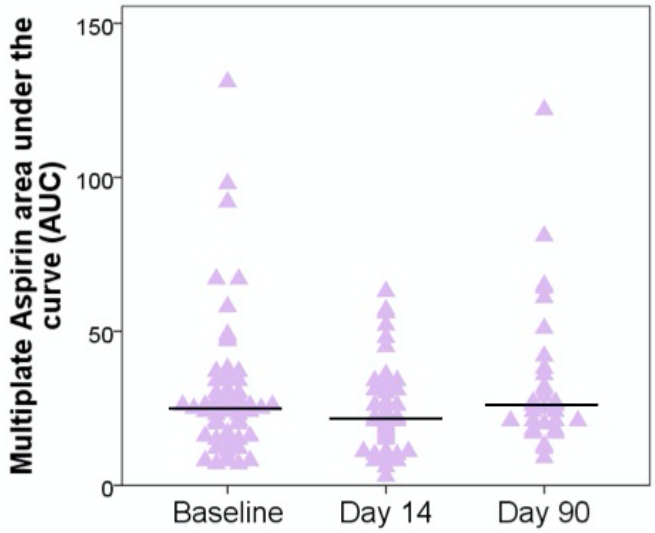


Multiplate® assays in patients commencing dipyridamole

Multiplate Aspirin Assay

There was no significant change in the Multiplate Aspirin AUC after commencing dipyridamole at 14d [24 U (range: 6 - 63) *vs*. 24 U (range: 7-131); p = 0.5, N = 35] or at 90d [27 U (range: 9 -122) *vs*. 26 U (range: 7 – 131); p = 0.3, N = 27] (table 8.5 and figure 8.10).

Figure 8. 10 Scatterplot of Multiplate® Aspirin assay AUC data at baseline, 14d and 90d in patients commencing dipyridamole



Multiplate ADP assay

There was no statistically significant change in the Multiplate ADP AUC after adding dipyridamole at 14d [89 U (range: 40 - 162) *vs*. 93 U (range: 24 -153); p = 0.5, N = 35] or at 90d [83 U (range: 47 -150) *vs*. 95.5 U (range: 24 – 153); p = 0.9, N = 27] (table 8.5 and figure 8.11).

However, using our novel, exploratory, longitudinal definition of dipyridamole-HTPR on the Multiplate ADP assay, the prevalence of dipyridamole-HTPR was 83.3% (30/36) at 14d and 81.5% (22/27) at 90d on this device. Two patients had dipyridamole-HTPR at 14d, but did not exhibit dipyridamole-HTPR at 90d. None of the patients who had dipyridamole-HTPR at 90d had been 'dipyridamole responsive' at 14d. Overall, amongst patients who had matched data at both 14d and 90d (N = 27), 24 (88.9%) retained the same dipyridamole-HTPR status during follow-up.

Figure 8. 11 Scatterplot of the Multiplate® ADP assay AUC data at baseline, 14d and 90d in patients commencing dipyridamole

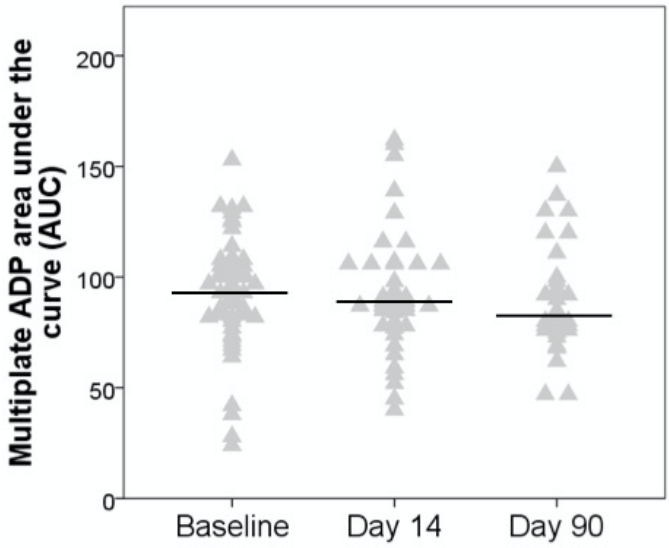


Table 8. 5 Median values (range: min-max) from different platelet function / reactivity assays at each time point.
P values refer to comparisons between data at baseline and at 14d and 90d, respectively

Time point	Baseline	14d	90d
Median Daily Dose of: Aspirin Dipyridamole MR	150 mg daily / 0 mg	75 mg daily / 200mg BD	75 mg daily / 200mg BD
PFA-100			
C-EPI (S) P value	301 (65-301)	301 (91-301) 0.36	292 (101-301) 0.1
C-ADP (S) P value	87 (50-301)	87.5 (60-174) 0.2	92 (60-172) 0.6
INNOVANCE P2Y (S) P value	71 (47-301)	67 (44-301) 0.3	74.5 (45-301) 0.1
VerifyNow			
Aspirin (ARU) P value	414 (350-662)	431.5 (350-590) 0.5	455 (380-670) 0.008
P2Y12 (PRU) P value	271 (107-453)	284 (186-368) 1	292.5 (156-418) 0.1
Multiplate			
ASPIRIN (U) P value	25 (7-131)	24 (6-63) 0.5	27 (9-122) 0.3
ADP (U) P value	92 (24-153)	89 (40-162) 0.5	83 (47-150) 0.9

8.4.1.3. Relationship between platelet activation status and dipyridamole-HTPR status on the PFA-100 C-ADP and Multiplate ADP assays

PFA-100 C-ADP assay:

The median %CD62P expression was significantly lower overall in the subgroup of patients with dipyridamole-HTPR than in those without dipyridamole-HTPR at 14d (3.01 *vs*. 6.17%; p = 0.03) (Table 8.6). There were no other significant differences in the expression of other platelet activation markers between those with *vs*. those without dipyridamole-HTPR at any timepoint (Table 8.6). There was no significant correlation between the % CD62P expression, % CD63 expression or the % circulating leucocyte-platelet complexes and C-ADP closure times ($p \geq 0.16$). However, the % monocyte-platelet complexes increased in the subgroup of patients with dipyridamole-HTPR (p = 0.02; figure 8.12), but not in those without dipyridamole-HTPR (p=0.61) at 14d compared with baseline. In addition, the % monocyte platelet complexes also increased in the subgroup of patients with dipyridamole-HTPR (p = 0.038; figure 8.13), but not in those without dipyridamole-HTPR (p=0.78) at 90d compared with baseline.

Figure 8. 12 Boxplot of % monocyte-platelet complexes at baseline vs. 14d in patients with dipyridamole-HTPR on PFA-100 C-ADP assay.
Values are medians (range: 25th -75th percentiles)

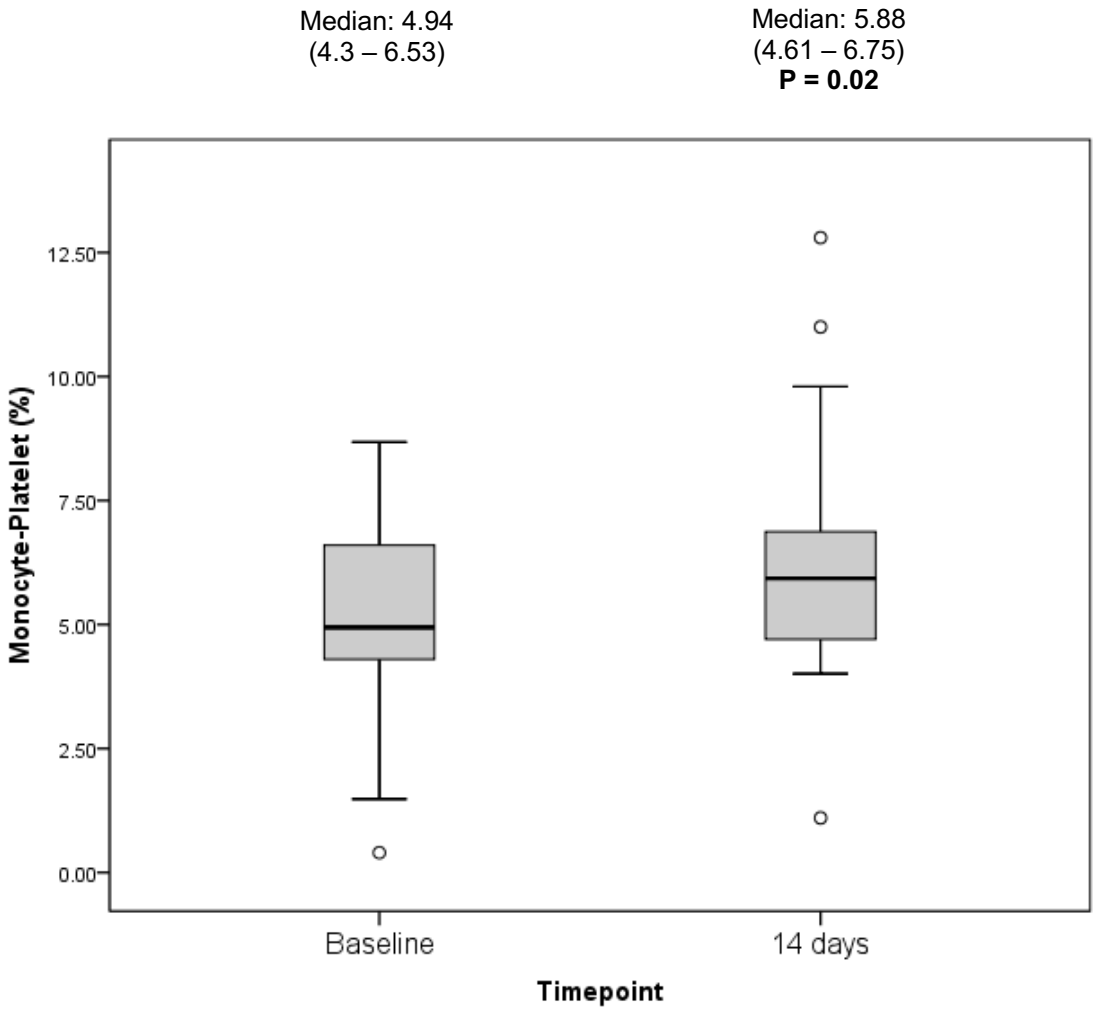
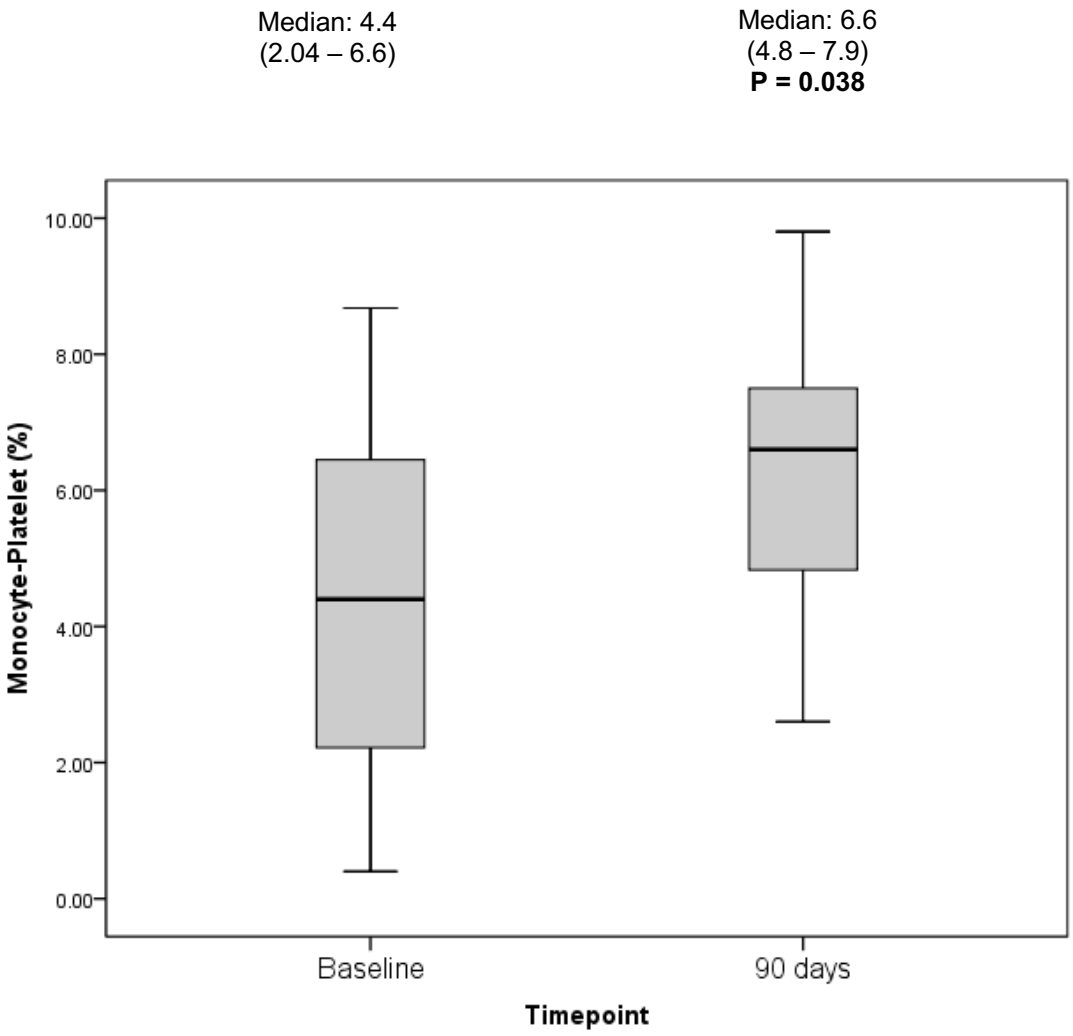


Figure 8. 13 Boxplot of % Monocyte-platelet complexes at baseline vs. 90d in patients with dipyridamole-HTPR on PFA-100 C-ADP assay
Values are medians (range: 25th -75th percentiles)



Multiplate ADP assay:

The median % neutrophil-platelet complexes was significantly lower overall in the subgroup of patients with dipyridamole-HTPR than in those without dipyridamole-HTPR at 90d (2.91 *vs*. 4.59%; p = 0.02). There were no other significant differences in the expression of other platelet activation markers between those with *vs*. those

without dipyridamole-HTPR at any timepoint (Table 8.6). There was no significant correlation between the % expression of CD62P, CD63 or leucocyte-platelet complexes and the Multiplate ADP AUC values ($p \geq 0.05$). There was no significant change in the % circulating monocyte-platelet complexes at 14d *vs*. baseline in either the subgroup of patients with dipyridamole-HTPR ($p = 0.089$) or in those without dipyridamole-HTPR on the Multiplate ADP assay ($p = 0.07$). Furthermore, there was no significant change in the % monocyte-platelet complexes at 90d *vs*. baseline in patients with dipyridamole-HTPR ($p = 0.15$) or in those without dipyridamole-HTPR ($p=0.59$) on the Multiplate ADP assay.

Table 8. 6 Comparison of platelet activation markers between those with vs. those without HTPR on the PFA-100 C-ADP assay
Values represent medians (25^{th} -75^{th} percentiles). Patients without dipyridamole-HTPR are designated as 'No HTPR' in the following 2 tables

Marker	14 days	90 days
% CD62P expression		
HTPR	3.01 (1.68-3.54)	2.85 (1.95-4.01)
No HTPR	6.17 (5.36-7.62)	4.56 (2.98-6.13)
P Value	**0.03**	0.07
% CD63 expression		

HTPR	13.7 (10.5-19.2)	15.0 (10.55-19.25)
No HTPR	11.4 (6.70-15.3)	15.2 (11.75-25.7)
P Value	0.32	0.39
% Neutrophil-Platelet Complexes		
HTPR	3.12 (2.32-3.88)	3.01(2.29-3.83)
No HTPR	2.65 (2.41-3.39)	2.99 (2.84-3.46)
P Value	0.69	0.93
% Monocyte-Platelet Complexes		
HTPR	5.88 (4.65-6.64)	6.60 (4.83-7.50)
No HTPR	7.5 (7.35-8.45)	6.05 (3.99-7.08)
P Value	0.08	0.73
% Lymphocyte-Platelet Complexes		
HTPR	2.145 (1.73-2.87)	2.42 (1.78-2.70)
No HTPR	2.06 (1.50-2.41)	2.24 (1.46-2.50)
P Value	0.5	0.64

Table 8. 7 Comparison of platelet activation markers between those with vs. those without HTPR on the Multiplate ADP assay.

Values represent medians (25^{th} -75^{th} percentiles)

Marker	14 days	90 days
CD62P %		
HTPR	3.34 (2.41-6.53)	3.17 (2.54-5.47)
No HTPR	1.8 (1.07-3.50)	3.77 (3.02-8.75)
P Value	0.124	0.538
CD63 %		
HTPR	12.9 (10.2-19.2)	11.95 (10.5-20.8)
No HTPR	8.94 (5.90-16.75)	21.25 (10.79-25.65)
P Value	0.31	0.48
Neutrophil-Platelet (%)		
HTPR	3.36 (2.40-3.88)	2.91 (2.33-3.69)

No HTPR	2.51 (1.55-3.01)	4.59 (3.90-4.95)
P Value	0.13	**0.02**
Monocyte-Platelet (%)		
HTPR	6.10 (4.70-7.55)	6.05 (3.97-7.10)
No HTPR	6.54 (4.55-8.94)	6.85 (5.60-8.55)
P Value	0.87	0.39
Lymphocyte-Platelet (%)		
HTPR	2.16 (1.58-2.87)	2.24 (1.57-2.54)
No HTPR	2.63 (2.15-5.93)	3.80 (2.13-5.91)
P Value	0.24	0.2

8.4.1.4. Relationship between Dipyridamole-HTPR status and risk of recurrent vascular events

One patient who was classified as having had a 'cryptogenic TIA / TIA of undetermined aetiology' at initial presentation in association with a PFO and an inter-atrial septal aneurysm had a recurrent TIA on 300 mg of aspirin daily and 200 mg of dipyridamole MR once daily, but dipyridamole MR had only been started 24 hours earlier. The dose of dipyridamole MR was subsequently increased by his treating physician to 200 mg BD six days later, but he had a further minor ischaemic stroke during follow-up on that same day. He was then empirically changed to clopidogrel monotherapy, so was no longer eligible for follow up in this arm of the OATS study and had not reached the 14d follow-up stage on full dose dipyridamole to enable us to assess his dipyridamole-HTPR status. None of the other patients had

any recurrent cerebrovascular or other vascular events during a follow-up period of up to 1 year after symptom onset.

8.5. Discussion

To our knowledge, this is the most comprehensive, pilot study to date to simultaneously assess the impact of adding dipyridamole to aspirin therapy *on ex-vivo* platelet activation status, and platelet function / on-treatment platelet reactivity at both high and low shear stress in the early and late phases after TIA or ischaemic stroke.

CD62P and CD63 expression did not change after adding dipyridamole to aspirin, in keeping with the findings from an earlier study by our research group (Tobin, Kinsella et al. 2011). These data confirm that one cannot use 'unstimulated whole blood flow cytometry assays' to reliably detect additional inhibition of platelet function *ex vivo* when dipyridamole is added to aspirin. Prior studies have found an increase in leucocyte-platelet complexes in the early and late phases after TIA or ischaemic stroke compared with controls, indicating excessive platelet activation in CVD (Garlichs, Kozina et al. 2003; McCabe, Harrison et al. 2004). We found a significant increase in the % circulating monocyte-platelet complexes at 14d on aspirin-dipyridamole combination therapy compared with baseline levels on aspirin monotherapy. However, the difference in the % monocyte-platelet complexes at 90d *vs*. baseline was no longer statistically significant (P = 0.06), possibly reflective of a type II error due to the smaller number of subjects who were followed up to 90d. Our research group previously reported increased monocyte-platelet complexes at

both 14d and 90d after the addition of dipyridamole to aspirin after TIA or ischaemic stroke, but this rise in monocyte-platelet complexes over time was driven by data from the patient subgroup with dipyridamole-HTPR (Tobin, Kinsella et al. 2011). The results of this aspect of the OATS study are in keeping with these prior data and independently validate the finding that monocyte-platelet complexes only significantly increase in the subgroup of patients with dipyridamole-HTPR. Marquardt *et al*. also reported an increased % monocyte-platelet complexes on day 2 following acute ischaemic stroke compared with age- and sex-matched controls, but the levels were not increased at any other timepoint during follow-up to 90 days after symptom onset (Marquardt, Anders et al. 2009). However, the precise antiplatelet regimens which were prescribed in patients in that study were not described in detail (Marquardt, Anders et al. 2009). We also observed a significant increase in the % neutrophil-platelet complexes on aspirin-dipyridamole at 90d, but not at 14d compared with baseline on aspirin monotherapy, so assessment of the profile of neutrophil-platelet complexes in patients commencing dipyridamole deserves further study. We also found that, compared with patients without dipyridamole-HTPR on the respective devices, patients with dipyridamole-HTPR on the PFA-100 C-ADP assay at 14d had lower median % CD62P expression, and patients with dipyridamole-HTPR on the Multiplate ADP assay at 90d had lower percentages of circulating neutrophil-platelet complexes. It is possible that these findings may reflect a type 1 error because the differences between groups were not consistent at each time point during follow up, so we should not conclude from this study alone that those without dipyridamole-HTPR actually exhibited an enhanced platelet activation status compared with those with dipyridamole-HTPR.

We did not detect any significant changes in median C-EPI, C-ADP or Innovance P2Y closure times on the PFA-100 after adding dipyridamole to aspirin. The C-EPI cartridge findings are in keeping with prior findings by our research group which also revealed that this cartridge does not detect additional inhibition of platelet function with dipyridamole (Tobin, Kinsella et al. 2011). Although there was a non-significant trend, we did not confirm prior findings from our research group of a statistically significant prolongation of median C-ADP closure times at 14d or 90d compared with baseline after adding dipyridamole to aspirin (Tobin, Kinsella et al. 2011). There was a lower proportion of stroke patients and a higher proportion of TIA patients in this study compared with the prior study by Tobin *et al.*; however, this does not explain the differences in findings between the studies which may, also in part, reflect a type II error with a smaller number of patients with follow-up data at both 14d and 90d in the OATS study. Furthermore, because there was a trend towards prolonged C-ADP closure times after the addition of dipyridamole to aspirin in this study, we think it is unlikely that the findings in the TRAP study represented a type I error (Tobin, Kinsella et al. 2011). The prevalence of dipyridamole-HTPR was also numerically higher in this study (71.4% - 83.3%) than in the study by Tobin et al (56-59%) (Tobin, Kinsella et al. 2011), but the proportion of patients with dipyridamole-HTPR was not actually significantly different between the two studies at 14d ($p=0.169$) or at 90d ($p=0.228$). We do not think that a greater degree of non-adherence to dipyridamole prior to retesting explained the slight disparity in the prevalence of dipyridamole-HTPR between these studies because each study employed a similar methodology of phoning patients in the week before their planned reassessment date to verbally confirm they were adhering to their treatment regimen.

The median ARU was significantly higher at 90d *vs*. baseline (p = 0.008) on the VerifyNow Aspirin assay. This finding may simply reflect less marked inhibition of platelet function on the VerifyNow Aspirin assay due to a reduction in the median daily dose of aspirin which was prescribed during follow up (150mg daily at baseline *vs*. 75mg daily at 14d and at 90d, respectively; table 8.8).

This carefully-designed longitudinal study has revealed the novel finding that one may detect additional inhibition of platelet aggregation in a clinically important proportion (17-21%) of patients with the Multiplate ADP assay, as well as additional inhibition of platelet adhesion and/or aggregation with the PFA-100 C-ADP assay in at least 23-26% of patients**.** Therefore, the Multiplate analyser can be added to the small list of devices which may be considered capable of detecting the additional antiplatelet effects of dipyridamole *ex vivo*. We did not systematically study inhibition of platelet function with the PFA-100 C-ADP or Multiplate ADP assays in patients who were being changed from no medication to dipyridamole because the numbers of patients who are prescribed dipyridamole monotherapy in clinical practice is very small.

Two large randomised controlled trials have shown that aspirin-dipyridamole combination therapy is superior to aspirin alone at preventing recurrent ischaemic stroke or vascular events during follow-up (Diener, Cunha et al. 1996; Halkes, van Gijn et al. 2006). In addition, a recent meta-analysis has shown that when dipyridamole is added to aspirin in the later phase following an ischaemic stroke, the risk of a further stroke is significantly reduced (Rothwell, Algra et al. 2016).

Unfortunately, none of the aforementioned trials routinely employed platelet function/reactivity testing to assess for Dipyridamole-HTPR during follow-up (see below).

This study had some limitations. Only a small proportion of patients with large artery atherosclerotic TIA or ischaemic stroke were recruited to this aspect of the OATS study because most of these patients at our recruiting centres underwent urgent carotid revascularisation, mainly with carotid endarterectomy. Because 'recent surgery' was an exclusion criterion, the majority of these patients were excluded from this study because they could not undergo repeat platelet function/reactivity testing 14d after changing antiplatelet therapy as part of our longitudinal study design. Only one patient in this aspect of the OATS study had a recurrent TIA/stroke during comprehensive study follow-up. This patient did not have data on Dipyridamole-HTPR status on the PFA-100 C-ADP or Multiplate ADP assays because his antithrombotic treatment regimen was changed by his treating physician prior to his scheduled 14d follow-up visit. Therefore, we cannot make any reliable comments on the value of dipyridamole-HTPR status at predicting recurrent vascular events; much larger studies are warranted to address this issue. This pilot study included a relatively small number of highly-phenotyped CVD patients, so some analyses may have been prone to type II errors and occasional type I errors, as clearly acknowledged above. Personal correspondence to our research group has indicated that the reagents for the Multiplate assays are not routinely available at present, thus limiting the current availability and applicability of this assay in the clinical or academic setting until this particular supply for this user- friendly device has been restored.

8.6. Conclusion

In conclusion, this aspect of the OATS study has enhanced our understanding of the ability of certain established and novel laboratory tests of platelet function/reactivity to identify patients with recent TIA or ischaemic stroke who have *ex vivo* dipyridamole-HTPR. Our data indicate that one can detect additional inhibition of platelet function/reactivity *ex vivo* with the PFA-100 C-ADP and Multiplate ADP assays when dipyridamole is added to aspirin after TIA or ischaemic stroke. We have confirmed that patients with dipyridamole-HTPR on the PFA-100 exhibit increasing monocyte-platelet complexes over time. Larger, longitudinal, multi-centre studies using the PFA-100 C-ADP and Multiplate ADP assays (if the Multiplate reagents become routinely available once again) are warranted to assess the potential value of these and other emerging devices at predicting the long-term risk of recurrent vascular events and outcomes on aspirin and dipyridamole in CVD. If HTPR status is shown to predict outcomes over time, one could opt to continue dipyridamole in those with *ex vivo* 'dipyridamole responsiveness', and change to an alternative, personalised antiplatelet regimen to optimise secondary preventive treatment following a TIA or ischaemic stroke in those with dipyridamole-HTPR.

9. Reticulated platelets in the early and late phases after TIA or ischaemic stroke

9.1. Introduction

'Reticulated platelets' are young, circulating platelets containing a residual amount of megakarocyte-derived RNA which are released into the circulation after fragmentation of megakaryocytes in the bone marrow and pulmonary circulation (Harrison, Robinson et al. 1997). Platelets normally have a lifespan in the circulation of around 7-10 days (Harker and Finch 1969; Deutsch and Tomer 2006; Mason, Carpinelli et al. 2007; Zhang, Nimmer et al. 2007), but reticulated platelets may only persist for less than a day based on data from animal studies (Ault and Knowles 1995). Therefore, measurement of the percentage of reticulated platelets can serve as a measure of increased platelet production and/or turnover, which could occur in subjects with elevated platelet activation (Robinson, Harrison et al. 1998). However, reticulated platelets may not increase unless the stimulus to platelet activation also promotes thrombopoiesis. Reticulated platelets have been reported to be elevated in cardiovascular disease patients with myocardial infarction compared with angina (Lakkis, Dokainish et al. 2004) and levels reduce significantly over time after a myocardial infarct (Eisen, Lerman-Shivek et al. 2015). It has also been reported that the *ex vivo* response to antiplatelet therapy in stable coronary artery disease is strongly associated with the % circulating reticulated platelets (Guthikonda, Alviar et al. 2008; Cesari, Marcucci et al. 2013).

To our knowledge, only three studies to date have assessed the % circulating reticulated platelets in patients after a TIA or stroke compared with controls (Nakamura, Uchiyama et al. 2002; Smith, Pathansali et al. 2002; McCabe, Harrison et al. 2004). One small study revealed that circulating reticulated platelets were increased in patients within 3 days of stroke onset compared with controls (Smith, Pathansali et al. 2002), and a further pilot study showed that reticulated platelets were only elevated in the subgroup of patients with cardio-embolic stroke *vs*. 'neurological controls' (Nakamura, Uchiyama et al. 2002). The largest case-control study on this topic to date revealed an increase in the percentage of reticulated platelets on whole blood flow cytometry in the early (< 28 days) and late (> 78 days) phases after TIA or ischaemic stroke only after adjusting for differences in age between controls and patients (McCabe, Harrison et al. 2004). More recently, Murphy *et al* reported a higher % reticulated platelet fraction in early (5.78%; $P < 0.001$) and late phase symptomatic (5.11%; $P = 0.01$) compared with asymptomatic moderate-severe carotid stenosis patients (3.48%) using an automated assay on a Sysmex XE 2100 haematology analyser (Murphy, Lim et al. 2018). Therefore, data on this topic in an overall ischaemic cerebrovascular (CVD) patient population are limited.

9.2. Aims and Hypotheses

The aims of this case-control study were to determine whether:

1) There was convincing evidence of an increase in the % circulating reticulated

platelets in patients with TIA or ischaemic stroke compared with healthy controls despite treatment with commonly-prescribed antiplatelet treatment and other secondary preventive medications;

2) There were any demographic or vascular risk factors, or TIA/stroke subtypes which influenced the % circulating reticulated platelets in our CVD patient population;

3) There was a correlation between the % reticulated platelets on flow cytometry and the mean platelet volume or platelet distribution width in CVD patients.

We hypothesised that:

1) Circulating reticulated platelets would be increased in the early or late phases after TIA or ischaemic stroke compared with controls;
2) Age and TIA/stroke subtype might influence the % circulating reticulated platelets in CVD patients;
3) There would be a positive correlation between the % circulating reticulated platelets and the mean platelet volume (MPV) or platelet distribution width (PDW), providing further evidence that 'younger', reticulated platelets are larger than more mature platelets in an overall CVD patient population.

9.3. Methods

9.3.1. Inclusion criteria for CVD patients:

Consecutive eligible patients, older than 18 years of age, with a recent diagnosis of TIA or ischaemic stroke within the preceding 4 weeks, whose treating physician

opted to start or change antiplatelet therapy were screened for inclusion in this prospectively-planned, single centre, observational analytical study. Patients were participants of one of 2 consecutive observational studies coordinated by our department: The **TR**inity **A**nti**P**latelet Responsiveness (**TRAP**) study which recruited and followed up patients between 4th September 2007 and February 2010 and the **O**ptimal **A**ntiplatelet **T**herapy in TIA and Ischaemic **S**troke (**OATS**) Study which recruited subjects between 26/10/2011 to 13/01/2016. Both studies had the same inclusion criteria and the OATS study design was based on the pilot outcome data from the TRAP study. Patients were recruited from the Rapid Access Stroke Prevention Service, and from the inpatient population of the Neurology, Age-Related Health Care, Stroke and Vascular Surgery Services at our secondary and tertiary referral university teaching hospital.

9.3.2. Exclusion criteria:

Patients were excluded if they had a myocardial infarction, DVT, PE or recent surgery within the preceding three months; ongoing unstable angina or unstable symptomatic peripheral vascular disease; platelet count < 100 x 10^9/L; known bleeding or clotting diathesis, including known platelet-related bleeding disorders; active proven vasculitis; active neoplasia; non-steroidal anti-inflammatory drug (NSAID) intake other than aspirin or aspirin in combination with dipyridamole in the preceding 11-14 days (Tobin, Kinsella et al. 2011; Tobin, Kinsella et al. 2013). We also excluded CVD patients unable to attend for clinical follow-up and repeat testing at 14 +/- 7 days, patients with active infection, renal impairment (e.g. urea > 10 mmol/L or GFR < 30 ml/min), or who had a prior history of intracranial haemorrhage.

9.3.3. Clinical assessment

All patients underwent detailed neurovascular assessment by one of the neurology research registrars (STL or SJM) or supervising consultant vascular neurologist (DJHM) according to ESO guidelines (Steiner, Al-Shahi Salman et al. 2014). Information regarding vascular risk factors, including hypertension, prior TIA or stroke, ischaemic heart disease, atrial fibrillation, valvular heart disease, diabetes mellitus, hyperlipidaemia, peripheral vascular disease, migraine, family history of stroke, medication intake (including anti-thrombotic therapy), smoking status, alcohol intake, illicit substance intake, and the method of detection of carotid stenosis and timing of any carotid intervention in patients with large artery atherosclerosis was collected prospectively. Details regarding antiplatelet regimens, dose and duration of therapy were recorded. Results of routine haematological (FBC), coagulation (PT/APTT), biochemical and blood glucose testing were collected prospectively. CT and/or MRI brain and CDUS of neck vessels was performed in all patients, as well as magnetic resonance angiography (MRA) or CT angiography (CTA) to establish concordance between CDUS and another non-invasive imaging modality in all recently symptomatic carotid stenosis patients. A chest radiograph, electrocardiograph (ECG), 24-hour ECG recording and transthoracic or trans-oesophageal echocardiograph were obtained in all patients. TIA and stroke subtyping was performed according to the TOAST classification system (Adams, Bendixen et al. 1993).

All patients underwent clinical and laboratory assessment before (baseline), 14 +/-7 days after (14d), and at least 90 days after (90d) starting or changing their antiplatelet regimen. In the large artery atherosclerotic subgroup of patients who had moderate-severe carotid stenosis, the 90d follow-up visit was performed at least 3 months following carotid surgery or endovascular treatment, unless intervention had been delayed for at least 3 months after symptom onset.

Because most patients in TRAP and OATS at baseline and most patients in the TRAP study at 14 +/-7d were assessed during their inpatient stay, adherence to antithrombotic therapy in these patients was confirmed by checking the inpatient prescription chart. Adherence in all outpatients was assessed by history taking alone, but all were phoned in the week prior to their scheduled follow-up visit to stress the importance of complete medication adherence before reassessment. If complete adherence was not initially confirmed, any issues potentially affecting adherence were discussed and the follow-up visit postponed for 14 days until full adherence was verbally confirmed.

9.3.4. Controls

Control subjects of similar age and gender were recruited from amongst the staff at AMNCH-TUH and from the local population; spouses of patients and control subjects were also recruited. The exclusion criteria for control subjects were the same as those for patients, with the exception that subjects were also excluded from the control group if they had a history of stroke or TIA in the past, if they had evidence of > 50% carotid or vertebral artery stenosis on colour Doppler ultrasound screening, or if they were on antiplatelet therapy.

Written informed consent, or ‘proxy consent’ where appropriate, was obtained from

all subjects. The TRAP (REC Ref: 2007/07/MA) and OATS (REC Ref: 2011/35/03) studies were approved by the St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee.

9.3.5. Blood Sampling and Laboratory Methods

Careful, atraumatic venepuncture was performed as outlined in chapter 7. Whole blood flow cytometric analysis was performed to calculate the % of TO-positive (reticulated) platelets in the whole blood sample in FL1 within 120 minutes of venepuncture, as described in chapter 7. A FBC measurement was performed in all subjects in EDTA- and 3.2% citrate-anticoagulated whole blood between 2 and 4 hours after venepuncture on a Sysmex XE-2100® haematology analyser (Sysmex UK Ltd, Milton Keynes, UK).

9.4. Statistical Analysis

The Wilcoxon signed rank test and the Mann-Whitney test were used for comparison of paired and unpaired non-parametric variables, respectively. Paired and unpaired t-tests were used for comparison of paired and unpaired parametric variables. Multiple linear regression analysis was performed to control for the potential influence of any differences in demographic or vascular risk profiles between groups on the results obtained, incorporating stepwise regression for the covariates. The assumptions for multiple linear regression were met, i.e. residuals were normally distributed. $P < 0{\cdot}05$ was considered to be statistically significant. All statistical calculations were performed with SPSS version 22.

9.5. Results

Data from 210 eligible patients with recent TIA or ischaemic stroke who had baseline data following recruitment to the TRAP and OATS studies were analysed. The median time interval from TIA or stroke onset to study inclusion was 8 days (minimum-maximum range: 0-28 days). Follow up data were available in 181 patients at 14d, and 145 patients at 90d. Follow-up data were not available in all patients because some had their anti-thrombotic regimen altered by their treating physician (e.g. following detection of paroxysmal atrial fibrillation which warranted a change to anticoagulation or due to intolerance of treatment), and were no longer eligible for follow-up in the TRAP and OATS studies; some were too unwell, unavailable for or lost to follow up. Thirty-four controls were recruited and assessed once. The baseline demographic and vascular risk factor profiles of study participants (Table 9-1), and the TIA/stroke aetiological subtypes in patients are outlined below (Table 9-2).

The prescribed antiplatelet regimens in CVD patients at baseline, 14d and 90d are outlined in Table 9-3. One hundred and fifty-nine patients were on aspirin monotherapy at baseline, 47 were on no antiplatelet medication, 4 were on aspirin-dipyridamole combination therapy. Two patients in the subgroup who changed from aspirin to clopidogrel had recurrent TIAs during follow up. One patient had a recurrent TIA due to embolisation from an aortic arch atheromatous plaque within a week of recruitment; one patient had possible vertebral artery dissection; one patient had a left hemispheric TIA due to possible embolism from a stenosed internal carotid artery within three months of recruitment. No other patients had recurrent TIA, stroke or any other vascular events during follow up.

9.5.1. % Reticulated Platelets on *Flow Cytometry* in CVD patients *vs*. Controls

Median % Gp1b expression was similar in CVD patients and controls (99.3 *vs*. 98.9%, P = 1.0), confirming that the vast majority of cell analysed on whole flow cytometry were platelets. The intra-assay co-efficient of variation (CV) for the whole blood flow cytometry assay quantifying the % reticulated platelets (%RP) was 6.64%, calculated using 3 separate samples taken from the same patient which were tested between 60 and 90 minutes after venepuncture on the same day.

There were no significant differences in the median %RP between the overall TIA / ischaemic stroke patient population at baseline or 14d compared with controls ($p \geq 0.2$). However, the median % RP was significantly higher in CVD patients at 90d than in controls ($p = 0.036$) (table 9-4 and figure 9-1). Due to differences in demographic and vascular risk factor profiles, multiple linear regression analysis was performed to individually control for the potential influence of age, a prior history of TIA or stroke, ischaemic heart disease, hypertension, diabetes, current smoking and statin use on the comparison of %RP between CVD patients and controls. None of these variables independently influenced the comparisons between CVD patients *vs*. controls at any stage during follow up ($p \geq 0.1$). The %RP profiles in the 2 patients in the subgroup who changed from aspirin to clopidogrel who had recurrent TIAs during follow up are described in table 9-5.

9.5.2. % RPs in TIA / Ischaemic Stroke subgroups versus Controls

The median %RP was significantly elevated in the subgroup of patients with TIA/ischaemic stroke due to small vessel disease at baseline ($p = 0.04$) and at 90d ($p = 0.01$), but not at 14d compared with controls (figure 9-2 a,b,c). Otherwise, there were no statistically significant differences in the median %RP between other TIA/stroke subgroups and controls ($P \geq 0.05$) (figure 9-2 a,b,c).

9.5.3. Simultaneously-collected FBC parameters in CVD patients *vs*. Controls

There were no significant differences in median platelet counts ($p \geq 0.07$), MPV ($p \geq 0.4$) or PDW in EDTA or citrate ($p \geq 0.4$), or haemoglobin concentrations in EDTA ($p \geq 0.1$) between CVD patients and controls at any time point (table x7). Total white cell count, neutrophil and monocyte counts were higher in CVD patients than in controls at each time point ($p < 0.0001$, table 9-6).

9.5.4. Correlation analysis:

There was no correlation between the median platelet count in either EDTA or citrate and the median %RP on flow cytometry in CVD patients at baseline, 14d or 90d ($p \geq 0.08$). However, there was a significant positive correlation between the MPV in citrate and EDTA and the %RP in citrate at baseline (rho ≥ 0.29, $p < 0.001$), 14d (rho ≥ 0.28, $p < 0.001$) and 90d (rho ≥ 0.3, $p < 0.001$) (Figure 3). There was also a significant positive correlation between the PDW in citrate and EDTA and the %RP in citrate at baseline (rho ≥ 0.26, $p < 0.001$), 14d (rho ≥ 0.22, $p <$

0.002) and 90d (rho ≥ 0.23, p ≤ 0.002) (figure 4)

9.6. Discussion

To our knowledge, this is the largest case-control study to prospectively assess the % circulating reticulated platelets using whole blood flow cytometry in a highly-phenotyped CVD population in the early, subacute and late phases after TIA/ischaemic stroke onset in patients who were starting or changing antiplatelet therapy. The %RP was not significantly increased in the early or subacute phases, but was significantly increased in the late phase after symptom onset in our TIA/ischaemic stroke population overall compared with controls. These results are consistent with the %RP data from patients in the late phase after TIA/ischaemic stroke after adjustment for age in one prior study (McCabe, Harrison et al. 2004), but do not confirm the findings of a pilot, case-control study which reported an increased %RP in the more acute phase (≤ 3 days) after an ischaemic stroke using fixed whole blood (Smith, Pathansali et al. 2002). The fact that our CVD patients were initially studied later, at a median of 8 days after TIA/ischaemic stroke onset, and that we did not include demographic and vascular risk factor-matched controls may explain some of the differences between the findings in our study and those of Smith *et al*. (Smith, Pathansali et al. 2002). However, Smith *et al*. fixed the samples before the addition of TO, they appear to have used a higher concentration of TO (800μl) which may well have contributed to non-specific labelling of platelet dense granules and possibly mitochondrial DNA, and they did not assess any patients in the late phase after symptom onset (Smith, Pathansali et al. 2002). The lack of a significant increase in %RP in the early and subacute phases after TIA/ischaemic

stroke in our study may also reflect a type II error due to the smaller number of controls compared with patients included in our study.

Prior studies have shown enhanced platelet activation in TIA/stroke patients in the early (Grau, Ruf et al. 1998; Meiklejohn, Vickers et al. 2001; Marquardt, Ruf et al. 2002; McCabe, Harrison et al. 2004) and late (Grau, Ruf et al. 1998; Meiklejohn, Vickers et al. 2001; Yamazaki, Harano et al. 2001; Cha, Jo et al. 2004; McCabe, Harrison et al. 2004) phases following symptom onset. Our data could potentially be interpreted as suggesting that increased platelet production/turnover occurs following symptom onset and in response to the ischaemic cerebrovascular event, rather than prior to the onset of the TIA/ischaemic stroke. However, as alluded to above, our early and subacute phase data may have been subject to a type II error, so further adequately-sized studies are required to test this hypothesis because circulating reticulated platelets have been shown to be increased in certain patients with underlying vascular disease who also have increased platelet activation. For example, the %RP has been shown to be elevated in patients with essential thrombocytosis (Arellano-Rodrigo 2009), following renal transplantation (Cesari, Marcucci et al. 2013), the metabolic syndrome (Guthikonda, Alviar et al. 2008), peripheral vascular disease (Esposito, Popescu et al. 2003) or coronary artery disease (Cesari, Marcucci et al. 2013), but not in patients with hypercholesterolaemia (Pathansali, Smith et al. 2001) or the antiphospholipid syndrome (Joseph, Donohoe et al. 1998). These studies imply that particular risk factors may lead to elevated reticulated platelet formation, whereas others may not.

Subgroup analysis according to TOAST subtypes revealed that patients with TIA/ischaemic stroke due to small vessel disease (SVD) had a higher %RP at

baseline and 90d *vs*. controls, but the differences between CVD patients with SVD at 14d and controls did not reach statistical significance (P=0.06). The differences in %RP between the other CVD TOAST subtypes and controls were not statistically significant. Because the bulk of patients who had a TIA and ischaemic stroke of 'known aetiology' had underlying SVD, with relatively small numbers of patients in the other subgroups, we acknowledge that the 14d data analysis in this SVD subgroup and the other subgroup analyses could also have been subject to a type II error. In contrast, as alluded to above, the %RP has been reported to be increased in cardio-embolic stroke patients *vs*. 'neurological controls', and subgroup analysis from that study indicated that the %RP in fixed, washed platelet preparations from PRP was increased in the acute (< 7 days, N = 4) compared with the later phase (≥ 31 days, N = 10) after a cardio-embolic stroke (Nakamura, Uchiyama et al. 2002). However, the same patients were not assessed at each time point and the number of patients included in the subgroup analysis in that study was very limited.

We did not identify an increase in the mean MPV in CVD patients *vs*. controls in either EDTA-or citrate-anticoagulated blood. These findings are similar to some prior studies (Smith, Pathansali et al. 2002; McCabe, Harrison et al. 2004), but not in agreement with others (O'Malley, Langhorne et al. 1995; Butterworth and Bath 1998). However, in keeping with prior data, our correlation analysis did confirm that reticulated platelets are larger than more mature platelets in CVD patients (McCabe, Harrison et al. 2004), with a positive correlation between both the MPV and PDW and %RP at baseline, 14d and 90d. Platelet counts were not significant increased in ischaemic CVD patients at any time point compared with controls, as noted previously (Smith, Pathansali et al. 2002; McCabe, Harrison et al. 2004).

These simultaneously-collected data on the %RP, platelet counts and MPV indicate that there is an ongoing stimulus to the formation of larger reticulated platelets, especially in the late phase after TIA or stroke onset in our cohort, even though the overall platelet count remains stable. This area of platelet biomarker research deserves further study because larger platelets have been reported to be 'more reactive', aggregate more rapidly in response to collagen, synthesise more thromboxane B_2, release more granules and serotonin, and express more adhesive surface receptors than smaller platelets (Guthikonda, Alviar et al. 2008; Murphy, Lim et al. 2018).

The median total white cell count, neutrophil and monocyte counts were significantly higher at baseline, 14d and at 90d days in CVD patients than in controls in the absence of any active infection or other 'inflammatory diathesis' aside from their recent TIA or ischaemic stroke. The duration of the elevated neutrophil and monocyte counts was much longer than the 3 days reported in one prior study (Marquardt, Anders et al. 2009), but fully in keeping with prior case-control data from a smaller group of CVD patients who were tested in the early and late phases after TIA or stroke onset (McCabe, Harrison et al. 2004). These findings indicate that there also appears to be an ongoing stimulus to the formation and release of certain leucocyte subsets in CVD patients compared with controls which may even predate the onset of their TIA or stroke, but this study was not specifically designed to test that hypothesis. One must stress that the median values for the total white cell count, neutrophil and monocyte counts were still within the overall normal ranges for our laboratory (normal ranges for total white cell count: 4 - 11 x 10^9/L; neutrophil counts: 4 - 7.5 x10^9/L; monocyte counts: 0.2 - 0.8 x 10^9/L). Further prospective studies in healthy controls would be required to determine

whether controls with levels of circulating neutrophils or monocytes above a specific threshold have a higher risk of developing a subsequent TIA/stroke than those with lower levels of these leucocyte subsets.

This study had some potential **limitations**. Although one must accept that there is no gold standard method to quantify the %RP on flow cytometry, we used a low concentration of thiazole orange (1 in 10 dilution with Isoton II) which has been shown to specifically stain mRNA in reticulated platelets and is considered to be a strength of this study (Robinson, Mackie et al. 1998; Robinson, MacHin et al. 2000; Robinson, MacKie et al. 2000). Recent data from our group in patients with symptomatic compared with asymptomatic moderate-severe carotid stenosis patients have shown that an automated assay on the Sysmex XE-2100® haematology analyser may be more sensitive at detecting differences in the % reticulated platelet fraction between groups (Murphy, Lim et al. 2018). Because the automated assay to quantify the % reticulated platelet fraction (%RPF) was not available in our lab at the time of the TRAP study, we cannot comment further on the relative sensitivies of the 2 assays in this combined case-control study from the TRAP and OATS studies. Further data on this topic are being collected in our lab and will be reported in due course. The number of subjects included in some of our TOAST subgroups was small, and as acknowledged above, some analyses may have been subject to a type II error. There were too few clinical outcome events during prospective follow-up to reliably comment on the potential value of the %RP at predicting the risk of recurrent vascular events over time. This case-control component of the TRAP-OATS studies was not designed to look at the impact of %RPs on antiplatelet-HTPR status in CVD patients, but this is the subject of an ongoing longitudinal study by our research group.

9.7. Conclusions

The percentage of circulating reticulated platelets was increased in the late phase (> 90 days), but not significantly increased in the early phase (≤ 4 weeks) after a TIA or ischaemic stroke compared with healthy controls in this study. Elevated levels of this important population of larger, recently-released reticulated platelets are observed in the absence of significant elevations in the total platelet count in CVD patients. The underlying pathophysiological mechanisms responsible for increased circulating reticulated platelets after TIA and ischaemic stroke need to be delineated. Further studies in larger cohorts of patients with different TIA/ischaemic stroke subtypes, ideally with simultaneous assessment of the %RP on flow cytometry and newer automated assays to quantify the %RPF are warranted to understand the profile of reticulated platelets in different TIA/ischaemic stroke subtypes. Ongoing work in our lab should inform us whether levels of circulating reticulated platelets influence on-treatment platelet reactivity status in CVD patients. These data have the potential to improve our understanding of the pathogenesis of recurrent vascular events in CVD patients on commonly-prescribed antiplatelet therapy.

Table 9. 1 Demographic and vascular risk profiles of study participants at enrolment.

Values are Means [±SD] or absolute values with percentages in parentheses (%), where appropriate. P values relate to comparisons between Patients and Controls by Chi-squared or Fisher's Exact tests, where appropriate.

Significant P values in bold.

IHD* = History of ischemic heart disease; DVT/PE** = Deep venous thrombosis or pulmonary embolism; ***Hyperlipidaemia = Total cholesterol > 5.0 mmol/L or LDL > 3.5 mmol/L at the time of the TRAP study design.

Parameter	CVD Patients (N = 210)	Controls (N = 34)	P Value
Mean Age (years)	60 (±13)	51 (±12)	**0.04**
Gender (M/F)	134/77	20/14	0.7
Index TIA at enrolment	160 (76%)	0	**N/A**
Index Stroke at enrolment	50 (24%)	0	**N/A**
Prior Stroke/TIA	49 (23%)	0	**N/A**
Index event occurred on antiplatelet agent	160 (76%)	0	**N/A**
IHD *	37 (17.5%)	0	**0.004**
Hypertension	100 (47%)	9 (26.5%)	**0.03**
Diabetes Mellitus	27 (13%)	0	**0.02**
Hyperlipidaemia ***	78 (37%)	2 (6%)	**< 0.001**
Atrial Fibrillation/Flutter at Enrolment	1 (0.5%)	0	1.0
Prior DVT/PE **	4 (2%)	0	1.0
Peripheral Vascular Disease	10 (5%)	0	0.4
Migraine	39 (19%)	3 (9%)	0.2
Current Smoker	54 (26%)	2 (7%)	**0.008**
Ex-smoker	68 (32%)	6 (18%)	0.1
Never smoker	89 (42%)	26 (76.5%)	**< 0.001**
Statin Therapy	110 (52%)	4 (12%)	**< 0.001**
Family History of Stroke	70 (33%)	7 (21%)	0.2

Table 9. 2 Aetiological subtyping of CVD patients at baseline by TOAST classification (Total N = 210)

TIA / Ischaemic Stroke Subtype	Numbers (%)
Large Artery Atherosclerotic	13 (6.1%)
Small Vessel Disease (Lacunar)	36 (17.1%)
Cardioembolic	24 (11.4%)
Other Determined	7 (3.3%)
Undetermined Aetiology	130 (61.6%)

Table 9. 3 Antiplatelet regimens in CVD patients during the study

Antiplatelet Regimen	Baseline	14d	90d
None	47 (22%)	0 (0%)	0 (0%)
Aspirin (75mg-300mg daily)	159 (76%)	34 (19%)	21 (14%)
Aspirin (75mg daily) + Dipyridamole MR (200 mg BD)	4 (2%)	86 (48%)	76 (52%)
Clopidogrel 75 mg daily	0 (0%)	61 (33%)	48 (33%)

Table 9. 4 Comparison of % Reticulated Platelets (%RP) between CVD patients overall at different time points and Controls.
Values represent medians (range: min-max). Significant P values highlighted in bold.

	CVD Patients at baseline	CVD Patients at 14d	CVD Patients at 90d	Controls
% RP	14.2 (1.4 – 33.8)	13.7 (1.3 – 32.9)	18.0 (0.5 – 38.4)	13.0 (1.2-25.7)
P value	0.2	0.3	**0.04**	

Table 9. 5 % Reticulated platelets on flow cytometry in patients who had recurrent vascular events during follow up

%RP in Controls simply displayed for comparative purposes (range: min-max)

	Stroke Mechanism	**Baseline (%)**	**14d (%)**	**Controls** (%)
Patient 1	Cardioembolic	24.5 %	21.1 %	13 (1.2 – 25.7)
Patient 2	Large artery atherosclerotic	12.7 %	6.66 %	13 (1.2 – 25.7)

Table 9. 6 Comparison of %RP between subtypes of CVD patients at different time points and Controls

Values represent medians (range: min-max). Significant P values highlighted in bold. 'N' in first column refers to number of patients in each subgroup at baseline

% RP (Flow Cytometry)	**CVD Patients at baseline (%)**	**CVD Patients at 14d (%)**	**CVD Patients at 90d (%)**	**Controls (%)**
Large Artery (N=13)	17 (4.45% - 26.9%)	12.8 (4.14 – 28.7)	12.5 (1.93 – 25.2)	13.0 (1.2–25.7)
P	0.3	0.8	0.8	
Small Vessel Disease (N = 36)	17.8 (5.74 -30.4)	17.1 (5.4 – 32.9)	19.6 (4.39 - 38.4)	13.0 (1.2–25.7)
P	**0.04**	0.06	**0.01**	
Cardio-Embolic (N = 24)	15 (4.9 – 25.5)	17.5 (4.1 – 30.4)	18.5 (8.75-27.2)	13.0 (1.2– 25.7)
P	0.4	0.3	0.05	
Other Determined (N = 7)	18.7 (6.0 – 28.2)	20.8 (6.2 – 27.2)	19.5 (4.2-31.9)	13.0 (1.2–25.7)
P	0.2	0.06	0.2	
Undetermined (N = 130)	13 (1.4 – 33)	12.6 (1.3 – 32.4)	16.9 (5.0 – 37.8)	13.0 (1.2–25.7)
P	0.5	0.6	0.09	

Table 9. 7 FBC parameters in CVD patients at baseline, 14d and 90d compared with Controls

Values represent medians (range: min-max) or means (±SD)

FBC Parameter	CVD Patients at Baseline	CVD Patients at 14d	CVD Patients at 90d	Controls
Platelet Count (x 10^9/L) (EDTA) P	242 (136-570) 0.07	241 (122-584) 0.08	235 (128-516) 0.2	220.5 (142-347)
Platelet Count (x 10^9/L) (Citrate P	176 (107-412) 0.8	175 (104-371) 0.8	175 (106-323) 0.8	168 (125-310)
Mean Platelet Volume (fl) (Citrate) P	9.9 (± 0.9) 0.96	9.7 (± 0.9) 0.4	9.8 (± 0.8) 0.6	9.8 (± 0.8)
Mean Platelet Volume (fl) (EDTA) P	10.8 (± 0.8) 0.23	10.5 (± 0.74) 0.41	10.7 (± 0.78) 0.34	10.6(± 0.75)
Platelet Distribution Width % EDTA P-value	13.2 (8.2-19.1) 0.44	12.8 (8.0-18.1) 0.88	12.8 (9.0-18.6) 0.98	12.6 (9.6-17.4)
Platelet Distribution Width (%) (Citrate) P	11.2 (4.8-18.1) 0.83	11.1 (4.1-16.8) 0.40	11.0 (8.1-15.3) 0.53	10.9 (8.3-16.4)
Haemoglobin (g/dl) (EDTA) P	14.0 (8.2-18.7) 0.79	13.8 (8.8-17.3) 0.11	13.9 (10.0-17.2) 0.31	14.0 (11.9-15.9)
WCC (x 10^9/L) (EDTA) P	7.44 (4.22-12.72) **< 0.0001**	7.07 (3.06-15.19) **< 0.0001**	6.89 (3.78-12.45) **< 0.0001**	5.36 (3.12-8.49)
Neutrophils (x 10^9/L) (EDTA) P	4.46 (2.07-10.09) **< 0.0001**	4.06 (1.51-12.68) **< 0.0001**	4.10 (1.61-8.87) **< 0.0001**	2.98 (1.47-5.26)
Monocytes (x 10^9/L) (EDTA) P	0.63 (0.12-1.97) **< 0.0001**	0.58 (0.25-1.89) **< 0.0001**	0.58 (0.27-1.63) **< 0.0001**	0.44 (0.27-0.90)
Lymphocytes (x 10^9/L) (EDTA) P	2.11 (0.67-4.36) 0.55	1.93 (0.67-4.12) 0.79	1.90 (0.53-3.95) 0.74	1.99 (0.87-3.22)

Eosinophils (x 10^9/L) (EDTA) P	0.17 (0.00-1.00) 0.33	0.17 (0.00-1.31) 0.19	0.16 (0.00-0.81) 0.37	0.17 (0.04-0.46)
MCH (fl) (EDTA) P	30.7 (20.7-37.0) 0.20	30.6 (24.0-36.7) 0.11	30.7 (22.7-35.3) 0.21	30.8 (26.7-33.1)
MCV (pg) (EDTA) P	90.3 (67.6-109.4) 0.64	89.8 (72.5-104.5) 0.37	90.3 (73.9-100.5) 0.81	90.0 (78.5-98.3)
HCT (L/L) (EDTA) P	0.41 (±0.04) 0.77	0.40 (±0.04) 0.14	0.41 (±0.04) 0.45	0.41 (±0.03)

Figure 9. 1 Scatterplot of circulating %RP in CVD patients overall at Baseline, 14d and 90d vs. Controls.

Each point represents a single patient. Red lines and numbers above figure indicate median values. P values refer to comparison of median %RP between CVD patients at different time points and Controls. Significant P values highlighted in bold.

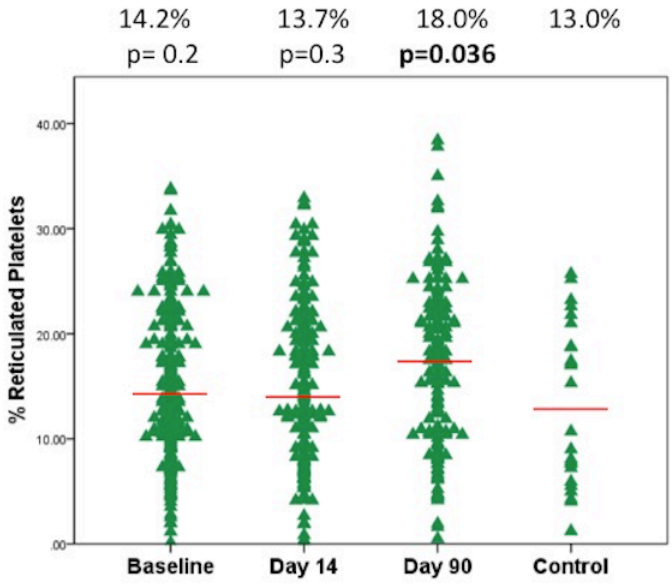


Figure 9. 2 Scatterplots of circulating %RP in CVD patient TOAST subgroups at (a) Baseline, (b) 14d and (c) 90d vs. Controls

Each point represents a single patient. Red lines and numbers above figure indicate median values. P values refer to pairwise comparisons of median %RP between CVD subgroups at the respective time points *vs.* Controls. Significant P values highlighted in bold.

Figure 9-2a: %RP in Baseline CVD patient subgroups *vs.* Controls

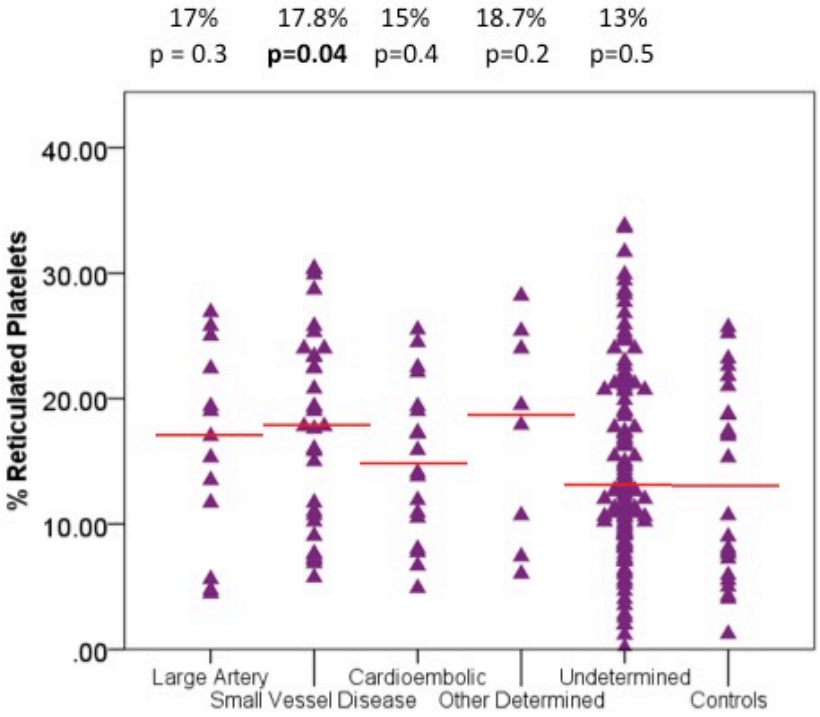


Figure 9-2b: %RP in 14d CVD patient subgroups *vs*. Controls

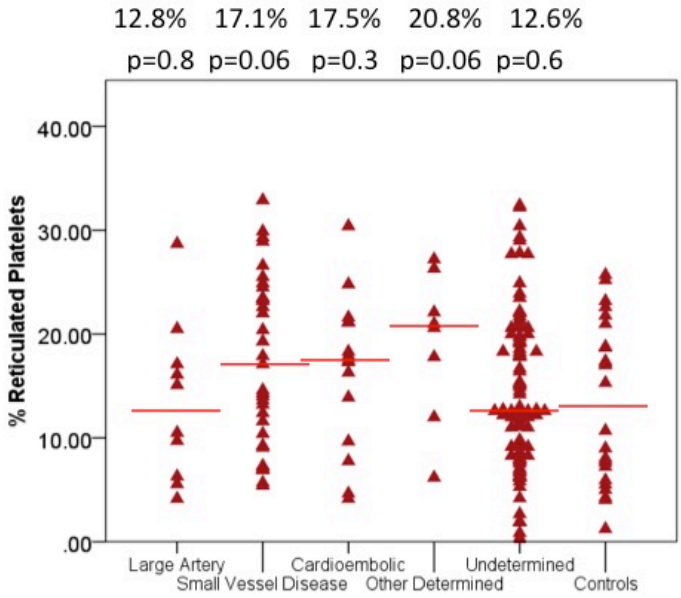


Figure 9-2c: %RP in 90d CVD patient subgroups *vs*. Controls

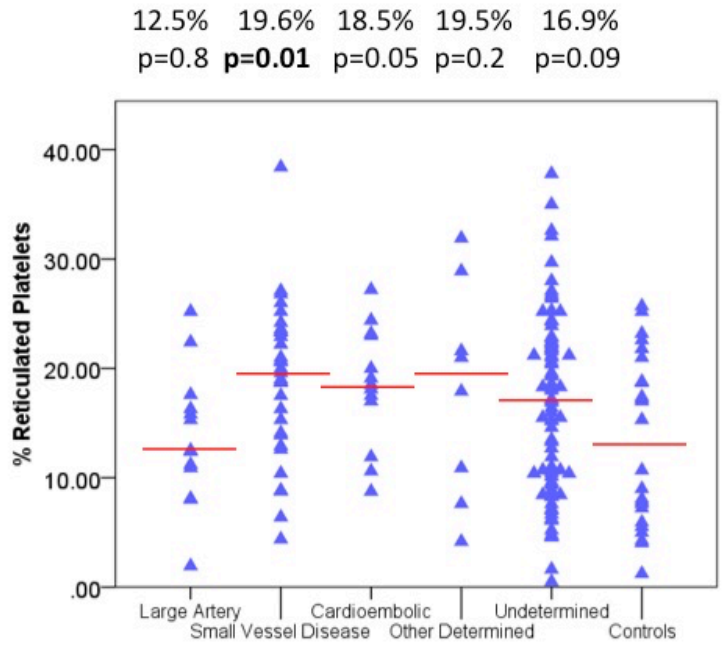


Figure 9. 3 Fitted line plot showing a positive correlation between the MPV (Citrate) and the %RP at baseline (a), 14d (b) and 90d (C)

Figure 9-3a: Fitted line plot showing a positive correlation between the MPV (Citrate) and the %RP at baseline (rho ≥ 0.29, p < 0.001)

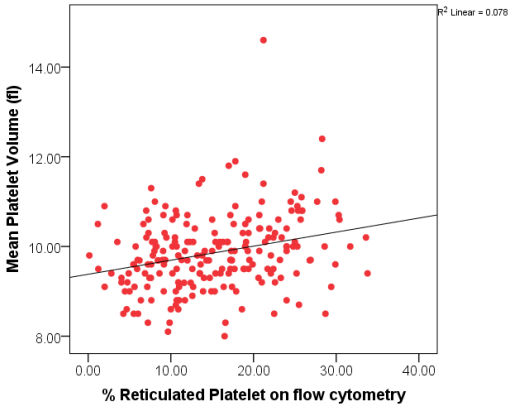


Figure 9-3b: Fitted line plot showing a positive correlation between the MPV (Citrate) and the %RP at 14d (rho ≥ 0.28, p < 0.001)

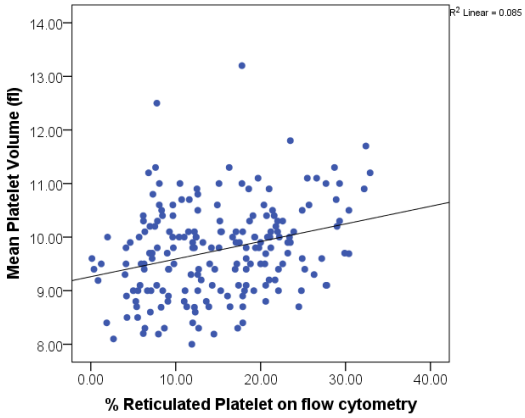


Figure 9-3c: Fitted line plot showing a positive correlation between the MPV (Citrate) and the %RP at 90d (rho ≥ 0.3, p < 0.001)

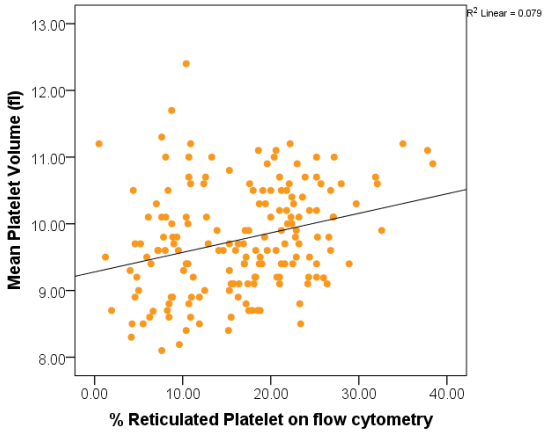


Figure 9. 4 Fitted line plot showing a positive correlation between the PDW (Citrate) and the %RP at baseline (a), 14d (b) and 90d (c)

Figure 9-4a: Fitted line plot showing a positive correlation between the PDW (Citrate) and the %RP at baseline (rho ≥ 0.26, p < 0.001)

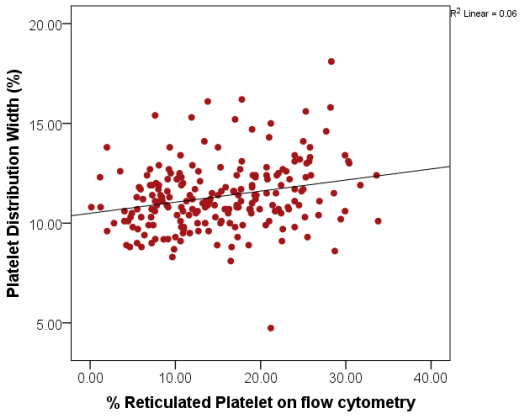


Figure 9-4b: Fitted line plot showing a positive correlation between the PDW (Citrate) and the %RP at 14d. (rho ≥ 0.22, p < 0.002)

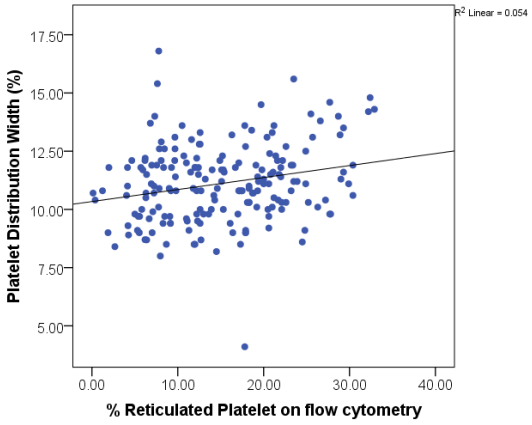
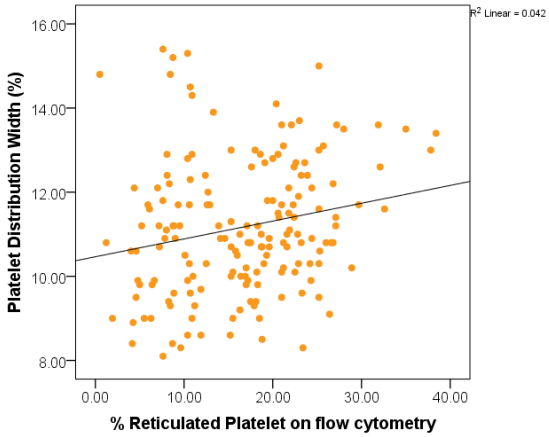


Figure 9-4c: Fitted line plot showing a positive correlation between the PDW (Citrate) and the %RP at 90d (rho ≥ 0.23, p ≤ 0.002)



10. Summary and Overview of Future Work

This thesis includes a clinically-informative introduction to ischaemic cerebrovascular disease (CVD) and aspects of platelet biology relevant to this body of work. I have completed a systematic review of the literature and innovative meta-analysis of data on the potential value of Antiplatelet-HTPR testing in CVD patients, in collaboration with world-experts in Vascular Neurology/Stroke Medicine. I have comprehensively assessed biomarkers of platelet activation and platelet production/turnover/secretion, and have conducted well-designed, prospective pilot studies of platelet function/reactivity in CVD patients who were starting or changing antiplatelet therapy. These research findings have led to the generation of new knowledge in the field of translational platelet science / haemostasis, and have enhanced our understanding of the potential clinical importance of Antiplatelet-HTPR testing in CVD patients.

10.1. Value of platelet function/reactivity testing at predicting risk of recurrent vascular events and outcomes after TIA or ischemic stroke: A Systematic Review and Meta-Analysis

Chapter 4 reported on the findings of an up-to-date systematic review and meta-analysis of the literature on the prevalence of *ex-vivo* antiplatelet-HTPR and the

relationship between antiplatelet-HTPR status and recurrent vascular events/outcomes in CVD patients. The potential clinical value of testing for Antiplatelet-HTPR to predict outcomes in TIA/ischaemic stroke patients on antiplatelet therapy was unclear prior to the conduct of this aspect of my thesis. The prevalence of Antiplatelet-HTPR was reported to vary between 3-65% with aspirin, 8-56% with clopidogrel and 1.8-35% with aspirin-clopidogrel therapy, and these wide ranges were partly dependent on the platelet testing platform employed and time after TIA/stroke onset at which patients were recruited. Our robust, innovative meta-analysis indicates that CVD patients with antiplatelet-HTPR on any regimen, based on HTPR definitions employed in individual studies, had 2-3 times the risk of experiencing the composite outcome of recurrent stroke/TIA, myocardial infarction or vascular death or recurrent ischaemic cerebrovascular events during prospective follow-up compared with patients without antiplatelet-HTPR. These findings also applied to patients on aspirin monotherapy, but there was high heterogeneity between studies. Clopidogrel-HTPR status did not predict risk of subsequent composite vascular outcomes or ischaemic stroke/TIA, but the number of included studies was much smaller, with moderate heterogeneity between studies. Data on antiplatelet-HTPR in patients on combination antiplatelet therapy, predominantly aspirin-clopidogrel were limited but were also clinically informative, so future studies are needed in patients on all commonly-prescribed antiplatelet regimens. The risk of having a severe stroke was higher in those with *vs*. those without antiplatelet-HTPR, but high-quality prospective data on this topic were very limited. These data suggest that identification of antiplatelet-HTPR predicts the risk of recurrent vascular events and outcomes in CVD patients, especially on aspirin. Given the moderate-high heterogeneity between studies, further prospective, multi-

centre studies are needed to address this issue. With the increasing use of dual antiplatelet treatment in acute ischaemic stroke and TIA, there is a clear need to establish the 'true efficacy' of antiplatelet agents and potentially utilise a platelet function testing-guided approach to personalise therapy. This may optimise protection for CVD patients and provide insights into the risks of subsequent ischaemic stroke and vascular events whilst weighing this up against the risk of bleeding complications.

10.2. Assessment of on-treatment platelet activation and platelet reactivity in the early and late phase following acute TIA or Ischaemic Stroke in patients commencing Aspirin

Chapter 6 revealed that measurement of unstimulated levels of platelet surface activation markers (CD62P and CD63) or leucocyte -platelet complexes on whole blood flow cytometry with a Beckmann-Coulter XL MCL is not a sensitive method to detect the effects of aspirin treatment *ex vivo* in patients after a recent TIA or ischaemic stroke. The inhibitory effects of aspirin were reliably detected by the PFA-100 C-EPI cartridge, VerifyNow Aspirin cartridge and Multiplate Aspirin assay, but up to one quarter to one third of patients exhibit 'cross-sectional' Aspirin-HTPR on certain platelet function assays despite the use of commonly prescribed doses of aspirin. The prevalence of aspirin-HTPR was 'numerically' but not statistically-significantly lower when one used a longitudinal definition *vs*. a cross-sectional of HTPR, as shown previously in an earlier study by our group, but this lack of significance represented a type II error due to the small numbers of patients

included in this aspect of my thesis. Using a cross-sectional definition of Aspirin-HTPR, there was 'moderate agreement' between the PFA-100 C-EPI and VerifyNow Aspirin assays, but only 'fair agreement between the PFA-100 C-EPI and Multiplate Aspirin assays and 'slight agreement' between the VerifyNow Aspirin and Multiplate Aspirin assays. There were no recurrent vascular events observed during comprehensive, prospective clinical and laboratory follow-up to at least 1 year after symptom onset in any of our patients who had available Aspirin-HTPR data. Therefore, we cannot reliably comment on the value of Aspirin-HTPR status at predicting the risk of recurrent vascular events over time from this pilot study. We concluded that larger, prospective studies are warranted to determine whether cross-sectional/case-control or relatively novel longitudinal definitions of Aspirin-HTPR may predict the risk of recurrent vascular events and outcomes in the clinical setting in individual CVD patients who are treated with aspirin.

10.3. Assessment of on-treatment platelet activation and platelet reactivity in the early and late phase following acute TIA or Ischaemic Stroke in patients commencing Clopidogrel.

Chapter 7 reported on the ability of established and novel laboratory tests of platelet activation and reactivity to identify patients with recent TIA or ischaemic stroke who have *ex vivo* Clopidogrel-HTPR according to established cross-sectional and novel longitudinal definitions of HTPR. Commencement of clopidogrel did not significantly affect platelet surface CD62P or CD63 expression, or the percentage of any circulating leucocyte-platelet complexes during follow-up at either 14d or 90d

vs. baseline. The PFA-100® Innovance P2Y cartridge, VerifyNow® P2Y12 cartridge and Multiplate® ADP assay were found to be sensitive at detecting the effects of clopidogrel treatment in stroke and TIA patients. The cross-sectional prevalence of clopidogrel-HTPR is low to moderate overall in the early phase (31.6 - 60.5%) and in the late phase (20.8 - 56%) after TIA or ischaemic stroke based on simultaneous assessment with all 3 platelet function/reactivity testing platforms. Comparison of data from all 3 assays shows that the cross-sectional prevalence of Clopidogrel-HTPR is significantly higher on low shear stress than on moderately high shear stress platforms. Clopidogrel-HTPR prevalence is also higher with cross-sectional than with novel longitudinal definitions of HTPR. The clinical predictive value of these findings is unclear from this study alone because only 3 patients had a recurrent TIA during follow up, but it is interesting that all patients with recurrent TIAs had Clopidogrel-HTPR according to one of the cross-sectional definitions on at least one device. At present, one cannot recommend altering antiplatelet therapy based on the results of Clopidogrel-HTPR testing from this thesis alone, but these findings do enhance the argument for simultaneously assessing Clopidogrel-HTPR with more than one platelet function/reactivity platform in CVD (see below). Furthermore, these data can contribute to future meta-analyses on this topic.

10.4. Assessment of on-treatment platelet activation and platelet reactivity in the early and late phase following acute TIA or Ischaemic Stroke in patients commencing Dipyridamole

Chapter 8 reported on the ability of established and novel laboratory tests of platelet function/reactivity to identify patients with recent TIA or ischaemic stroke who have dipyridamole-HTPR *ex vivo* after the addition of dipyridamole to aspirin using 'novel longitudinal definitions' of HTPR. There were no statistically significant changes in platelet surface CD62P or CD63 expression at 14d or 90d *vs*. baseline after commencing dipyridamole, or consistent changes in the % of circulating leucocyte-platelet complexes in this patient group overall. However, our data indicate that one can detect additional inhibition of platelet function/reactivity *ex vivo* with the PFA-100 C-ADP and Multiplate ADP assays when dipyridamole is added to aspirin after TIA or ischaemic stroke. This confirms prior innovative work by Tobin *et al*. from our research group on the PFA-100 C-ADP assay, but to our knowledge, similar findings have not previously been reported on the Multiplate ADP assay. This longitudinal study also confirmed a prior interesting observation by our group that the% monocyte-platelet complexes increased in the subgroup of patients with Dipyridamole-HTPR, but not in those without Dipyridamole-HTPR at both 14d and 90d compared with baseline, improving our understanding of the biological mechanism responsible for Dipyridamole-HTPR on the PFA-100 C-ADP assay. Larger, longitudinal, multi-centre studies using the PFA-100 C-ADP and Multiplate ADP assays (if the Multiplate reagents become routinely available once again) are warranted to assess the potential value of these and other emerging devices at predicting the long-term risk of recurrent vascular events and outcomes

on aspirin and dipyridamole in CVD. If HTPR status is shown to predict outcomes over time, one could opt to continue Dipyridamole in those with *ex vivo* 'Dipyridamole-responsiveness', and change to an alternative, personalised antiplatelet regimen to optimise secondary preventive treatment following a TIA or ischaemic stroke in those with Dipyridamole-HTPR.

10.5. Reticulated platelets in patients on antiplatelet therapy in the early and late phases after TIA or ischaemic stroke

Chapter 9 reported on the profile of circulating reticulated platelets in patients with TIA or ischaemic stroke who were on treatment with commonly-prescribed antiplatelet agents compared with healthy controls. Data were derived from a combined analysis of the TRAP and OATS studies. Prior to the conduct of this thesis, there were very few data on this topic in CVD, and to our knowledge, this is the largest case-control study to assess reticulated platelets in CVD patients to date. The percentage of circulating reticulated platelets was increased in the late phase (> 90 days), but not significantly increased in the early phase (≤ 4 weeks) after a TIA or ischaemic stroke in the overall CVD population compared with healthy controls. It was of interest that the median %RP was significantly elevated only in the TOAST patient subgroup with TIA/ischaemic stroke due to small vessel disease at baseline and at 90d, but not at 14d compared with controls. We acknowledge that the lack of significant findings in the small vessel disease subgroup at 14d compared with controls could reflect a type II error. Nevertheless, these data emphasise the importance of careful phenotyping of CVD patients in any future studies to assess the profile of reticulated platelets in different TIA/stroke subtypes

within a larger CVD cohort to determine whether these findings can be replicated. Our research group have planned to address this issue in an independent CVD population with a more sensitive, updated flow cytometry protocol, and with simultaneous assessment of the 'reticulated platelet fraction' on a newer 'automated assay' in whole blood.

In keeping with prior data, our correlation analysis confirmed that reticulated platelets are larger than more mature platelets in CVD patients, with a positive correlation between both the MPV and PDW and %RP at baseline, 14d and 90d. The simultaneously-collected data on the %RP, platelet counts and MPV indicate that there is an ongoing stimulus to the formation of larger reticulated platelets, especially in the late phase after TIA or stroke onset in our cohort, even though the overall platelet count remains stable. This area of platelet biomarker research deserves further study because larger platelets have been reported to be 'more reactive' and may be partly responsible for the phenomenon of Antiplatelet-HTPR described in some of the above chapters. Future studies have been prospectively-planned to assess the relationship between the % of circulating reticulated platelets and antiplatelet-HTPR status, but were beyond the scope of this thesis. Furthermore, the underlying pathophysiological mechanisms responsible for increased circulating reticulated platelets after TIA and ischaemic stroke need to be delineated. These data have the potential to improve our understanding of the pathogenesis of recurrent vascular events in CVD patients on commonly-prescribed antiplatelet therapy.

In summary, the data presented in this thesis support the hypothesis that well-designed case-control and longitudinal studies improve our understanding of the platelet, haemostatic and thrombotic profiles in patients with TIA or ischaemic stroke who are on commonly prescribed antiplatelet regimens. This work has already led to the design of larger multicentre studies by our Clinical Academic Vascular Neurology Research group to advance this field even further, with a view to facilitating optimised, personalised medicine in patients following TIA or ischaemic stroke.

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