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Assessment of Platelet Activation and Function in the
Early and Late Phases Following Acute TIA and
Ischaemic stroke

A Thesis submitted to University of Dublin, Trinity College 2010
for examination towards the award of
Doctor in Philosophy (Ph.D.)

by

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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 control subjects and 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, and FBC measurements and prepared the platelet poor plasma for further analysis. I carried out some of the ELISA assays, but was assisted with these experiments by my colleagues who are acknowledged below. I entered all of the data onto a database and performed the statistical analysis of the data with the help and supervision of Dr. Dominick J.H. McCabe.

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Dr. William Oliver Tobin
May 6th 2011
Summary

Aims: The purpose of this thesis was to comprehensively assess the impact of commencing commonly prescribed antiplatelet regimens on platelet activation, platelet function and platelet turnover after TIA or ischaemic stroke, and to perform preliminary experiments to investigate coagulation system potential and endothelial activation in these patients. We also aimed to improve our understanding of the clinical and cellular mechanisms influencing non-responsiveness to antiplatelet therapy in the laboratory.

Methods: A longitudinal observational study was performed to assess patients who changed from no medication to aspirin, aspirin to aspirin-dipyridamole combination therapy, or aspirin to clopidogrel ≤ 4 weeks of onset of a TIA or acute ischaemic stroke. Platelet activation and turnover were quantified with whole blood flow cytometry to assess the ‘unstimulated expression’ of platelet activation markers (CD62P and CD63), leucocyte-platelet complex formation and the percentage of circulating reticulated platelets. Inhibition of platelet function at moderately high shear stress was assessed with the platelet function analyser (PFA-100® collagen-ADP [C-ADP] and collagen-epinephrine [C-EPI] cartridges). A novel, ‘longitudinal definition’ of non-responsiveness on the PFA-100® was devised to overcome the limitations of ‘cross-sectional, case-control’ definitions of ex vivo antiplatelet non-responsiveness. Coagulation system potential was measured, and endothelial +/- platelet activation was quantified with von Willebrand factor and von Willebrand factor propeptide levels in platelet poor plasma in a subgroup of our patients.

Results: Unstimulated platelet surface CD62P or CD63 expression were not influenced by altering antiplatelet therapy. Collagen-epinephrine (C-EPI) closure times, but not C-ADP closure times, were prolonged after commencing aspirin monotherapy. The prevalence of aspirin non-responsiveness was 24% at 14d and 18% at 90d. C-ADP closure times were prolonged following the addition of dipyridamole MR to aspirin monotherapy reflecting additional inhibition of C-ADP-induced platelet adhesion and aggregation with dipyridamole. However, 59% of patients at 14 days (14d) and 56% of patients at >90 days after commencing therapy (90d) were defined as ‘dipyridamole non-responders’ on the PFA-100®. Monocyte-platelet complexes increased in these patients during follow-up, especially in dipyridamole non-responders, suggesting that elevated monocyte-platelet complexes may contribute to ex vivo dipyridamole non-
responsiveness. Withdrawal of aspirin was detected with the C-EPI cartridge in patients who changed treatment from aspirin to clopidogrel, but 41% of patients were non-responsive at 14d, and 35% were non-responsive to clopidogrel at 90d on the C-ADP cartridge. Circulating reticulated platelets were not increased in the early phase, but were increased in the late phase after TIA or stroke compared with controls. There was an elevated number of circulating reticulated platelets in patients who were non-responsive to aspirin in the early phase, but this effect did not persist into the late phase, although subject numbers were very limited. Circulating reticulated platelets were not affected by the addition of dipyridamole MR to aspirin, but increased after changing from aspirin to clopidogrel monotherapy. We hypothesise that this finding arose due to withdrawal of aspirin rather than in response to commencing clopidogrel monotherapy. We found some evidence of enhanced coagulation system potential in the early phase after TIA or stroke, with an apparent reduction in coagulation system potential after adding dipyridamole MR to aspirin. We also identified further evidence of excessive endothelial +/- platelet activation after TIA or stroke (elevated VWF antigen levels), and demonstrated that VWF antigen levels may reduce following the addition of dipyridamole MR to aspirin monotherapy, although one could not exclude resolution of the acute phase response.

Conclusions: Quantification of the expression of platelet surface activation markers with unstimulated whole blood flow cytometry is not likely to be useful in predicting the response to antiplatelet therapy in the clinical setting, but flow cytometric assessment of ‘inducible platelet reactivity/activation’ deserves further study. For the first time, we have identified that additional inhibition of platelet adhesion and aggregation with dipyridamole can be identified with the PFA-100® C-ADP cartridge, which has the potential to predict the response to dipyridamole in the clinical setting. However, a substantial proportion of ischaemic CVD patients exhibit ex vivo non-responsiveness to all commonly prescribed antiplatelet regimens. Elevated leucocyte-platelet complexes and increased circulating reticulated platelets appear to contribute to ex vivo antiplatelet non-responsiveness on the PFA-100®. There is some evidence of enhanced coagulation system potential and endothelial activation following ischaemic CVD that may also be influenced by dipyridamole. Based on these data, an adequately powered, longitudinal, multicentre translational study assessing the clinical predictive value of established and novel high and low shear stress tests of platelet function in CVD patients commencing antiplatelet therapy has been planned by our research group.
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To whom I owe the leaping delight.
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<td>ACA</td>
<td>Anterior Cerebral Artery</td>
</tr>
<tr>
<td>ACAS</td>
<td>Asymptomatic Carotid Atherosclerosis Study</td>
</tr>
<tr>
<td>ACE Trial</td>
<td>Aspirin and Carotid Endarterectomy Trial</td>
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<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>βTG</td>
<td>β-thromboglobulin</td>
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<tr>
<td>C-ADP</td>
<td>Collagen-ADP</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3', 5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy</td>
</tr>
<tr>
<td>CAST</td>
<td>Chinese Acute Stroke Trial</td>
</tr>
<tr>
<td>CAVATAS</td>
<td>Carotid and Vertebral Artery Transluminal Angioplasty Study</td>
</tr>
<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
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<tr>
<td>CD</td>
<td>Cluster Differentiation</td>
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<td>Carotid Endarterectomy</td>
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<td>COX</td>
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<td>eGMP</td>
<td>Guanosine 3', 5'-cyclic monophosphate</td>
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<tr>
<td>CT</td>
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<tr>
<td>CTA</td>
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<tr>
<td>CVD</td>
<td>Cerebrovascular Disease</td>
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<tr>
<td>Cy5</td>
<td>Cyanine 5</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DP</td>
<td>Dipyridamole</td>
</tr>
<tr>
<td>DSA</td>
<td>Digital Subtraction Angiography</td>
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<tr>
<td>DWI</td>
<td>Diffusion Weighted Imaging</td>
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<tr>
<td>EAFT</td>
<td>European Atrial Fibrillation Trial</td>
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<tr>
<td>ECA</td>
<td>External Carotid Artery</td>
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<tr>
<td>ECST</td>
<td>European Carotid Surgery Trial</td>
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<tr>
<td>EDV</td>
<td>End Diastolic Velocity</td>
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<tr>
<td>E-selectin</td>
<td>Endothelial-leucocyte adhesion molecule</td>
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<tr>
<td>ESPS-2</td>
<td>Second European Stroke Prevention Study</td>
</tr>
<tr>
<td>FBC</td>
<td>Full Blood Count</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FISS</td>
<td>Fraxiparin in Stroke Study</td>
</tr>
<tr>
<td>FISS bis</td>
<td>Fraxiparine in Ischaemic Stroke Study</td>
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
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<tr>
<td>FS</td>
<td>Forward Scatter</td>
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<tr>
<td>G Proteins</td>
<td>Guanine Nucleotide Binding Regulatory Proteins</td>
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<tr>
<td>Gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>HAESt</td>
<td>Heparin in Acute Embolic Stroke Trial</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks Balanced Saline Solution</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>HS</td>
<td>Haemorrhagic Stroke</td>
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<tr>
<td>H2O</td>
<td>Water</td>
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<tr>
<td>ICA</td>
<td>Internal Carotid Artery</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ICH</td>
<td>Intracerebral Haemorrhage</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IST</td>
<td>International Stroke Trial</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-Associated Membrane Proteins</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low Molecular Weight Heparin</td>
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<tr>
<td>Mac-1</td>
<td>Macrophage 1 antigen</td>
</tr>
<tr>
<td>MAST-I</td>
<td>Multicentre Acute Stroke Trial-Italy</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle Cerebral Artery</td>
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<tr>
<td>MCL</td>
<td>Multi-tube Carousel Loader</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
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<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
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<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
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<tr>
<td>MMP-9</td>
<td>Matrix Metalloproteinase-9</td>
</tr>
<tr>
<td>MPV</td>
<td>Mean Platelet Volume</td>
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<tr>
<td>MRA</td>
<td>Magnetic resonance angiography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NASCET</td>
<td>North American Symptomatic Carotid Endarterectomy Trial</td>
</tr>
<tr>
<td>NVAF</td>
<td>Non-valvular Atrial Fibrillation</td>
</tr>
<tr>
<td>OCS</td>
<td>Open Canalicualr System</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Arterial Disease</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PARs</td>
<td>Protease Activated Receptors</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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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
PIP₂  Phosphatidylinositol 4,5-biphosphate
PMT  Photomultiplier Tube
PPP  Platelet Poor Plasma
PRP  Platelet Rich Plasma
PS  Phosphatidylserine
PSGL-1  P-selectin glycoprotein ligand-1
PSV  Peak Systolic Velocity
PTE  Phosphatidylethanolamine
RGD  Arginine-Glycine-Aspartic Acid
RP  Reticulated platelets
RPE  R-phycoerythrin
RS  Recurrent Ischaemic Stroke
SALT  Swedish Aspirin Low-dose Trial
SLAM  Signalling Lymphocyte Activation Molecule
SPIRIT  Stroke Prevention in Reversible Ischaemia Trial
SS  Side Scatter
TEG  Thrombelastography Platelet Mapping
TIA  Transient Ischaemic Attack
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-α</td>
</tr>
<tr>
<td>TO</td>
<td>Thiazole Orange</td>
</tr>
<tr>
<td>TOAST</td>
<td>Trial of ORG 10172 in Acute Stroke Treatment</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>VWF:Ag</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>von Willebrand factor propeptide</td>
</tr>
<tr>
<td>WARSS</td>
<td>Warfarin-Aspirin Recurrent Stroke Study</td>
</tr>
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1. Overview of Transient Ischaemic Attack (TIA) and ischaemic stroke

1.1. Definition of TIA and stroke

The most commonly quoted definition of stroke is: "A neurological deficit of sudden onset; with focal rather than global neurological dysfunction; with symptoms lasting more than 24 hours or resulting in death before 24 hours; and in which, after adequate investigation, symptoms are presumed to be of a non-traumatic vascular origin" (Bamford, 1992). The aetiology of stroke may be complicated however, and more so in some groups than in others (Kristensen et al, 1997). In his original paper, Bamford was attempting to introduce a rational definition and classification scheme which would be important for guiding investigations, treatment and informing prognosis. Such an approach excludes some of the rarer causes of stroke, which by their aetiology, fall outside the relatively rigid standard definition. For example, cervical artery dissection, can be related to neck trauma and was the leading cause of stroke in one large young cohort study (Kristensen et al, 1997). Patients presenting with subarachnoid haemorrhage may present with a severe headache and meningism without focal or global neurological dysfunction. Stroke can occasionally cause global neurological dysfunction from the outset, e.g. in patients with extensive subarachnoid haemorrhage or bilateral cerebral hemispheric or brain stem involvement. The definition does not include neurological dysfunction caused by subdural or extradural haemorrhage (Whisnant et al, 1990; Klijn et al, 1997), traumatic intracranial contusional haemorrhage, and some authors also exclude cerebral infarction or haemorrhage caused by an infection or a tumour (Warlow et al, 1996). These decisions are practical, because the prognosis and treatment of these types of neurological disorders are often very
different from that of an ischaemic stroke which fits well into the standard definition. Therefore, one needs to be flexible in including these cases within the definition of stroke, as deemed appropriate. For the purpose of this thesis, Banford’s definition of ischaemic stroke was employed, although patients with global neurological dysfunction caused by brainstem ischaemia or bilateral cerebral ischaemia/infarction were eligible for inclusion, as were patients e.g. with non-penetrating neck injury that led to cervical artery dissection (see later).

A Transient Ischaemic Attack (TIA) can be defined as a clinical syndrome characterised by a sudden onset of focal neurological or monocular dysfunction, with symptoms lasting for less than 24 hours, that are presumed to be vascular in origin (Warlow et al, 1996). Most TIAs last for minutes rather than hours, with the majority lasting between six and 30 minutes in one hospital-based series (Warlow et al, 1996). The distinction between a TIA and a stroke is purely based on the duration of the symptoms, because approximately 5% of patients with a TIA will have abnormal but functionally unimportant neurological signs for longer than 24 hours e.g. an extensor plantar response (Warlow et al, 1996). This clinical definition of TIA was endorsed by many authors until relatively recently. Between 20-50% of patients with TIAs have been shown to have acute ischaemic lesions on diffusion weighted imaging (DWI) (Ay et al, 2002; Crisostomo et al, 2003; Coutts et al, 2005; Calvet et al, 2009). It also has been shown that the presence of DWI-lesions correlates with several clinical features known to predict stroke risk after TIA (Redgrave et al, 2007). However, there are only limited data demonstrating an increased risk of stroke recurrence in ‘DWI-positive’ patients when compared with patients with normal brain imaging (Purroy et al, 2004; Prabhakaran et al, 2007). This has lead to debate in the international community
as to what should constitute a TIA for epidemiological and clinical studies, and this issue has not yet been resolved because diagnostic classification of a TIA will depend on the extent of evaluation that the individual patient receives.

The European Stroke Organisation (ESO) has not changed its definition of a TIA due to the lack of a 'perfect biomarker' for permanent ischaemic brain damage (Karolinska Stroke Update Consensus Statement, 2008). The American Stroke Association revised its definition to define a TIA as a 'transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia, without acute infarction' (Easton et al, 2009). The American guidelines go on to state that the typical duration of a TIA is <1 or 2 hours, but occasionally, prolonged episodes occur. If the American definition of a TIA were to be globally adapted, this would necessitate a revision of the definition of ischaemic stroke i.e. an ischemic stroke would be defined as 'an infarction of central nervous system tissue'. This revised definition removes the differentiation between a TIA and a stroke on the basis of an arbitrary 'duration cut point' of 24 hours, or even the presence or absence of residual neurological dysfunction which was proposed by Bamford (Bamford, 1992); i.e. patients with acute diagnostic imaging changes would not necessarily need ongoing clinical symptoms or signs to be diagnosed with a stroke. However, a tissue-based definition clearly has limitations: some patients with TIAs with potentially reversible ischaemic changes on DWI could be inadvertently categorised as having had an acute ischemic stroke if they were imaged very early after symptom onset before the DWI changes resolve; some patients with acute ischaemic stroke do not have acute imaging changes at the time of presentation, and the presence or absence of imaging changes depends on the imaging modality employed.
It may well be proposed that one should dually classify TIA's according to (a) the clinical definition, as outlined above, to allow one to consistently perform international epidemiological studies in different countries, and (b) a 'tissue based definition', as proposed by others if imaging data are available in the neurovascular stroke centre in question.

For the purpose of this thesis, the European Stroke Organisation definition of stroke and TIA were used, unless explicitly stated in the text.
1.1. Epidemiology of stroke (emphasis on ischaemic stroke)

Stroke is the second to third leading cause of death and the commonest cause of acquired disability in adults in Europe (Warlow C.P, 1998; Petersen et al, 2005).

The incidence of acute cerebrovascular events (transient ischaemic attack and stroke combined) currently exceeds the incidence of acute coronary heart disease in Europe (Rothwell et al, 2005). Stroke is also the commonest cause of acquired disability in adults (Warlow et al, 2001). A recent meta-analysis has shown that the incidence of stroke (ischaemic and haemorrhagic) in 'high-income' countries in the last decade was 85-94 per 100,000 overall, rising to 1151-1216 per 100,000 in the >75 year old age group, with a case fatality following stroke of 17-30% in 'high-income' countries and 18-35% in 'low-income' countries (Feigin et al, 2009).

By the year 2050, the percentage of the population who are over 65 years of age is expected to increase by almost 240%, and the number of patients aged 80 years or older is expected to increase by almost 450% (Department of Economic and Social Affairs.Population Division, 2004). Therefore, the impact of stroke on society is likely to increase because of the increasing age of the population at risk.

The majority of patients survive their first ischaemic stroke with 95% alive at seven days, 80-90% alive at 30 days, and 77% alive at one year (Warlow et al, 1996; Feigin et al, 2009), these patients are at risk of stroke recurrence and also have an increased risk of serious coronary events of about 3% per annum over the following five years (Warlow C.P, 1998). Recurrence may occur 'early' or 'late' after the index stroke, and
the reported rate of recurrence varies depending on the aetiology of the initial stroke, the
duration of follow up, and the study design (Rothwell et al, 2005; Feigin et al, 2009).

Overall, early recurrence occurs in 1.4% to 18.5% of patients in the first month after
ischaemic stroke (Petty et al, 2000). The risk of recurrent ipsilateral stroke in medically
treated patients with severe (> 70%) symptomatic extracranial carotid stenosis is
particularly high in the first year, at approximately 18%, with an overall risk of 26%
over two years (North American Symptomatic Carotid Endarterectomy Trial
Collaborators, 1991). Early recurrence appears to be less frequent in the first one to
three months after lacunar stroke (1% to 2%) (North American Symptomatic Carotid
Endarterectomy Trial Collaborators, 1991), but the long term outcome is less certain
with a recurrence rate of 2-12% in the first year (Bamford et al, 1991; Norrving, 2003),
and as high as 28% after 5 years (Norrving, 2003). Atrial fibrillation is the most
common source of cardiogenic brain embolism (EAFT (European Atrial Fibrillation
Trial) Study Group, 1993) with a stroke recurrence rate of up to 5% in the first 30 days
(Petty et al, 2000). Patients with non-valvular atrial fibrillation (NVAF) have an overall
risk of stroke recurrence of 12% in the first year, with this risk decreasing to 5% per
annum thereafter (EAFT (European Atrial Fibrillation Trial) Study Group, 1993).

Although the combined rate of death or dependency after a first ischaemic stroke is
approximately 50%, it is likely that this risk of death or dependency will increase with
recurrent cerebrovascular events (Warlow et al, 1996).
In summary, acute cerebrovascular events are common, and can be debilitating or fatal. However, the opportunities that exist to modify risk factors, provide acute and long-term rehabilitation, enhance secondary prevention and improve long term prognosis in patients who survive their first or subsequent cerebrovascular event cannot be underestimated.
1.2. Aetiology of TIAs and ischaemic stroke

Most TIAs or ischaemic strokes are caused by thromboembolism to, or thrombotic occlusion of an intracranial artery (Sacco et al, 1989). In the case of thromboembolism, the thromboemboli may arise from anywhere in the arterial tree proximal to the occlusion i.e. from an atherosclerotic plaque in a large extracranial or intracranial artery, intracranial small vessel disease, or embolism from the heart. In the remaining patients, less common causes for the ischaemia are identified (e.g. arterial dissection, sickle cell disease or vasculitis) or the origin of the infarction is not established despite extensive investigation.

The relative frequencies of the different ischaemic stroke subtypes in any clinical series depends on the criteria used for classification (Amarenco et al, 2009a). Several different classification schemes have been proposed, most for research purposes. The system most commonly in use at the time of conduct of this thesis was the TOAST classification, although several others have been proposed (Amarenco et al, 2009a; Amarenco et al, 2009b).
The TOAST classification was proposed by the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) investigators (Adams, Jr. et al, 1993). This classification defined 5 categories of stroke:

1. Large-artery atherosclerosis (embolic/thrombotic)
2. Cardioembolism (high-risk/medium-risk)
3. Small-vessel occlusion (lacunar)
4. Stroke of other determined aetiology
5. Stroke of undetermined aetiology
   - Two or more causes identified
   - Negative evaluation
   - Incomplete evaluation

The first four categories were subclassified into 'probable' or 'possible', based on the results of ancillary investigations.

The TOAST classification system has been criticised for its failure to take into account the exhaustive extent of a modern investigative work up, and conversely by its tendency to mask a less intense investigative work up (Amarenco et al, 2009a). However, it does allow a 'mechanistic classification' of TIA and ischaemic stroke in contrast to other classification systems (Bamford et al, 1991), thus allowing one to guide clinical management to suit individuals, and facilitating multicentre research studies in patients with ischaemic cerebrovascular disease.

In the following section, I will outline the underlying pathogenic mechanisms responsible for, and the clinical features associated with, the different aetiological categories of ischaemic stroke or TIA.
1.2.1. Large-artery atherosclerosis

Most patients with atherothrombotic stroke or TIA in association with extracranial carotid artery stenosis are believed to have initial atherosclerotic plaque rupture with subsequent ipsilateral distal thromboembolism of platelet emboli and/or plaque fragments (Golledge et al, 2000). The incident stroke or TIA, may be preceded by or followed by micro-emboli, only some of which may be symptomatic (Markus et al, 2005). In a minority of cases, acute thrombosis can occur on the plaque surface and cause carotid occlusion. This may be asymptomatic if there is adequate collateral circulation, or cause distal haemodynamic compromise especially in the presence of severe contralateral carotid stenosis or poor collateral intracerebral circulation (Whisnant et al, 1990; Klijn et al, 1997). Less frequently, patients with internal carotid artery occlusion may have emboli which arise (i) from the occluded internal carotid stump (Quill et al, 1989), (ii) from the ipsilateral common or external carotid arteries via external carotid collaterals, or (iii) by transhemispheric passage from a contralateral internal carotid stenosis via the circle of Willis (Klijn et al, 1997).

Clinical features supportive of a diagnosis of stroke or TIA of large-artery atherosclerotic aetiology are ocular or cortical symptoms or signs in the territory of the internal carotid artery, previous TIA or stroke in the same vascular territory, and absence of symptoms, signs or radiological features in other vascular territories (Adams, Jr. et al, 1993). While patients with higher grades of carotid stenosis are at higher risk of stroke and TIA (The European Carotid Surgery Trialists Collaborative Group, 1995; Halliday et al, 2004) and may require carotid revascularisation, patients with lower grades of carotid stenosis may also experience stroke or TIA of large artery
aetiology but do not benefit from carotid endarterectomy or endovascular treatment (Halliday et al, 2004).

Colour Doppler ultrasound imaging, magnetic resonance angiography (MRA), computed tomography angiography (CTA), or intra-arterial digital subtraction angiography (DSA) can be used to quantify the severity of carotid stenosis.

The degree of carotid stenosis on carotid angiography may be quantified via one of three methods: The Common Carotid method (CAVATAS Investigators, 2001), the European Carotid Surgery Trial (ECST) method (European Carotid Surgery Trialists' Collaborative Group, 1998), and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) method (Rothwell et al, 2003). With the Common Carotid method, the percent stenosis = 100 (C-A/C), where A = the diameter of the residual lumen at the point of maximal stenosis, and C = the width of the disease free common carotid artery below the bifurcation at a point where the walls are approximately parallel. With the ECST method, the percent stenosis = 100 (E-A/E), where A = the diameter of the residual lumen at the point of maximal stenosis, and E = the estimated normal diameter of the ICA at the point of maximal narrowing; therefore, one needs to 'guess' how wide the carotid bulb was before it became narrowed by the atherosclerotic plaque. The NASCET method calculates the percent stenosis as 100 (N-A/N), where A = the diameter of the residual lumen at the point of maximal stenosis, and N = the diameter of the disease free distal ICA at a point where the walls are approximately parallel. The Common Carotid and ECST methods give rise to similar estimates of the degree of carotid stenosis (CAVATAS Investigators, 2001). However, the NASCET method produces a lower estimate of the degree of carotid stenosis than
the other two methods. Therefore, when interpreting the results of studies in patients with carotid stenosis, it should be remembered that 50 and 70% stenosis by the NASCET method are equivalent to 65% and 82% stenosis, respectively, by the ECST method (Rothwell et al., 2003). To facilitate data interpretation and to guide best international clinical practice, it has been suggested that the NASCET method be adopted for grading ICA stenosis (Rothwell et al., 2003).
1.2.2. Lacunar ischaemic stroke

Lacunar infarcts are small infarcts in the deep subcortical regions of the cerebrum or brainstem that result from occlusion of small, deep penetrating branches of the large cerebral arteries. When the infarcts heal, they may leave behind a small cystic fluid-filled cavity called a lacune, and some collapse of the cavity is the rule (Fisher, 1982; Whisnant et al, 1990; Klijn et al, 1997). The term lacune is derived from the Latin, ‘lacuna’ (a pit or hole), or the French, ‘la lacune’ (a gap or empty space). The vascular territories involved are those supplied by the middle cerebral, posterior cerebral or basilar arteries, and less commonly the anterior cerebral or vertebral arteries (Fisher, 1982). The size of the infarct varies depending on the diameter of the occluded artery and the pathogenesis of the vessel occlusion, although Fisher reported that approximately 90% of patients with lacunar infarcts in his series had hypertension (Fisher, 1982). Small lacunar infarcts (3 to 7 mm in diameter) result from occlusion of small penetrating arteries of 40 to 200 μm in diameter and are often asymptomatic unless strategically located in the sensory or motor tracts (Fisher, 1982). The pathological process involved is lipohyalinosis, characterised by vessel occlusion, with fibrinoid necrosis and infiltration of the vessel wall with haemosiderin-laden macrophages (Fisher, 1982; Lammie, 2000). Larger lacunar infarcts that are more commonly symptomatic are usually caused by intracranial atherosclerosis involving arteries 200 to 900 μm in diameter (Fisher, 1982; Lammie, 2000); all radiological definitions indicate that the maximum diameter of a lacunar infarct is < 15 mm (see below) (Adams, Jr. et al, 1993). The atheromatous plaque can involve the proximal portion of the perforating artery (microatheroma), the origin of the artery from the parent vessel (junctional atheroma), or the parent artery itself (mural atheroma) (Lammie, 2000). In some pathologically proven cases, thrombus was seen in the
stenotic vessel (Lammie, 2000), but in others, neither lipohyalinosis nor intracranial atherosclerosis were identified and the artery supplying the region of the infarct was normal (Fisher, 1982). It has been assumed that these ‘lacunar-type infarcts’ were secondary to embolism from the heart or the proximal feeding artery, and this hypothesis is supported by the occurrence of lacunar stroke syndromes in a proportion of patients with severe ipsilateral carotid stenosis (Inzitari et al, 2000). Some patients may also present with ‘lacunar TIAs’, presumably secondary to occlusion and subsequent recanalisation of a deep perforating artery (Warlow et al, 2001), and some of these events may be secondary to thromboembolism from the heart or proximal feeding artery.

Although over 20 have been reported (Fisher, 1982), five classic lacunar stroke syndromes are widely recognised: (i) pure motor hemiparesis, (ii) pure sensory stroke, (iii) sensorimotor stroke, (iv) ataxic hemiparesis, and (v) the dysarthria-clumsy hand syndrome (Fisher, 1982; Whisnant et al, 1990; Klijn et al, 1997; Inzitari et al, 2000).

Brain CT may be normal or show an anatomically compatible infarct of <15 mm in maximum diameter (Whisnant et al, 1990; Adams, Jr. et al, 1993). MRI of brain is much more likely to identify the responsible lesion, especially if it is located in the posterior fossa, but the maximum infarct diameter should also be < 15 mm on MRI. Neurovascular and cardiac investigations should not identify any potential proximal source of embolism, e.g. severe ipsilateral carotid stenosis or a left atrial thrombus, and no other cause for the infarction should be identified on diagnostic neurovascular imaging.
1.2.3. Cardioembolic ischaemic stroke

Patients can be classified as having cardioembolic ischaemic stroke if they have a potential cardiac or transcardiac source of embolism with no other obvious cause for stroke identified (Whisnant et al, 1990). Cardiac conditions that may cause emboli include continuous or paroxysmal atrial fibrillation or flutter, recent myocardial infarction, left atrial appendage or left ventricular thrombus, dilated cardiomyopathy, infective endocarditis or significant mitral or aortic valve disease. ‘Paradoxical embolism’ may also occur in patients with deep venous thrombosis who have a right to left intracardiac shunt e.g. an atrial septal defect or patent foramen ovale (Adams, Jr. et al, 1993). In patients with patent foramen ovale treated with aspirin, the presence of both a PFO and an atrial septal aneurysm has been shown to increase the risk of recurrent stroke compared with the presence of either abnormality alone, or compared with patients with no evidence of an atrial septal abnormality (Mas et al, 2001). The mechanisms potentially responsible for paradoxical or cardiac embolism in patients with a PFO with or without an interatrial septal aneurysm have been reviewed elsewhere, but in the absence of identifying an intra-cardiac thrombus or a DVT in such cases, it may be impossible to determine whether the presence of the cardiac abnormality actually caused the TIA or stroke in the first instance (McCabe & Rakhit, 2007). Ongoing studies are assessing the safety and efficacy of PFO closure in patients with TIA or stroke associated with a PFO, with or without an inter-atrial septal aneurysm (Mas, 2008; Hansen, 2010).
In some cases, it may also be difficult to be certain whether a TIA or stroke is cardioembolic in origin, because approximately one third of patients with stroke in association non-valvular atrial fibrillation (NVAF) have another potential underlying cause for their ischaemic symptoms (Bogousslavsky et al, 1990).

Most cardioembolic infarcts involve the cortex, and although the clinical presentation is dependent on the area of brain affected, patients who present with isolated homonymous hemianopia or isolated dysphasia often have stroke of cardioembolic origin (Whisnant et al, 1990). However, as mentioned above, some ‘lacunar-type’ infarcts may also be secondary to cardiac embolism. Investigation with electrocardiography, 24-hour cardiac rhythm (holter) monitoring, and where appropriate, echocardiography (trans-oesophageal is much more informative than trans-thoracic echocardiography) should reveal a cardiac source of embolism (McCabe & Rakhit, 2007). CT or MRI of brain should reveal an infarct in a clinically appropriate location (Adams, Jr. et al, 1993). MRI of brain with DWI sequences may be very informative, and may prompt one to do further cardiac investigations if one identifies simultaneous, multifocal cerebral ischaemia or infarction in different vascular territories. For example, if initial 24-hour holter monitoring is uninformative, prolonged cardiac rhythm monitoring for 48 hours-5 days should be considered in an attempt to identify a rhythm abnormality, especially paroxysmal atrial fibrillation, that would prompt treatment with anticoagulation rather than antiplatelet therapy (Jabaudon et al, 2004).
1.2.4. Ischaemic stroke of other determined aetiology

Patients whose stroke aetiology does not fit into the previous three categories, but for whom a cause of stroke is found, are classified as having a stroke of 'other determined aetiology'. This category includes patients with rarer causes of stroke, such as arterial dissection, non-atherosclerotic vasculopathies including inherited stroke syndromes (e.g. CADASIL or Fabry disease,) or haematologic disorders (e.g. essential thrombocytosis). This group tends to have a higher proportion of younger patients because stroke aetiologies vary more in younger age groups (Kristensen et al, 1997).

1.2.5. Ischaemic stroke of undetermined aetiology

Patients who cannot be categorised as having any of the above TIA or ischaemic stroke subtypes on the basis of the clinical findings or results of investigations can be classified as having TIA or ischaemic stroke of indeterminate aetiology. This group also includes patients who have two potential aetiologies for their TIA or stroke, and for whom the exact source of symptoms is unclear; for example, in patients with severe carotid stenosis and atrial fibrillation. This subgroup may be somewhat unsatisfactory from a research viewpoint when one is attempting to collate data from different centres, because the reasons for non-determination of stroke aetiology may range from inappropriate or insufficient investigations during stroke work up, to the lack of identification of a cause for stroke despite extensive investigations. In this thesis, patients were only included in this category if they had more than one potential aetiology for their symptoms, or if the aetiology was unclear despite extensive laboratory, neurovascular and cardiac workup at AMNCH.
1.3. Thrombus Formation in TIA and Ischaemic Stroke

As described above, several different mechanisms may be responsible for TIA or ischaemic stroke. In the following section, I will give a brief overview of thrombus formation under different conditions, and in particular, focusing on the interaction between platelets and the coagulation system.

1.3.1. Overview

There are two major physiological pathways which are thought to be important in mediating platelet thrombus formation: (a) the ‘collagen pathway’ which requires exposure of sub-endothelial collagen, and (b) the ‘tissue factor pathway’ which is independent of subendothelial collagen (Furie & Furie, 2008). The exact contribution of the two pathways to platelet activation in vivo is unknown, and their relative contribution depends on the underlying disease process (Furie & Furie, 2008). Furthermore, the pathways involved in platelet activation are also dependent on the levels of shear stress to which platelets are exposed in vivo.

(a) The collagen pathway may be activated when subendothelial 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 thrombosis. In the resting state, platelets ‘survey’ the endothelium and roll on the endothelial surface without adhering to it (Ruggeri, 2000). Following endothelial disruption, platelets adhere to exposed subendothelial collagen directly via the platelet glycoprotein (Gp) VI receptor, and via interaction of the platelet GpIb-V-IX receptor with von Willebrand factor bound to subendothelial collagen. These interactions also result in conversion of Factor XII to XIIa, initiating the process of thrombin formation, however platelet activation in this collagen-initiated pathway is
initially independent of thrombin. Of interest, inhibition or deficiency of factor XII protects mice from ischemic brain injury, without causing a concurrent increase in rates of intracerebral haemorrhage (Kleinschnitz et al, 2006). The GpVI-collagen interaction predominates in settings of low shear stress (e.g. venous thrombosis, left atrial appendage thrombus), and conversely, the GpIb-V-IX / VWF / collagen interaction predominates under high shear stress conditions (e.g. following rupture of a stenosing carotid artery plaque). In addition to its role in mediating the adhesion of platelets to collagen, GpVI receptor binding also promotes further platelet activation and dense granule release (Stoll et al, 2008). The platelet integrin $\alpha_2\beta_1$ (GpIIb/IIIa) plays a supportive, but not essential role in the interaction between platelets and collagen.

(b) The tissue factor pathway does not require disruption of the endothelium and is independent of von Willebrand factor and glycoprotein VI. Inactive tissue factor on the endothelial surface is activated by protein disulfide isomerise, which is released from activated endothelial cells and platelets. Activated tissue factor can subsequently activate factor X, which leads to the conversion of prothrombin to thrombin, and ultimately the conversion of fibrinogen to fibrin. It is unclear how, or if, platelets are recruited to a site of vessel injury when activated via the tissue factor pathway. Thrombin is a potent platelet agonist. Thrombin cleaves protease-activated receptor 1 (PAR1) on the platelet surface in humans (Furie & Furie, 2008), thereby activating platelets and promoting platelet degranulation with the release of adenosine diphosphate (ADP), serotonin, and thromboxane A$_2$. Thrombin and these other agonists subsequently activate other platelets in a positive feedback loop (Furie & Furie, 2008).
In addition to reacting to proteins generated by the coagulation cascade, which may lead to platelet-platelet aggregation as outlined above, platelets also contribute to the clotting cascade (Monroe et al., 2002). These two events occur simultaneously, possibly initiated by the same agonists. Elevation in platelet cytosolic calcium during platelet activation leads to the exposure of procoagulant phosphatidylserine (PS) on the platelet surface (van der Meijden et al., 2005). The platelet surface, with exposed phosphatidylserine, serves as an assembly site for tenase and prothrombin complexes, thus promoting accelerated thrombin generation (van der Meijden et al., 2005). Despite the fact that thrombin is a potent stimulator of platelets, and that during thrombus formation a saturating amount of thrombin should be generated to fully activate platelets, activation of the platelet P2Y12 ADP receptor actually increases the time taken to achieve peak thrombin formation (van der Meijden et al., 2005). This suggests that platelet activation by ADP may modulate the rate of thrombin formation, and this could theoretically, in turn, limit the extent of thrombin-induced platelet activation in settings with high levels of circulating ADP.
Two distinct pathways, which may act in parallel or separately to activate platelets. With intact endothelium, protein disulphide isomerase 'decrypts' circulating Tissue Factor which then activates Factor VII, and the activated Tissue Factor-Factor VIIa complex ultimately catalyses the conversion of Prothrombin to Thrombin. Thrombin then cleaves PAR-1 on the platelet surface to activate it, initiating platelet activation. In some disease states, Protein Disulphide Isomerase may not be required, as Tissue Factor may already be present in a decrypted form. With a disrupted endothelium, for example due to trauma, or atherosclerotic plaque rupture, shear-dependant platelet surface receptors bind collagen (low shear) or VWF (high shear) leading to 'outside in' platelet activation.
1.3.2. Platelet Thrombus Formation and Propagation

A developing platelet thrombus recruits circulating unstimulated platelets. Within the thrombus, platelet activation only occurs in a subset of recruited platelets (Furie & Furie, 2008). Other platelets may remain loosely associated with the thrombus, do not undergo marked activation, and may ultimately disengage from the thrombus depending on the degree of shear stress and the strength of platelet integrin binding with relevant agonists. The platelet GpIIb/IIIa receptor (also called integrin α_{2}β_{1}) mediates recruitment of platelets to the thrombus, as well as platelet–platelet interactions. Activation of GpIIb/IIIa, similar to tissue factor, requires protein disulfide isomerise (Furie & Furie, 2008). Activation of platelets bound to the wall of the injured vessel causes a conformational transition in GpIIb/IIIa that increases it’s affinity for fibrinogen and von Willebrand factor. At low shear rates, fibrinogen is the predominant GpIIb/IIIa ligand, whereas von Willebrand factor is the predominant ligand at higher shear rates (Furie & Furie, 2008). However, neither von Willebrand factor nor fibrinogen are absolutely required for platelet adhesion or thrombus formation (Ni et al, 2000). Platelets of mice deficient in von Willebrand factor and fibrinogen have been shown to accumulate fibronectin in their α-granules, suggesting that fibronectin could be the ligand supporting platelet aggregation in these mice (Ni et al, 2000). During platelet activation, degranulation and release of the contents of platelet alpha and dense granules occurs, thus further promoting thrombus formation (see Chapter 2.2 - Platelet Structure and Signalling). Late signalling events also enhance platelet–platelet aggregation. Growth-arrest-specific gene 6, CD40 ligand, ephrin-Eph, and signalling lymphocyte activation molecule (SLAM) enhance platelet–platelet affinity by participating in the platelet-platelet synapse to create a protected environment within the clot that stabilizes the platelet thrombus (Furie & Furie, 2008).
Delivery of tissue factor to the interior of the platelet thrombus is thought to be achieved via circulating platelet microparticles which express tissue factor on their surface. Activated platelets, express CD62P following platelet activation which binds to P-selectin glycoprotein ligand 1 (PSGL-1) on the surface of tissue factor-expressing microparticles. Recruitment of microparticles to the platelet thrombus increases the concentration of locally available membrane-bound tissue factor, which is then activated or “decrypted” by protein disulfide isomerase (Stoll et al, 2008).

1.4. Summary
Ischaemic stroke is a heterogeneous disease involving thrombosis or thromboembolism in the majority of cases. A better understanding of the haemostatic/thrombotic mechanisms involved in TIA or ischaemic stroke, and how these processes are affected by currently prescribed antiplatelet treatment regimens should lead to improvements in secondary preventative therapy in individual CVD patients.
2. Platelets

2.1. Historical Background

Alfred F. Donné was a French Histologist who is usually credited as being the first person to recognise platelets as distinct particles in the blood in 1842 (Owen, 2001). He called them the ‘globulins of chyle’. However, others have questioned whether Donné actually saw platelets, or simply identified ‘fat globules’ in the blood. In 1865, Max Schultze described grey, colourless, spherical bodies, one sixth to one eight the size of erythrocytes, that were occasionally clumped together in masses of up to 100 individual components (Owen, 2001). The clumped cells tended to have crenated margins and distinct internal granules, and protoplasm streamed out of these small cells during coagulation. However, Schultze proposed that these cells arose from the disintegration of leucocytes (Owen, 2001). In 1875 Zhan observed cells deposited on the inner wall of a vessel injured by pressure or salt. However he mistook the cells for leukocytes, since the platelets in frog’s blood have nuclei.

In 1881, Professor Bizzozero of Turin repeated Zahn’s experiment, this time using guinea-pigs or young rabbits, whose platelets are non-nucleated. He proposed the term ‘Blut Plättchen’ (blood platelet) to describe the third corpuscle in blood. He showed that the first step in thrombus formation in vivo was the adhesion of blood platelets to a damaged area of vessel wall, and described the formation of platelet ‘plaques’ (aggregates) and the subsequent plugging of small vascular punctures by the ‘white platelet thrombus’ (Owen, 2001; Meade T, 2005).

Although the original descriptions of the gross morphological appearances of platelets and the changes they undergo during the process of activation were remarkably
accurate, our understanding of the molecular mechanisms involved in platelet activation has advanced considerably.
2.2. Platelet Structure and Signalling

2.2.1 Platelet Structure

Resting anucleate platelets circulate as discoid cells that are 2 to 4 μm in diameter (Leeson et al, 1985) and 1 μm thick (Blockmans et al, 1995). Electron microscopy has revealed that the platelet ultrastructure can be divided into four zones: (a) the peripheral zone, (b) the sol-gel zone, (c) the organelle zone, and (d) the membrane zone (Figure 2-1) (Shapiro, 1999).

(a) The peripheral zone: This zone contains the exterior coat or glycocalyx of the platelet that is composed of membrane glycoproteins, glycolipids, mucopolysaccarides, and adsorbed plasma proteins (Ware & Coller, 1995). Sialic acid residues in these glycoproteins and glycolipids confer a net negative surface charge on the glycocalyx that is believed to minimise the attachment of circulating platelets to one another. Beneath the glycocalyx lies the outer platelet membrane that is composed of a phospholipid bilayer (Blockmans et al, 1995), and about half of the platelet phospholipid content is contained within the plasma membrane. The negatively charged phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PTE) residues are almost exclusively confined to the inner cytoplasmic layer, where they may serve as substrates for phospholipases involved in prostaglandin (PG) synthesis (Blockmans et al, 1995; Shapiro, 1999). These negatively charged phospholipids (especially PS) are also able to accelerate several steps in the coagulation cascade, especially the conversion of factor X to Xa, and of prothrombin to thrombin, if they are exposed during the process of platelet activation (Colman et al, 1994; Monroe et al, 2002). The outer layer of the membrane bilayer is rich in sphingomyel in and phosphatidylcholine (Sims & Wiedmer, 2001), and the remaining phospholipids are
more evenly distributed throughout the membrane and associated platelet structures. This zone also includes the sodium and calcium ATPase pumps that control the platelet ionic environment, and various transmembrane glycoprotein receptors. A submembrane skeleton of actin filaments exists that is crosslinked by actin-binding proteins, spectrin, and dystrophin-related protein (Fox, 2001). This submembrane skeleton, that is linked to the plasma membrane mainly through glycoprotein (Gp) Ib and Gpla/Ila, stabilises the phospholipid bilayer, regulates the shape of the plasma membrane (Blockmans et al, 1995), lines the channels of the surface-connected open canicular system (OCS) (Shapiro, 1999) and participates in the transport of alpha and dense granules during platelet activation assisted by α-dystrobrevins (Cerecedo et al, 2010). The OCS is an elaborate series of conduits that begin as indentations of the plasma membrane, course throughout the interior of the platelet and greatly increase its surface area (Blockmans et al, 1995). The OCS facilitates the entry of plasma substances to the interior of the platelet, and the release of products from the interior of the cell (Shapiro, 1999). It also serves as an extensive internal store of membrane that may be used in the process of platelet spreading and pseudopod formation after platelet adhesion.

(b) The sol-gel zone: This zone consists of a number of different components, lies beneath the peripheral zone, and forms the cytoskeletal framework of the platelet that comprises between 30 to 50% of the total platelet protein content (Shapiro, 1999). A circumferential band of microtubules, composed of tubulin, lies beneath the submembrane skeleton of actin filaments, and is involved in maintaining the discoid shape of the unstimulated platelet (Blockmans et al, 1995).
In addition to the integrated membrane actin filaments mentioned above, cytoplasmic actin filaments are also present within the platelet and they comprise the bulk of the platelet cytoskeleton (Blockmans et al, 1995). The organisation of these actin filaments is maintained by their association with other proteins, such as actin-binding protein, tropomyosin and α-actinin (Blockmans et al, 1995). During the process of platelet activation, actin may bind to myosin (Blockmans et al, 1995), and this contractile system mediates platelet shape change, pseudopod formation, internal contraction and release of granules contents (Shapiro, 1999).

(c) The organelle zone: This zone contains a small number of mitochondria, and a pool of glycogen granules that play an essential role in platelet metabolism (Shapiro, 1999). In addition, young platelets that have been recently released into the circulation contain a residual amount of megakaryocyte-derived mRNA and can be identified using supravital dyes, such as thiazole orange (Ingram & Coopersmith, 1969; Ault & Knowles, 1995) or more recently using a combination of polymethine and oxazine (Briggs et al, 2007). These reticulated platelets have been shown to be unstable and to undergo degradation within 24 hours in the circulation in animal studies (Ault & Knowles, 1995).

There are also four distinct populations of granules within the organelle zone: α-granules, dense granules, lysosomes, and peroxisomes (Blockmans et al, 1995). α-granules are the most abundant granules (Blair & Flaumenhaft, 2009). They contain membrane bound proteins, although most of these are already present on the resting platelet membrane, including 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). Not all membrane-associated α-granule proteins, however, are present on the unstimulated platelet plasma membrane (e.g., the integral membrane proteins fibrocystin L, CD109, CD62P) (Blair & Flaumenhaft, 2009), but are expressed on platelet activation. α-granule VWF:Ag can contribute to, but is not necessary for, normal haemostasis and thrombus formation (Blair & Flaumenhaft, 2009). Platelet α-granules also contain a number of coagulation factors and co-factors which participate in haemostasis. Factors V, XI, and XIII each localize in α-granules and are secreted upon platelet activation. Factor V and XI are involved in activation of factor X, a reaction which is catalysed on the platelet surface. α-granules also store inhibitory proteases, such 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. α-granules store antithrombin, which cleaves activated clotting factors in both the intrinsic and extrinsic pathways, and C1-inhibitor, which degrades plasma kallikrein, factor Xla, and factor Xlla. Platelets secrete tissue factor pathway inhibitor (TFPI), protein S, and protease nexin-2 (amyloid β-A4 protein), which inhibits factors Xla and IXa. Although the definition of an α-granule is morphological, there are many variations in morphology that can be considered to represent an α-granule (Blair & Flaumenhaft, 2009). It is possible that this heterogeneity in α-granule morphology means that there are different subtypes of α-granule, as yet not described, each of which may perform a predominant function or set of functions, depending on their structure or constituents.
Many of the proteins found in α-granules are present in plasma. This raises the question as to whether the α-granule counterparts of plasma proteins differ in structure or function. Plasma proteins, such as fibrinogen and immunoglobulin, are taken up into α-granules by either endocytosis or pinocytosis, and this is thought to continue throughout the life of the platelet (Blair & Flaumenhaft, 2009). Also, while many important bioactive proteins are present, concentrated, and even modified in platelet α-granules, establishing the physiologic importance of a particular α-granular protein is challenging. Nonetheless, there is evidence that secreted α-granule proteins function in coagulation, inflammation, atherosclerosis, antimicrobial host defense, angiogenesis, wound repair, and malignancy (Blair & Flaumenhaft, 2009).

**Dense granules** contain CD62P, CD63 (Israels *et al*, 1992), ATP, ADP, serotonin and calcium, although a cytoplasmic pool of adenine nucleotides also exists (Shapiro, 1999).

**Lysosomes** are small vesicles that contain β-glucuronidase, cathepsins, collagenase and elastase (Ware & Coller, 1995). They also contain lysosomal-associated membrane proteins 1 and 2 (LAMP 1 and LAMP 2) and CD63 (Grau *et al*, 1998) that are expressed on the plasma membrane after platelet activation. Although CD63 may serve to protect the plasma membrane from degradation by lysosomal proteins, its exact biological function is not sufficiently understood (Grau *et al*, 1998).

**Peroxisomes** are very small organelles, and relatively few in number, that are thought to contribute to lipid metabolism in platelets (Ware & Coller, 1995).
(d) The membrane zone: This includes the OCS and the dense tubular system (Shapiro, 1999). The dense tubular system is a closed system of membrane bound tubules that is equivalent to the smooth endoplasmic reticulum in other cells. It is the site where calcium and protein disulfide isomerase is sequestered, and where the enzymes involved in prostaglandin synthesis are localised (Blockmans et al, 1995; Shapiro, 1999; van Nispen tot Pannerden et al, 2009). Platelets may secrete thromboxane A$_2$ and platelet activating factor (in response to stronger stimuli) from the dense tubular system during the activation process.
Figure 2-1 Diagrammatic Representation of the Ultrastructure of a Platelet in Cross Section
(adapted from Figure 1 in Monograph by Shapiro, 1999)
2.3. **Signalling mechanisms and second messengers in platelets**

Platelets can be activated or inhibited by a variety of physiological and pharmacological agents that exert their effects through their interaction with specific receptors on the platelet surface (Blockmans *et al*, 1995). Some of these are glycoprotein receptors e.g. the GpIIb/IIIa receptor, whereas others are not e.g. the ADP receptors (Fitzgerald, 2001). Binding of agonists to these receptors may generate a molecular signal within the platelet in a process referred to as ‘outside-in’ signalling (Du & Ginsberg, 1997). In addition, molecular signals within the platelet may also affect the activation status and binding affinity of the surface receptor – a process referred to as ‘inside-out’ signalling (Blockmans *et al*, 1995). Before dealing with these platelet receptors, the signalling mechanisms involved in receptor dependent platelet activation and inhibition will be described.

### 2.3.1. Guanine nucleotide binding regulatory (G) Proteins

Many platelet receptors are coupled to the second messenger generating enzymes, phospholipase C, phospholipase A$_2$, and adenylate cyclase via G proteins (Blockmans *et al*, 1995). G proteins are composed of 3 subunits: $\alpha$, $\beta$, and $\gamma$. The $\alpha$ subunit is mainly responsible for mediating the interaction between receptors and effectors within the platelet, whereas the other 2 subunits anchor the G protein to the cell membrane, and mediate the activation of ion channels, inhibition of adenylate cyclase, and activation of phospholipase A$_2$ (Blockmans *et al*, 1995).
2.3.2. Second messenger generating enzymes and second messengers in platelets

The main second messengers involved in platelet activation/inhibition are calcium, inositol 1,4,5-triphosphate (IP$_3$), diacylglycerol (DAG), TxA$_2$, PGs, cAMP and cGMP. The main enzymes involved in generating these second messengers will now be described.

Phospholipase C catalyses the breakdown of phosphatidylinositol 4,5-biphosphate (PIP$_2$) into IP$_3$ and DAG, and this enzyme is predominantly stimulated by agonists linked to G proteins (Blockmans et al, 1995). IP$_3$ binds to specific receptors and induces the release of calcium from the dense tubular system, and promotes the influx of external calcium into the platelet (Blockmans et al, 1995). ADP may induce calcium influx via receptor mechanisms that are independent of IP$_3$. Of note, all known platelet agonists increase intraplatelet calcium concentrations, and this will ultimately lead to phosphorylation of myosin and platelet shape change.

Calcium mobilisation leads to activation of phospholipase A$_2$, although this enzyme may also be activated directly by G protein mediated mechanisms (Blockmans et al, 1995). Phospholipase A$_2$ liberates arachidonic acid from the dense tubular system and plasma membrane, with the subsequent formation of PGG$_2$, PGH$_2$, and the potent platelet agonist TxA$_2$. PGG$_2$, PGH$_2$, and TxA$_2$ can all induce platelet aggregation, but PGH$_2$, and TxA$_2$ are the main analogues involved in mediating platelet aggregation and secretion. PGH$_2$ can be converted to the inhibitory PGD$_2$, to the inactive or minimally inhibitory PGF$_2\alpha$, and to inhibitory or stimulatory PGE$_2$. 
These prostaglandins may also leave the platelet, and enter endothelial cells where they are converted into the platelet inhibitor and vasodilator, PGI$_2$ (Blockmans et al, 1995).

DAG can induce platelet aggregation and secretion of serotonin. DAG promotes the translocation of protein kinase C from the platelet cytoplasm to the membrane, and activates this enzyme. Protein kinase C in turn phosphorylates certain proteins, including myosin, in the presence of phosphatidylserine (PS) and calcium, but induces little platelet shape change.

Adenylate cyclase uses ATP to form cAMP, and increased cAMP levels inhibit platelet activation by leading to decreased calcium mobilisation, sequestration of calcium in the dense tubular system, and inhibition or reversal of platelet aggregation (Blockmans et al, 1995). In addition, cAMP may inhibit phospholipase C, thus preventing the breakdown of PIP$_2$ into the second messengers IP$_3$ and DAG (Blockmans et al, 1995). Adenylate cyclase is activated by binding of adenosine, PGI$_2$, PGE$_1$, and PGD$_2$, to their respective $G_s$ regulatory proteins. In contrast, thromboxane, ADP, epinephrine, and thrombin stimulate $G_i$ regulatory proteins, with inhibition of adenylate cyclase. This leads to a resultant decrease in cAMP levels in platelets where cAMP was initially elevated (Blockmans et al, 1995).

Soluble guanylate cyclase catalyses the conversion of guanosine triphosphate (GTP) to guanosine 3', 5'-cyclic monophosphate (cGMP). cGMP is another inhibitory second messenger in platelets, and cGMP synthesis is stimulated by nitric oxide and other vasodilators (Blockmans et al, 1995). In addition, cGMP levels rise in platelets in response to stimulation by aggregating agents, thus providing a negative feedback loop
during platelet aggregation (Canobbio et al, 2004). Both cAMP and cGMP are degraded by phosphodiesterases.

2.3.3. Platelet membrane glycoprotein receptors

Several platelet surface glycoprotein receptors traverse the outer platelet membrane, so that their interior tails are located within the cytoplasm of the platelet. The main glycoprotein receptors that were studied either directly or indirectly in this thesis, and that will be discussed in greater detail in the following section, are the Gp Ib-IX-V complex, the GpIIb/IIIa receptor, and the Gpla/Ila and GpVI receptors.

The GpIb-IX-V complex is a unique plasma membrane glycoprotein complex that consists of four types of transmembrane subunits: GpIbα, GpIbβ, GpIX, and GpV (Andrews et al, 1997). Each receptor contains two GpIbα, two GpIbβ and two GpIX subunits, but only one GpV subunit (Escolar & White, 2000). With the exception of patients with Bernard-Soulier syndrome, in whom this receptor may be deficient, there are approximately 25,000 copies of GpIb-IX (Andrews et al, 1997) per platelet, and approximately half as many copies of GpV (Canobbio et al, 2004). The GpIbα (CD42b) subunit of the GpIb-IX-V complex is the predominant receptor for VWF on platelets, and this receptor mediates VWF-dependent platelet adhesion to the subendothelium (Gardiner et al, 2010) and platelet activation (Berndt et al, 2001) at high shear rates. GpIbα also possesses binding sites for thrombin, Mac-1, CD62P, high molecular weight kininogen and Factor XII (Canobbio et al, 2004). The cytoplasmic tail of GpIbα contains a binding site for actin-binding proteins (Andrews et al, 1997; Berndt et al, 2001), and the GpIbβ cytoplasmic tail contains a protein kinase A phosphorylation site that inhibits platelet actin polymerisation in response to agonist stimulation (Andrews et
al, 1997). In addition to these functions of the receptor subunits, GpIbα, GpIbβ and GpIX are all required for stable surface expression of the receptor complex (Berndt et al, 2001). Until relatively recently, the exact physiological function of GpV was not known, but it appears that it is has a regulatory and inhibitory role in thrombin-dependent platelet activation when the agonist binds to this receptor (Berndt et al, 2001; Clemetson & Clemetson, 2001). Thrombin firstly cleaves GpV near the platelet membrane surface, releasing an extracellular soluble fragment, thus facilitating thrombin binding to GpIbα with the subsequent initiation of intraplatelet signalling responses and platelet activation (Berndt et al, 2001). Overall, the signalling events induced by VWF (and perhaps other agonists) binding to GpIb-IX-V include opening of the transmembrane calcium channels, elevation of intraplatelet calcium levels, and activation of protein kinase C and tyrosine kinase(s) (Berndt et al, 2001; Canobbio et al, 2004). These signalling events ultimately result in 'inside-out' activation of the GpIIb/IIIa receptor and platelet aggregation (Cosemans et al, 2008).

GpIIb/IIIa (αIIbβ3 integrin) is the predominant platelet receptor, with up to 80,000 copies on each platelet surface (Cosemans et al, 2008). Some GpIIb/IIIa receptors are also located in the OCS and in platelet α-granules (Blockmans et al, 1995), but can be translocated to the platelet membrane surface upon platelet activation (Du & Ginsberg, 1997). The GpIIb subunit consists of a heavy and a light chain linked by a disulphide bond, and this subunit is non-covalently bound to the GpIIIa subunit to form a heterodimer (Calvete, 1995). The association between the GpIIb and GpIIIa subunits depends on a submicromolar concentration of extracellular calcium (Abrams & Shattil, 1991; Moran N & FitzGerald GA, 1994; Du & Ginsberg, 1997). All of the ligands that bind to GpIIb/IIIa contain an arginine-glycine-aspartic acid (RGD) in their primary
amino acid sequence, and the ligand binding pocket within the receptor is believed to contain a recognition site for RGD (Du & Ginsberg, 1997). Thus, the receptor complex can bind several different ligands including fibrinogen, VWF, fibronectin, vitronectin and thrombospondin (Moran N & FitzGerald GA, 1994). The surface binding ligand varies depending on the stimulus to activation e.g. fibrinogen is the predominant ligand in the presence of thrombin, ADP or collagen, but VWF is the predominant ligand when aggregation is induced by high shear stress (Litjens et al, 2000). In the resting platelet, GpIIb/IIIa is unable to bind fibrinogen or VWF in solution, but can adhere to these ligands if they are coated on a surface (Bennett et al, 2009). However, platelet activation leads to a conformational change in this receptor that facilitates binding of soluble ligands, and this change is the final common pathway leading to platelet aggregation (Shattil & Leavitt, 2001). When GpIIb/IIIa is activated and ligand binding occurs, this may trigger ‘outside-in’ signalling and induce profound changes in platelet shape and adhesiveness by interaction with the platelet cytoskeleton (Shattil & Leavitt, 2001). These outside-in signals may also potentiate agonist-induced platelet secretion and procoagulant activity (Shattil, 2009). However, the second messenger systems mediating the effects of ligand binding to GpIIb/IIIa are not fully understood (Shattil & Leavitt, 2001). In addition, a variety of excitatory and inhibitory platelet receptors may exert their effect on platelets by ‘inside-out’ signalling that regulates the affinity and avidity of GpIIb/IIIa for ligands (Clemetson & Clemetson, 2001). The involvement of the GpIIb/IIIa receptor in the platelet adhesion and aggregation process is described in more detail below.
GpIa/IIa ($\alpha_2\beta_1$ integrin) and GpVI are the two main direct receptors for collagen on platelets (Cosemans et al, 2008). There are between 2000 and 4000 copies of GpIa/IIa (Chen et al, 2002), and approximately 1250 GpVI receptors per platelet (Chen et al, 2002). Under conditions of low shear stress, GpIa/IIa may bind to subendothelial collagen that is exposed during the process of vessel injury. GpVI also appears to be involved in the initial binding of platelets to collagen (Arthur et al, 2010). Recently, downstream signalling has been described via TRAF4 and other redox relevant signalling proteins, and data suggest that GpVI exerts downstream effects on TRAF4/Nox2-dependent redox pathways (Cosemans et al, 2008).

Under conditions of high shear stress, neither GpIa/IIa nor GpVI can mediate platelet adhesion to collagen. During platelet activation, $\alpha_2\beta_1$ converts to an active state (Clemetson & Clemetson, 2001). Subsequently, the GpIb-VWF complex may bind to subendothelial collagen via a receptor for collagen on VWF. In this sense, the GpIb-IX-V complex acts as an indirect platelet receptor for collagen. The role of other potential platelet collagen receptors in the process of platelet adhesion e.g. GpIV (CD36) and CD31 is unclear (Cosemans et al, 2008). Activated $\alpha_2\beta_1$ reinforces the GPVI-induced signaling to phospholipase C$\gamma$2 activation, and Ca$^{2+}$ release and secretion, in addition to integrin activation (Clemetson & Clemetson, 2001). After the initial adhesion process, signalling events may facilitate more firm platelet adhesion, with further collagen binding to GpIa/IIa, activation of GpIIb/IIIa with subsequent fibrinogen binding, and perhaps binding of fibronectin to GpIc/IIa ($\alpha_5\beta_1$), and laminin to GpIc'/IIa ($\alpha_6\beta_1$) (Cosemans et al, 2008).
Procoagulant properties of integrins

Profound platelet stimulation, such as evoked by the Gp-VI interaction with collagen, or by the combination of thrombin and ADP, may cause a marked loss in platelet adhesion, loss of pseudopods and lamellipods, platelet disintegration, membrane bleb formation and microparticle formation (Cosemans et al, 2008). These platelets characteristically show prolonged, high intracellular Ca^{2+} rises, and exposed phosphatidylserine on their surface, which stabilises tenase complexes, and accelerates thrombin formation (Cosemans et al, 2008). Surface expression of GpIIb/IIIa is retained while fibrinogen and PAC-1 binding ability is lost in these platelets suggesting an additional conformational change in this integrin, which allows these non-adherent platelets to become separated from adherent platelets, allowing adequate access to plasma factor X (Blockmans et al, 1995).

2.4. Excitatory ‘non-glycoprotein’ platelet receptors

The main ‘non-glycoprotein’ platelet receptor agonists that were studied directly or indirectly in this thesis were thromboxane A₂, ADP, and epinephrine. The responses to platelet stimulation with thrombin, platelet activating factor, serotonin, or vasopressin were not studied, and these agonists and receptors will receive limited attention in the following section.
2.4.1. Thromboxane A$_2$ receptor(s)

Thromboxane A$_2$ (TxA$_2$) is a potent platelet agonist and one of the most important mediators involved in amplifying the response to other platelet agonists in humans. Phospholipases may release arachidonic acid from membrane phospholipids in response to platelet stimulation by thrombin, collagen, or the platelet aggregation process itself. Arachidonic acid is metabolised to form thromboxane A$_2$, which has a short half-life of less than 1 minute before being rapidly metabolised to form inactive thromboxane B$_2$. TxA$_2$ induces platelet shape change, aggregation, and secretion of granule contents via G protein-coupled platelet membrane receptors that are linked to phospholipase C breakdown of PIP$_2$, and elevation of intraplatelet free calcium (Shapiro, 1999; Gachet, 2001).

2.4.2. ADP Receptors

ADP is present at near molar concentrations in platelet dense granules (Gachet, 2001). Although ADP itself is considered to be a weak aggregating agent, it is released when platelets are stimulated by other agents, such as thrombin or collagen, and it reinforces their stimulatory effects (Gachet, 2001). It also amplifies platelet activation induced by other weak agonists, like epinephrine or serotonin (Gachet, 2001).

Adenine nucleotides (ADP and ATP) interact with P2 receptors that are widely distributed in many different cell types including endothelial, smooth muscle, epithelial, haematopoietic cells and neurons (Jin et al, 1998; Hollopeter et al, 2001; Gachet, 2001). The P2 receptors are divided into two main groups: (i) the G protein-coupled P2Y ADP receptors, and (ii) the ligand-gated, ion channel P2X ATP receptors which also bind ADP.
The P2Y ADP receptors are termed P2Y₁ (Gq-coupled) and P2Y₁₂ (Gi-coupled). They mediate different aspects of the response to this agonist (Jennings, 2009). ADP must bind to both P2Y₁ and P2Y₁₂ receptors for normal ADP-induced platelet aggregation, but each receptor mediates its effects via different second messenger systems (Jin et al., 1998). When ADP binds to P2Y₁, calcium is mobilised from intraplatelet stores, thus triggering platelet shape change and pseudopod formation (Gachet, 2001), and the GpIIb/IIIa receptor is activated with resultant transient platelet aggregation (Gachet, 2001). In contrast, ADP binding to P2Y₁₂ leads to inhibition of adenylate cyclase with a decrease in intraplatelet cyclic AMP (cAMP) levels, potentiation of the secretion of granule contents, and stabilisation of platelet aggregates through full activation of GpIIb/IIIa (Kahner et al., 2006). Clopidogrel exerts its antiplatelet effects by selective and irreversible inhibition of the P2Y₁₂ ADP receptor.

The P2X₁ receptor is activated by binding of ATP. The effect of ATP is antagonised by binding of ADP (Kahner et al., 2006). ATP-induced activation of the P2X₁ receptor results in influx of both divalent calcium and monovalent sodium at a ratio of 4:1 (Kahner et al., 2006). Activation of the receptor alone causes shape change, transient centralisation of granules, and formation of small microaggregates which may be insufficient alone to cause platelet activation, but important in amplifying the response to other agonists. P2X₁ receptor activation has been shown to be essential for enhanced platelet adhesion and thrombus formation under high shear rates (Blockmans et al., 1995).
2.4.3. Epinephrine (Adrenaline) receptors

The term epinephrine will be used throughout this thesis to describe adrenaline, because this is the nomenclature used in the PFA-100® system. Platelets have stimulatory α2 adrenoceptors and inhibitory β2 adrenoceptors, and in most patients, the α2 adrenoceptors predominate (Blockmans et al, 1995). There are approximately 200 to 300 copies of the α2 adrenoceptor per platelet, and binding to this receptor may inhibit adenylate cyclase with a decrease in intraplatelet cAMP, and activate protein kinase C via G protein-mediated mechanisms (Helgason et al, 1993). It is also likely that epinephrine induces platelet aggregation using TxA2 as a second messenger, because epinephrine-induced platelet aggregation is primarily dependent on intact cyclooxygenase function and is inhibited to a significant degree by aspirin (Blockmans et al, 1995). Epinephrine may also sensitise platelets to the effects of other agonists (Blockmans et al, 1995). However, because epinephrine only induces platelet aggregation in the presence of subphysiological calcium concentrations, and at epinephrine concentrations that are much greater than those found in the circulation, the importance of epinephrine-induced platelet aggregation in vivo is unclear (Rivera et al, 2009).

2.4.4. Thrombin receptors

Thrombin is the most effective platelet agonist ex vivo, inducing shape change, secretion of the contents of platelet granules, synthesis and release of TxA2, mobilisation of CD62P to the platelet surface, and activation of GpIIb/IIIa (Rivera et al, 2009). It also induces the expression of procoagulant factors on platelets, which stimulates further thrombin generation, although this does not appear necessary for the development of a mature thrombus (Coughlin, 2005; Furie & Furie, 2008).
Thrombin-induced platelet activation is mediated at least partially by the GpIb-IX-V complex, and mainly by protease activated receptor 1 (PAR1), but also by PAR4 (Coughlin, 2005). Protease activated receptors are activated by an intriguing mechanism. When thrombin binds to PAR1 on platelets, it cleaves part of the receptor and un masks a new amino terminal domain, which in turn binds to the body of the receptor itself and activates it (Coughlin, 2005). It has been suggested that PAR1 and PAR4 mediate thrombin-induced platelet activation at low and high agonist concentrations, respectively, but that both receptors influence intraplatelet calcium signalling (Rivera et al, 2009). Blockade of the thrombin-GpIba interaction with antibodies impairs the cleavage of PAR1 (Rivera et al, 2009).

GpIba may serve as a co-factor that localises thrombin to the platelet surface, allowing its proteolytic action over PAR1 and PAR4. Despite this, PAR cleavage and auto-binding alone is sufficient to fully activate platelets (Mazzucato et al, 1998). The GPlba subunit of the GpIb-IX-V complex contains a moderate affinity, high capacity binding site for α-thrombin (Rivera et al, 2009) and patients with Bernard Soulier Syndrome exhibit reduced platelet responsiveness to thrombin (Blockmans et al, 1995).

After thrombin-induced platelet activation, desensitisation to thrombin occurs secondary to internalisation of PAR receptors in endosomes, and the majority are then transferred to and undergo degradation by lysosomes (Blockmans et al, 1995).
2.4.5. **Platelet activating factor (PAF) receptor**

PAF is formed in certain cells, including platelets, by the consecutive actions of phospholipase A$_2$ and acyltransferase on platelet membrane phospholipids (Blockmans *et al*, 1995). PAF causes platelet shape change and aggregation, but because it is dependent on TxA$_2$ to mediate the secretion of granule contents, it is unlikely to play a major role in amplifying the response to other platelet agonists in humans. PAF is internalised during platelet activation, and this may be related to reorganisation of the plasma membrane (Blockmans *et al*, 1995).

2.4.6. **Serotonin (5-HT) receptor**

Serotonin may be taken up from the circulating blood by active and passive diffusion, and is stored in platelet dense granules. The platelet 5-HT$_2$ receptor is a G protein-coupled receptor, and binding of 5-HT$_2$ to its receptor leads to platelet activation involving breakdown of phosphatidylinositol, increase in intraplatelet calcium levels, and protein phosphorylation (Blockmans *et al*, 1995).

2.4.7. **Vasopressin receptor**

Platelets possess vasopressin (V1) receptors that mediate platelet aggregation via phosphatidylinositol metabolism and an increase in the intraplatelet calcium concentration (Blockmans *et al*, 1995).
2.5. Inhibitory ‘non-glycoprotein’ platelet receptors

2.5.1. Adenosine receptor

Adenosine inhibits platelet shape change, aggregation and secretion by its action on the G protein-linked adenosine A2 receptor (Blockmans et al, 1995). Receptor activation stimulates adenylate cyclase activity, with a subsequent increase in intraplatelet cAMP levels. Adenosine is ultimately taken up by platelets, phosphorylated by an adenosine kinase to form adenosine monophosphate (AMP), and further phosphorylated to form ADP and ATP that is released into the cytoplasmic pool in platelets (Blockmans et al, 1995).

2.5.2. PGI$_2$ and PGE$_1$ receptor

Binding of PGI$_2$ and perhaps PGE$_1$ to this receptor also activates adenylate cyclase via a G protein-coupled mechanism, with an increase in cAMP production and inhibition of platelet function (Blockmans et al, 1995).

2.5.3. PGD$_2$ receptor

This receptor is also coupled via G proteins to adenylate cyclase, and binding of PGD2 increases cAMP levels (Blockmans et al, 1995).

2.5.4. PGE$_2$ receptor

It has been proposed that PGE$_2$ may inhibit platelet activation either by binding to its own receptor or the PGI$_2$ receptor (Blockmans et al, 1995). However, depending on the experimental conditions, PGE$_2$ may also potentiate platelet activation by activating protein kinase C or inhibiting cAMP formation.
3. Overview of Platelet Function Testing in Whole Blood

3.1. Overview
Assessment of inhibition of platelet function and reactivity has the potential be extremely important in predicting the response to antiplatelet therapy in patients with ischaemic CVD. Laboratory tests that are performed in whole blood have the potential advantage of allowing one to assess platelet function \textit{ex vivo} in the physiological milieu of whole blood, compared with tests which require preparation of PRP with the attendant higher risk of artefactual \textit{in vitro} platelet activation, and potential loss of important platelet subpopulations during sample preparation and analysis (Michelson, 1996).

At present, there is still no widely accepted, and clinically-validated, laboratory test that has been shown to predict the response to antiplatelet agents in patients with ischaemic CVD, and there are relatively fewer studies in this patient population (Grau \textit{et al}, 2003; Alberts \textit{et al}, 2004; Serebruany \textit{et al}, 2004a; Serebruany \textit{et al}, 2004b; Serebruany \textit{et al}, 2005; McCabe \textit{et al}, 2005a; Serebruany \textit{et al}, 2007; Serebruany \textit{et al}, 2008b) compared with the larger body of data in patients with ischaemic heart disease (Krasopoulos \textit{et al}, 2008; Glauser \textit{et al}, 2010; Fuchs \textit{et al}, 2010; Jakubowski \textit{et al}, 2010).

Some of the commercially available tests use stimuli that are specific to the antiplatelet drug of interest (VerifyNow® (Harrison \textit{et al}, 2008), PFA-100® (Kundu \textit{et al}, 1994; Kundu \textit{et al}, 1995), Plateletworks (Mark \textit{et al}, 2004; Joseph E. Mobley \textit{et al}, 2004)), whereas others use more non-specific stimuli to activate the haemostatic system (Thrombelastography Platelet Mapping (TEG®) (Craft \textit{et al}, 2004), Cone and Plate(let) analyzer (IMPACT-R®) (Panzer \textit{et al}, 2007)). Table 3-1 summarises the currently available commercial platelet function tests. Some platelet function testing paradigms
focus solely on platelet aggregation (VerifyNow®, Plateletworks®), whereas others assess platelet adhesion and aggregation (PFA-100®). Furthermore, an optimal, clinically-informative, predictive ‘cut-point’ is yet to be established on these platelet function tests in patients with ischaemic CVD. Even within test systems, the cut point at which patients are defined as being antiplatelet non-responders or responders is often arbitrarily based on comparisons with a group of health laboratory control subjects. Such a ‘cross sectional’ definition of non-responsiveness may be very informative, but does not take into account that some patients may have significant inhibition of platelet function compared with their own baseline platelet function before the antiplatelet agent in question was commenced. ‘Longitudinal studies’ that assessed platelet function in ischaemic CVD patients before and after changing antiplatelet therapy were very few prior to commencement of this thesis (Grau et al, 2003; Raman & Jilma, 2004; Serebruany et al, 2004a), and did not always assess patients on commonly prescribed antiplatelet regimens.
<table>
<thead>
<tr>
<th>Test</th>
<th>Stimulus</th>
<th>Shear Stress</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>VerifyNow® (Harrison et al, 2008)</td>
<td>Arachidonic acid OR ADP, iso-TRAP* and PAR-4 activating peptide OR iso-TRAP alone</td>
<td>Low</td>
<td>Aggregation of Fibrinogen-coated microparticles</td>
</tr>
<tr>
<td>PFA-100® (Kundu et al, 1994; Kundu et al, 1995)</td>
<td>Collagen +ADP or Collagen +Epinephrine</td>
<td>Moderate-High</td>
<td>Aperture occlusion</td>
</tr>
<tr>
<td>IMPACT-R® (Panzer et al, 2007)</td>
<td>Polystyrene ± ADP</td>
<td>Low</td>
<td>Platelet adhesion and aggregation to polystyrene surface</td>
</tr>
<tr>
<td>Plateletworks® (Mark et al, 2004; Joseph E. Mobley et al, 2004)</td>
<td>ADP or collagen or arachidonic acid</td>
<td>Low</td>
<td>Change in platelet count</td>
</tr>
<tr>
<td>Thrombelastography Platelet Mapping (Craft et al, 2004)</td>
<td>Kaolin ADP</td>
<td>Low</td>
<td>Strength of fibrin-platelet bonding</td>
</tr>
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*Iso-TRAP – iso-thrombin receptor activating peptide
4. Is Platelet Activation Increased Following TIA or Ischaemic Stroke?

4.1. Introduction
Platelets play a pivotal role in arterial thrombus formation (Shapiro, 2000). Although previously thought to be bystanders in the coagulation cascade, there is now evidence that platelets also play a key role in coordinating thrombus formation via interaction with the coagulation cascade, and interaction with leukocytes (Schonbeck & Libby, 2001).

It remains unclear whether arterial thrombus formation in ischaemic stroke is the cause of, or a result of excessive platelet activation. This review aims to summarise the evidence relating to platelet activation in patients with TIA or ischaemic stroke.

4.2. Urinary Markers
Early studies of platelet activation in patients with ischaemic stroke included urinary assays of indirect markers of platelet activation. Five studies have assessed 11-dehydro thromboxane B₂ - a metabolite of thromboxane A₂, as an indirect marker of platelet activation in urine following TIA or ischaemic stroke (Uyama et al, 1992; Koudstaal et al, 1993; van Kooten et al, 1994; van Kooten et al, 1999; McConnell et al, 2001).

Elevated urinary 11-dTB₂ levels were found in patients with ischaemic stroke compared with controls (p = 0.004) (Koudstaal et al, 1993) and in patients between 3 and 9 months following TIA, ischaemic stroke or haemorrhagic stroke compared with controls (p < 0.01) (van Kooten et al, 1999). Elevated 11-dTB₂ was also reported in patients with
previously symptomatic carotid stenosis compared with patients with stroke not associated with carotid stenosis or with healthy controls (p < 0.01) (Uyama et al, 1992). However a more robust study found no difference in urinary 11-dTB$_2$ between CVD patients and age-matched controls, although there was a significant difference between patients with stroke and non-age-matched controls (p < 0.05) (McConnell et al, 2001). No consistent pattern of urinary 11-dTB$_2$ excretion was found over time in patients within the first 48 hours of onset of an acute TIA or ischaemic stroke (van Kooten et al, 1994), but patients on aspirin had significantly lower urinary 11-dTXB2 levels than controls in two studies (van Kooten et al, 1999) (McConnell et al, 2001).

The number of patients in all of these studies was small (12 - 25 patients), and the same control group, which included patients with head injuries, was used for 3 of the studies (Koudstaal et al, 1993; van Kooten et al, 1994; van Kooten et al, 1999).

**Conclusion**

These studies provided some evidence of increased platelet activation, as measured indirectly with urinary 11-dTXB$_2$ levels, in both the acute and late phases after TIA or ischaemic stroke, but the results of different studies were conflicting. These small studies, with heterogeneous control groups, could not definitively conclude whether platelet activation varied over time in the hyperacute phase after symptom onset.
4.3. Plasma Markers

Urinary assays of metabolites of thromboxane A₂ potentially reflected platelet activation over hours to days, and only provided an indirect measure of platelet activation in these subjects. In an attempt to assess platelet activation in a more dynamic and specific manner, soluble markers of platelet activation in plasma were investigated.

4.3.1. Beta-Thromboglobulin and Platelet Factor-4

Platelet aggregation is associated with platelet degranulation and the release of a number of proteins, including beta-thromboglobulin (βTG). βTG is a small protein stored in the α-granules of platelets (Woo et al., 1988), and was one of the earliest markers of platelet degranulation to be studied in ischaemic stroke. Platelet Factor 4 (PF-4) is also released from alpha granules of platelets during platelet activation and increases ADP-dependent platelet aggregation (Tombul et al., 2005). It is believed to be an indirect marker of platelet activation, and is normally not found in plasma. Because of the more rapid clearance of PF-4 than βTG in vivo, the ratio of βTG:PF-4 may be higher in patients with in-vivo platelet activation; on the contrary, in vitro activation releases PF-4 which is not cleared rapidly enough, and the βTG:PF-4 ratio is correspondingly lower. Measurement of βTG and concurrent PF-4 may help distinguish between artefactual in vitro platelet activation, and actual platelet aggregation in vivo (Kaplan & Owen, 1981).
The earliest study in the literature assessing these plasma components was in 1982 (Fisher et al, 1982). Several studies have demonstrated elevated βTG levels in patients at any time following TIA or ischaemic stroke compared with controls (Shah et al, 1985), and in the early (Landi et al, 1987; Woo et al, 1988; Iwamoto et al, 1995; Tombul et al, 2005) and late phases (Iwamoto et al, 1995) after symptom onset. However, a small study which assessed βTG and PF-4 in arterial samples, and excluded patients with a βTG:PF-4 ratio of < 2, did not find any difference in βTG or PF-4 between patients with acute cardioembolic stroke associated with nonvalvular atrial fibrillation and controls (Nagao et al, 1995).

A study of patients who had a history of atrial fibrillation, both with and without prior ischaemic stroke, demonstrated elevated βTG levels in patients with atrial fibrillation compared with controls, regardless of whether these patients had a history of stroke (Gustafsson et al, 1990), suggesting that preceding platelet activation in patients with atrial fibrillation may increase their risk of stroke. Several studies failed to show any correlation between infarct size on CT and βTG levels (Shah et al, 1985; Landi et al, 1987; Woo et al, 1988), suggesting that elevated βTG in patients following ischaemic stroke is not a consequence of the infarct itself.

In contrast to the data on βTG, several studies failed to show evidence of elevated PF-4 levels in patients with TIA or ischaemic stroke (Shah et al, 1985; Nagao et al, 1995; Tombul et al, 2005). However, patients with atrial fibrillation and a history of ischaemic stroke were found to have higher PF-4 levels than healthy controls in sinus rhythm (Gustafsson et al, 1990).
Three studies have assessed the time course of βTG in patients with ischaemic stroke (Landi et al, 1987; Woo et al, 1988; Kurabayashi et al, 2000). Two studies involving 70 patients (Landi et al, 1987) and 264 patients (Woo et al, 1988), with follow-up for 5 days and 6 weeks, respectively, showed no change in βTG levels over time.

A small study investigating 18 patients within 2-12 hours of TIA or ischaemic stroke, and subsequently at 7 days and 6 months after symptom onset indicated that plasma levels of βTG and PF-4 at stroke onset were higher than those both at 7 days and at 6 months (Kurabayashi et al, 2000).

A study of 186 Japanese patients with a history of stroke, transient ischaemic attack orBinswanger's disease investigated the ratio of βTG in the jugular vein to that drawn from the cubital vein (Iwamoto et al, 1995). Of the 75 non-CVD control subjects in this study, at least 25 had atrial fibrillation alone and others had a history of myocardial infarction. Serum βTG concentrations were elevated within 7 days of TIA or lacunar or cardioembolic stroke, as well as in controls with atrial fibrillation, compared with healthy older control subjects. βTG remained elevated at >28 days after TIA or stroke onset. The mean ratio of βTG in the jugular vein to that of the cubital vein was higher than controls during the early phase following lacunar stroke (p < 0.05), and during the late phase following lacunar and atherothrombotic stroke subtypes (p < 0.01). There was no correlation between βTG ratio and the size of the infarct. The βTG ratio was also significantly elevated in patients with extracranial or intracranial arterial stenoses or occlusions found on cerebral arteriography compared with those with normal angiographic findings (p < 0.05).
**Conclusion**

There is some evidence that beta-thromboglobulin and platelet factor-4 levels are elevated following acute TIA or ischaemic stroke, and may remain elevated long after the event, but the data are more convincing for βTG. However, the results of these studies are conflicting, and overall, 4 studies found elevated levels of βTG (Landi *et al.*, 1987; Woo *et al.*, 1988; Iwamoto *et al.*, 1995; Tombul *et al.*, 2005) and 1 study found normal levels compared with controls (Nagao *et al.*, 1995). Although one of the published studies supported the hypothesis that βTG is mainly derived from the cerebral circulation in patients following ischaemic stroke, there was no correlation between infarct size on CT and βTG concentration. It is possible that elevated plasma markers of platelet activation precede the ictus in some stroke subtypes, based on the data from non-CVD patients with atrial fibrillation, but larger studies are required to confirm these hypotheses and to assess platelet activation status in different stroke subtypes.
4.3.2. Soluble P-selectin (CD62P)

CD62P or P-Selectin expressed on the platelet surface membrane mediates the adhesion of platelets to leukocytes, including neutrophils, monocytes and lymphocytes (de Bruijne-Admiraal et al, 1992). The predominant receptor for CD62P on leukocytes is P-selectin glycoprotein ligand-1 (PSGL-1) (Furie et al, 2001), and the percentage of leukocytes that are bound to platelets can be measured by flow cytometry (Li et al, 1997; Furman et al, 1998; Joseph et al, 2001). Because CD62P is rapidly shed from the surface of circulating degranulated platelets (Michelson, 1996), 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). However, CD62P which is cleaved from the surface of platelets can be measured by ELISA techniques in plasma (sP-selectin), and provides an indirect measure of CD62P expression, and hence, platelet activation.

Several papers have demonstrated elevated soluble p-selectin in patients at any timepoint (Anzej et al, 2007), or in the acute (Frijns et al, 1997; Cherian et al, 2003; Nadar et al, 2004; Wang et al, 2005) and late phase (Frijns et al, 1997) following TIA or ischaemic stroke. However, others have not demonstrated any significant differences between patients and controls (Bath et al, 1998; McCabe et al, 2004a), although one of these studies included patients between 0 and 28 days following ictus (McCabe et al, 2004a). Two studies also demonstrated lower sP-selectin levels in patients compared with controls in the late phase following TIA or ischaemic stroke (Cherian et al, 2003). Furthermore, longitudinal studies have shown that sP-selectin is lower in the late phase after TIA or ischaemic stroke compared with the acute phase.
(Cherian et al, 2003; Nadar et al, 2004), suggesting that resolution of the acute phase response may lead to a decrease in platelet activation over time following the acute event. One of these studies also quantified intra-platelet p-selectin in the acute phase and late phase following ischaemic stroke, in tandem with plasma p-selectin (Nadar et al, 2004). Intra-platelet p-selectin levels positively correlated with soluble p-selectin levels, adding to the evidence that elevated soluble p-selectin in patients with acute TIA and ischaemic stroke is predominantly platelet-derived, rather than being predominantly derived from the endothelium.

**Conclusion**

These studies provide some evidence regarding the pattern of sP-selectin secretion following an ischaemic cerebrovascular event, and there is some evidence that sP-selectin in ischaemic CVD may be predominantly derived from platelets. However, the conflicting findings in the literature indicate that sP-selectin does not appear to be a very sensitive or specific marker of platelet activation in ischaemic CVD.
4.4. Other Markers of Platelet Activation

4.4.1. Soluble CD154 + Membrane bound CD154

CD154 is a trans-membrane protein related to tumour necrosis factor-α (TNF-α) (Peitsch & Jongeneel, 1993). While it was originally thought to be confined to immune cells, it was later identified on stimulated mast cells, basophils, and platelets (Schonbeck et al., 2000). Its ligand is CD40. Experimental models in mice using an anti-CD154 antibody have shown that although the anti-CD154 antibody does not prevent the initiation of atherosclerosis, treatment with an anti-CD154 antibody results in the development of a stable, lipid-poor, collagen-rich plaque phenotype (Henn et al., 1998; Andre et al., 2002). Thus CD154/CD40 interactions, and in particular platelet CD154, have been postulated as being of pivotal importance in the link between inflammation and atherosclerosis (Pignatelli et al., 2004).

Membrane-bound CD154 expressed on activated platelets is quickly cleaved and released into the plasma as soluble CD154. Platelet membrane bound CD154 has been investigated as an immediate marker of platelet activation, and soluble CD154 could represent a more long term stable marker of platelet activation. Elevated plasma soluble CD154 has been shown to predict an increased risk of stroke and myocardial infarction in patients with atrial fibrillation (Ferro et al., 2007).

Few studies have investigated platelet membrane-bound CD154 in patients with TIA or ischaemic stroke. Two studies have shown elevated platelet-bound CD154 in patients in the acute phase following TIA or ischaemic stroke compared with controls (Garlichs et al., 2003) and with patients with asymptomatic carotid stenosis (Cha et al., 2003); this elevation in platelet bound CD154 persists into the subacute (Cha et al., 2003) and late...
phases (Garlichs et al, 2003) following TIA or ischaemic stroke. One of these studies measured concurrent soluble CD154, and also demonstrated elevated plasma levels of soluble CD154 in the acute and late phases following TIA or ischaemic stroke when compared with controls (Garlichs et al, 2003).

**Conclusion**
There is some evidence that both platelet-bound CD154 and soluble plasma CD154 are elevated following acute TIA and ischaemic stroke, and that these findings persists into the late phase. The fact that soluble CD154 levels predict future vascular events in ‘at risk individuals with atrial fibrillation’ indicates that preceding platelet activation may be a contributing factor to these vascular events.

4.5. **Flow Cytometry**
CD62P is expressed on the platelet surface membrane after α- or dense-granule secretion, and the CD63 antigen is expressed on the platelet surface after the release of lysosomes or dense granules (Israels et al, 1992; Grau et al, 1998). By adding ‘activation-dependent’ monoclonal antibodies that are conjugated to a fluorochrome and specific for CD62P or CD63, whole blood flow cytometry can be used to quantify the sample fluorescence and hence the expression of these activation markers on the platelet surface. Because CD62P is ultimately shed into the circulation by proteolysis (Frijns et al, 1997), whole blood flow cytometric analysis of platelets will only identify CD62P expressed on the platelet surface. Platelet surface expression of CD62P and CD63 in PRP and whole blood was quantified by some research groups as potentially more sensitive and specific measures of ongoing platelet activation in ischaemic CVD.
4.5.1. Platelet CD62P Expression

Platelet CD62P expression has been found to be elevated in platelet rich plasma in the acute (Garlichs et al, 2003; Yip et al, 2004) and late phases (Garlichs et al, 2003; Yip et al, 2004) after TIA or ischaemic stroke compared with controls, and in whole blood from patients with TIA or ischaemic stroke compared with controls in the acute (Garlichs et al, 2003; Yip et al, 2004) and late phases (Garlichs et al, 2003; Yip et al, 2004) after TIA or ischaemic stroke compared with controls in the acute (Grau et al, 1998; Meiklejohn et al, 2001; Markel et al, 2002; Marquardt et al, 2002; Cha et al, 2003; Karakantza et al, 2003; McCabe et al, 2004a), subacute (Cha et al, 2003; Cha et al, 2004) and late phases (Grau et al, 1998; Yamazaki et al, 2001; Meiklejohn et al, 2001; Cha et al, 2004; McCabe et al, 2004a) after symptom onset.

Earlier longitudinal studies demonstrated no differences in platelet CD62P expression in whole blood between the subacute (Grau et al, 1998; Cha et al, 2003; Cha et al, 2004) or late phases compared with the acute phase after TIA (Cha et al, 2004), or both TIA and ischaemic stroke (Meiklejohn et al, 2001; McCabe et al, 2005b). However, one robustly designed study using whole blood, demonstrated reduced platelet CD62P expression in the late phase after TIA or ischaemic stroke compared with the acute phase (Marquardt et al, 2002). This was apparently confirmed in a smaller study, although the p values for inter-group differences were not published by the authors (Karakantza et al, 2003).

Subsequent longitudinal studies also demonstrated a reduction in platelet CD62P expression in PRP in the subacute and late phases following TIA or ischaemic stroke compared with the acute phase (p < 0.01) (Yip et al, 2004).

Conclusion

There is strong evidence that platelet CD62P expression is elevated in the early stages following an acute TIA or ischaemic stroke, and that this increased expression is still present in the late phase (>3 months) following the event. There is some evidence to
suggest that the degree of expression in the acute phase is higher than later time points. Whether CD62P expression in patients with ischaemic CVD can be influenced by pharmacotherapy, and whether monitoring of CD62P levels would be of value in predicting the response to antiplatelet agents in the clinical setting has not been resolved.

4.5.2. Platelet CD63 Expression

Some authors have found that platelet CD63 expression is elevated in the acute phase after TIA or ischaemic stroke compared to controls (Grau et al, 1998; Marquardt et al, 2002; Cha et al, 2003; Karakantza et al, 2003; Cha et al, 2004), but others have not confirmed these findings (McCabe et al, 2004a). Most studies have found that platelet CD63 expression is elevated in the subacute (Markel et al, 2002; Cha et al, 2003) and late phase (Markel et al, 2002; Cha et al, 2004) following TIA or ischaemic stroke when compared to controls. However there is some evidence to the contrary (Grau et al, 1998; McCabe et al, 2004a). Longitudinal assessment of platelet CD63 expression in CVD patients did not demonstrate any change over time following the ictus in a large study of all stroke subtypes (Markel et al, 2002; Karakantza et al, 2003) or in a study specifically investigating patients with large artery atherosclerotic TIA or stroke compared with controls with asymptomatic severe carotid stenosis (McCabe et al, 2005b). However, a smaller study demonstrated lower platelet CD63 expression 30 days following the ictus when compared with the acute phase (Karakantza et al, 2003).

Conclusion

There is some evidence that platelet CD63 expression is up-regulated in the acute phase following TIA or ischaemic stroke. It is unclear whether this up-regulation definitely
persists into the late phase, but, in any case, CD63 appears to be a less sensitive marker of platelet activation than CD62P in some studies in ischaemic CVD.

4.5.3. Activated GpIIb/IIa receptor (PAC1) Expression on Platelets

Platelet activation leads to a conformational change in the GpIIb/IIIa receptor (GpIIb/IIIa) that facilitates binding of fibrinogen and the PAC1 monoclonal antibody. This change is the final common pathway in platelet aggregation (Moran N & FitzGerald GA, 1994). PAC1 only binds to activated platelets, and quantification of PAC1 binding may be used to assess the degree of activation of GpIIb/IIIa on platelets.

Few papers have investigated changes in PAC1 binding in patients following TIA and ischaemic stroke. McCabe et al. found that although the median percentage PAC1-binding was similar in acute TIA or stroke patients and controls, PAC1-binding was reduced in the convalescent patient group compared with controls (McCabe et al, 2004a). Because the authors identified evidence of platelet activation in their patient population otherwise (elevated CD62P and monocyte-platelet complexes), this reduction in PAC1 expression in the late phase CVD patients may have been secondary to reduced availability of GpIIb/IIIa receptor binding sites following platelet activation, or may have reflected the fact that GpIIb/IIIa receptor activation occurs at a relatively late stage in the platelet activation process and may be reversible (McCabe et al, 2004a).

McCabe et al. found no significant difference in PAC1 binding between acute or convalescent symptomatic and asymptomatic patients with severe extracranial carotid artery stenosis (McCabe et al, 2005b).
Conclusion
There are no convincing data to suggest that quantification of PAC1 binding in unstimulated whole blood samples is informative of the degree of platelet activation in TIA or ischaemic stroke patients. However, it must be emphasised that many of the patients in the aforementioned published studies were not assessed in the hyperacute phase (< 24 hours) after TIA or ischaemic stroke.

4.5.4. Platelet Fibrinogen binding
As mentioned earlier, platelet activation leads to a conformational change in the GpIIb/IIIa receptor which facilitates binding of fibrinogen. Detecting fibrinogen on the surface of platelets using monoclonal antibodies to fibrinogen has thus been used as a surrogate for detecting platelet activation.

Two studies have investigated platelet fibrinogen binding in patients with TIA or ischaemic stroke. One study that employed FITC-conjugated rabbit polyclonal anti-human fibrinogen antibodies found that platelet fibrinogen binding was elevated in patients in the acute and late phase following TIA or ischaemic stroke compared with controls (Meiklejohn et al., 2001). The authors also reported that platelet fibrinogen binding was lower at ≥ 3 months than in the acute phase after symptom onset.

A larger, but perhaps less robustly-designed study demonstrated elevated platelet fibrinogen binding in patients with atherothrombotic stroke and lacunar stroke when compared with either patients with cardioembolic stroke or controls (Yamazaki et al., 2001).
Conclusion
There is some evidence that platelet fibrinogen binding is increased following TIA or ischaemic stroke, but further studies are required to confirm whether definite differences in fibrinogen binding between stroke subtypes exist.

4.5.5. Leucocyte-Platelet Complexes

CD62P expressed on the platelet surface membrane mediates the adhesion of platelets to leucocytes, including neutrophils, monocytes and lymphocytes (de Bruijne-Admiraal et al, 1992). The predominant receptor for CD62P on leucocytes is P-selectin glycoprotein ligand-1 (PSGL-1) (Furie et al, 2001), and the percentage of leucocytes that are bound to platelets can be measured by flow cytometry (Li et al, 1997; Joseph et al, 2001). Because CD62P is rapidly shed from the surface of circulating degranulated platelets (Michelson, 1996), 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). Earlier studies in patients with acute myocardial infarction or in those undergoing percutaneous coronary intervention suggested 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 et al, 2000; Furman et al, 2001; Michelson et al, 2001).

Subsequent studies have consistently shown that circulated monocyte-platelet complexes are increased in patients with TIA or ischaemic stroke compared with controls in the acute (Garlichs et al, 2003; McCabe et al, 2004a; Marquardt et al, 2009) and late phases after symptom onset (Garlichs et al, 2003; McCabe et al, 2004a). One
study also revealed increased monocyte-platelet complex formation in the late phase compared to the acute phase following ischaemic stroke, but not TIA (Garlichs et al, 2003), but these findings were not replicated by others (McCabe et al, 2004a; Marquardt et al, 2009). One study also demonstrated an elevation in the percentage of neutrophils bound to platelets at days 1, 2 and 3 following TIA or ischaemic stroke (Marquardt et al, 2009), but the percentage of neutrophil-platelet complexes was not increased in CVD patients studied at any stage within 1-27 days of symptom onset in another case-control study (McCabe et al, 2004a). McCabe et al. identified elevated neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes in patients with acute symptomatic compared with asymptomatic severe (≥ 70% ) carotid artery stenosis. In the convalescent phase, the median percentages of all leucocyte-platelet complexes in the symptomatic group had dropped to levels similar to those found in the asymptomatic group (McCabe et al, 2005b).

**Conclusion**

There is strong evidence that monocyte-platelet complexes are increased in the early stages following acute TIA or ischaemic stroke; this may be of particular pathophysiological relevance in patients with large artery disease as discussed elsewhere (McCabe et al, 2005b). There is some evidence that neutrophil-platelet complexes are elevated in the first 3 days after TIA or ischaemic stroke, but data beyond the hyperacute phase are less convincing. The time course of monocyte-platelet complex formation in the late phase following an acute cerebrovascular event is unclear. The available data do not suggest that the percentage of circulating lymphocyte-platelet complexes is a sensitive measure of platelet activation in ischaemic CVD.
4.6. Discussion

There is clear evidence that platelet activation is increased following acute TIA or ischaemic stroke, as demonstrated by data on platelet surface marker expression, platelet surface protein cleavage, platelet degranulation and platelet binding to cellular and plasma protein components. There is some evidence to suggest that platelet activation precedes TIA and ischaemic stroke, and does not occur as a consequence of the ictus. Some studies have clearly shown that enhanced platelet activation persists in the late phase after TIA or ischaemic stroke despite treatment with antithrombotic therapy. It is reasonable to hypothesise that the beneficial clinical effects of antiplatelet agents for secondary prevention following TIA and ischaemic stroke (Diener et al., 1996; Halkes et al., 2006; Sacco et al., 2008) must relate to reduction in platelet activation status, but robust data supporting this hypothesis were not available prior to the commencement of this thesis.
5. Overview of antiplatelet therapy in TIA and ischaemic stroke

5.1. Introduction
There are several commonly used antiplatelet medications for the secondary prevention of TIA and ischaemic stroke. In this section, I will briefly describe the currently available medications, along with their mechanisms of action. I will also outline potential pathways that may be future targets for secondary prevention of vascular events following TIA and ischaemic stroke.

5.2. Aspirin
Aspirin is the most commonly used antiplatelet agent worldwide for the secondary prevention of ischaemic stroke and the first to be discovered. It was patented in Berlin on 6th March 1899 by Bayer, although who exactly discovered it first is debated (Rinsema, 1999). Although an increased bleeding tendency was considered to be an unwanted adverse effect with aspirin for some time, its potential usefulness as a therapeutic antiplatelet agent was subsequently realised (Roth & Calverley, 1994).

Mechanism of action as an antiplatelet agent
Cyclooxygenase is the key enzyme involved in prostaglandin (PG) biosynthesis (Roth & Calverley, 1994). The enzyme possesses both cyclooxygenase and hydroperoxidase activities. Two isoforms exist: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Roth & Calverley, 1994; Weber et al, 1999; FitzGerald & Patrono, 2001). COX-1 is constitutively expressed in most cells, including platelets, and determines the physiological functions of PGs, including the control of local tissue perfusion and haemostasis (Schror, 1997). However, its expression can also be regulated by certain
stimuli (FitzGerald & Patrono, 2001). In contrast, COX–2 is normally undetectable in most tissues, but its expression can be rapidly induced by exposure to cytokines, immunologic stimuli and growth factors with subsequent PG synthesis (Schorr, 1997; Weber et al, 1999). COX–2 is also present 10% of platelets of healthy controls (Guthikonda et al, 2007), and up to 60% of platelets in patients with high platelet turnover (Rocca et al, 2002). The relative contribution of COX–2 to PGE$_2$ and TXB$_2$ is higher in patients with high platelet turnover than in controls (Rocca et al, 2002). COX-2 mRNA is also present in platelets (Weber et al, 1999), and platelet COX–2 expression can be transiently induced by coronary artery bypass surgery (Zimmermann et al, 2003).

Aspirin is rapidly absorbed from the stomach and upper intestine, with peak plasma levels detected in 30 to 40 minutes, and inhibition of platelet function detectable within 1 hour after ingestion (Harker, 1998). It is noteworthy that peak plasma levels are delayed for 3 to 4 hours after oral administration of enteric-coated aspirin (Harker, 1998), and this is associated with a reduced inhibition of platelet aggregation when compared to soluble aspirin (Cox et al, 2006). Aspirin selectively and irreversibly inhibits the cyclooxygenase-mediated breakdown of arachidonic acid (Harker, 1998), thus inhibiting the subsequent formation of thromboxane A$_2$ (a potent platelet aggregator and vasoconstrictor). Orally administered aspirin enters the portal blood at about the same rate as platelets arrive from the pre-portal circulation, and the direct contact between platelets and newly absorbed aspirin facilitates the rapid inhibition of platelet COX-1 in the portal blood (Roth & Calverley, 1994). Because platelets are anucleate cells, the inhibitory effects of aspirin on platelet function should last for the lifespan of the platelet (8 to 11 days) (Ault & Knowles, 1995; Butterworth & Bath, 1998). During its passage through the liver, aspirin is deactivated to a significant
degree, but the drug can still inhibit cyclooxygenase activity in cells in the systemic circulation. This partially explains why the antiplatelet effects of aspirin are seen with lower doses of the drug than are required to produce an analgesic or anti-inflammatory effect (Roth & Calverley, 1994). In addition, endothelial cells can rapidly resynthesise COX-1 and may continue to synthesise PGs via COX-2 mediated pathways after administration of aspirin (Harker, 1998). It has been shown that aspirin is approximately 170-fold less potent at inhibiting COX-2 than COX-1 (Weber et al., 1999; Reiter et al., 2001). However, it is clear that cyclooxygenase inhibition is not always a positive phenomenon because selective and non-selective COX-2 inhibitors have been shown to increase the risk of vascular events in some patients (Kearney et al., 2006). More recently, COX-independent antiplatelet actions of aspirin have been suggested. Nitric oxide (NO)-donating properties of aspirin have been demonstrated in porcine coronary arteries (Taubert et al., 2004), and patients with metabolic syndrome have been shown to have enhanced nitric oxide formation in response to 12 weeks of aspirin therapy (Hennekens et al., 2010). The effects of NO on vascular smooth muscle cells and endothelium may partly explain some of the beneficial of aspirin in reducing vascular events.
5.3. Dipyridamole

There are several postulated mechanisms of action of dipyridamole that may explain its beneficial effects in secondary prevention following TIA and ischaemic stroke (see below). The relative importance of each potential mechanism is clear, although the additive effects of the different potential inhibitory effects may play a role. While steady state plasma levels are achieved after 14 days of therapy, levels of dipyridamole in leucocytes increase up to at least 30 days after commencing therapy (Serebruany et al, 2009), suggesting that the antithrombotic effects may not purely be related to its effects on platelets. The fact that treatment with dipyridamole was associated with a reduction in the risk of subsequent vascular events, without an increase in bleeding risk in 2 large clinical trials supports this hypothesis (Diener et al, 1996; Halkes et al, 2006).

Adenylate cyclase catalyses the conversion of adenosine triphosphate (ATP) to adenosine 3', 5'-cyclic monophosphate (cAMP), and soluble guanylate cyclase catalyses the conversion of guanosine triphosphate (GTP) to guanosine 3', 5'-cyclic monophosphate (cGMP). cGMP in turn stimulates adenylate cyclase via cGMP-dependent protein kinases, thus increasing the formation of cAMP (Haslam et al, 1999). Both adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are degraded by nucleotidases to form adenosine. Adenosine is a platelet-inhibiting vasodilator (FitzGerald, 1987; Muller, 2001), mediating its antiplatelet effects by stimulation of platelet adenylate cyclase via adenosine receptors (Haslam et al, 1999). The three predominant phosphodiesterase (PDE) enzymes present in platelets are PDE2, PDE3A and PDE5 (Haslam et al, 1999). PDE2 and PDE3A catalyse the breakdown of cAMP to 5'-AMP, whereas PDE5 catalyses the breakdown of cGMP to 5'-GMP. In addition to stimulating cAMP formation, cGMP also regulates the physiological breakdown of
cAMP by stimulating PDE2 and inhibiting PDE3A (Haslam et al, 1999). Any compound that increases the intraplatelet concentration of cAMP inhibits platelet aggregation (FitzGerald, 1987).

The recognised mechanisms of action of dipyridamole to date are:

**(a)** Dipyridamole is a phosphodiesterase inhibitor that increases the intracellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanine monophosphate (cGMP) by preventing their conversion to AMP and GMP, respectively (Kim & Liao, 2008). By increasing cAMP and cGMP levels in platelets, dipyridamole reversibly inhibits platelet aggregation, and enhances cGMP-dependent smooth muscle vasodilation (Kim & Liao, 2008). The ability of dipyridamole to increase intracellular cAMP and cGMP in a time- and dose-dependent manner through phosphodiesterase (PDE) inhibition is also associated with the antiproliferative effects of dipyridamole on venous and arterial vascular smooth muscle cells, a phenomenon attributed more to its effects on cGMP than cAMP (Chakrabarti & Freedman, 2004).

**(b)** In circulating blood, free adenosine is rapidly removed from plasma by a specific adenosine carrier that transports it into red blood cells and vascular endothelial cells. Dipyridamole inhibits adenosine uptake by red blood cells by >90%, and increases plasma adenosine levels by 60% (Kim & Liao, 2008).
(e) There is *in vitro* evidence to suggest that dipyridamole mediates some of its antithrombotic effect by its action on the endothelium itself, perhaps by enhancing the effects of nitric oxide (NO) (Eisert, 2001); NO increases cGMP by stimulating soluble guanylyl cyclase. Endothelium-derived NO is an important regulator of vascular tone, blood flow, and tissue perfusion. Endothelial NO synthase-deficient mice exhibit elevated systemic blood pressure and have larger myocardial and cerebral infarct size after experimental ischaemic injury.

(d) Dipyridamole protects erythrocyte membranes from oxidation and spares the antioxidant power of erythrocytes, along with suppressing oxygen free radical formation in platelets and endothelial cells, and improving 'cellular redox status' (Kim & Liao, 2008). These antioxidant effects of dipyridamole may extend the half-life and increase the bioavailability of endothelium-derived NO.

(e) There are also data to suggest interaction between these mechanisms affecting NO and cyclic nucleotides. Adenosine may increase platelet cGMP concentrations, whereas NO may inhibit adenosine formation (Chakrabarti & Freedman, 2004).

(f) Dipyridamole may also exert direct anti-inflammatory effects through inhibition of platelet-monocyte interactions, or inhibition of secretion of compounds from monocytes when they form complexes with platelets. Activated platelets may adhere to and stimulate monocytes, causing monocytes to secrete monocyte chemotactic protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) (Kim & Liao, 2008). Furthermore, dipyridamole has been shown to suppress TNF-α and increase IL-10 in monocyte cultures from patients with Crohn’s disease (Poturoglu *et al.*, 2009).
Dipyridamole has also been shown to inhibit the adhesion of neutrophils to the vascular endothelium in ischaemic stroke patients via a specific downregulation of Mac-1 (Macrophage 1 antigen) (Diener et al, 1996). This could be particularly important in the large artery atherosclerotic stroke patient subgroup.

Evidence for the effects shown above is gleaned mostly from *in vitro* models, with some indirect *ex vivo* evidence. The multitude of potential antithrombotic properties of dipyridamole makes it somewhat difficult to study single pathways that might be inhibited by the drug.

### 5.4. Clopidogrel

Clopidogrel is a thienopyridine derivative that selectively and irreversibly inhibits the P2Y12 ADP receptor on platelets, thus interfering with ADP-induced activation of the GpIIb/IIIa receptor complex. Binding to this receptor prevents ADP-induced inhibition of adenylate cyclase, thus increasing intraplatelet levels of cAMP (Brass, 2001). Although the antiplatelet effects of clopidogrel have been reported to be maximal after 3 to 5 days of therapy (Quinn & Fitzgerald, 1999), use of a loading dose (150 to 300 mg) produces a more rapid and stable inhibitory effect than that seen with 75 mg daily (Savcic *et al*, 1999).

Clopidogrel is a prodrug that requires biotransformation to an active metabolite. Esterases convert the majority of clopidogrel to SR26334, an inactive metabolite and the remaining prodrug is converted to 2-Oxo-clopidogrel and subsequently to R-130964 which is the final active metabolite (Mega *et al*, 2009). This two step biotransformation process is catalyzed by multiple CYP isotypes, including CYP3A, CYP2B6, CYP1A2,
CYP2C9 and CYP2C19 (Oyetayo & Talbert, 2010). Polymorphisms in the CYP2C19 gene have been associated with reduced levels of R-130964 and reduced inhibition of platelet aggregation in patients treated with clopidogrel following myocardial infarction (Mega et al, 2009), suggesting a mechanism responsible for clopidogrel antiplatelet resistance in these patients. A significant interaction between co-administration of proton pump inhibitors (PPIs) and clopidogrel has recently been reported, with PPIs reducing the apparent efficacy of clopidogrel, both ex-vivo (O'Donoghue et al, 2009), and in the clinical setting (Oyetayo & Talbert, 2010). The hypothesis that this interaction may by driven by competition for hepatic enzymes between the drugs is supported by laboratory evidence of competitive inhibition of CYP2C19 by omeprazole and other PPIs (Oyetayo & Talbert, 2010).
6. Review of the literature on von Willebrand factor and von Willebrand factor propeptide in TIA and ischaemic stroke

6.1. Introduction

Von Willebrand factor (VWF:Ag) is a multimeric plasma glycoprotein that is synthesised in vascular endothelial cells and megakaryocytes (Nishio et al, 2004; Bongers et al, 2006). Endothelial cells secrete VWF:Ag constitutively into the circulating blood or into the subendothelial matrix, and also release VWF:Ag stored in Weibel-Palade bodies in response to endothelial cell activation (Ruggeri, 1997). VWF:Ag may bind to the platelet surface glycoprotein (Gp) Ib-IX-V receptor complex, thus mediating platelet adhesion to exposed subendothelial collagen and subsequent platelet-rich thrombus formation (Nishio et al, 2004; Bongers et al, 2006). Elevated VWF:Ag levels have been identified in both the early and late phases following an ischaemic cerebrovascular event, (Kozuka et al, 2002; McCabe et al, 2004a) and following an ischaemic or haemorrhagic stroke compared with healthy controls (Catto et al, 1997).

Von Willebrand Factor propeptide (VWF:Ag II) is produced by cleavage of pro-VWF into VWF:Ag and VWF:Ag II, and is also released from the endothelium (van Mourik et al, 1999; Frijns et al, 2006). Recent reports have clearly demonstrated that the ratio of von Willebrand factor antigen (VWF:Ag) to von Willebrand factor propeptide (VWF:Ag II) in plasma provides an accurate measure of the degree of acute endothelial cell activation (van Mourik et al, 1999; Hollestelle et al, 2006). However, to date, VWF:Ag II has not been investigated in patients with ischaemic cerebrovascular vascular disease.
Studies have shown VWF:Ag to be elevated in the early (Catto et al, 1997; Bath et al, 1998; Kozuka et al, 2002; Lynch et al, 2004; McCabe et al, 2004a; Nadar et al, 2005; Bongers et al, 2006; Kotzailias et al, 2007) and late (Catto et al, 1997; Kozuka et al, 2002; Kain et al, 2002; McCabe et al, 2004a) phases following ischaemic stroke or TIA. Studies of patients with atrial fibrillation without a history of stroke are conflicting, with some demonstrating elevated VWF:Ag in patients with atrial fibrillation when compared to controls (Freestone et al, 2005) and others not supporting this finding (Yip et al, 2004). Although some studies have contradictory findings (Dai et al, 2001). I will now discuss these studies in more detail.

6.2. Cross sectional studies of von Willebrand factor in the Early Phase Following Ischaemic Stroke or TIA

Nadar et al measured VWF:Ag levels with ELISA in 29 hypertensive patients within 24 hours of acute stroke, 30 controls with hypertension and 30 normotensive controls (Nadar et al, 2005). VWF:Ag levels were higher in patients with acute stroke than those with hypertension \(p = 0.01\), and were also higher in hypertensive patients than in controls \(p = 0.03\).

Lynch et al investigated 44 patients \(\leq 24\) hours of ischaemic stroke, 13 \(\leq 24\) hours of onset of a TIA, and 157 controls without vascular disease (Lynch et al, 2004). The authors found that VWF:Ag levels were significantly higher in patients following acute stroke compared with controls \(<0.001\).

Freestone et al studied VWF:Ag levels in 28 patients with chronic atrial fibrillation who had not been hospitalised over the prior 3 months, 63 patients within 48 hours of
hospital admission who had electrocardiographic evidence of atrial fibrillation (22 with MI, 20 with acute left ventricular failure, 21 with acute stroke), and 20 healthy controls (Freestone et al, 2005). VWF:Ag levels were significantly higher in patients with atrial fibrillation with no recent cardiovascular or cerebrovascular event than in controls (p < 0.001). There was no significant difference in VWF:Ag levels between patients with chronic stable atrial fibrillation alone and those with atrial fibrillation in association with an acute MI, ischaemic stroke or left ventricular failure (p = 0.4). Once again, patient numbers in each subgroup were very small, so this type of subgroup analysis needs to be interpreted with extreme caution.

Bongers et al demonstrated elevated plasma VWF:Ag levels in 124 patients within 7 – 14 days following a first-ever ischaemic stroke compared with 125 age- and gender-matched controls without a history of stroke (Bongers et al, 2006).

Bath et al also demonstrated elevated plasma VWF:Ag levels in acute (< 48 hours) ischaemic (n = 163) and haemorrhagic (n = 40) stroke compared with controls (n = 33), but found no difference in levels between different clinical subtypes ischaemic stroke (Bath et al, 1998).

Kotzailias et al performed a study with the primary aim of assaying the response to clopidogrel on the PFA-100®, as discussed in Chapter 10 [Assessment of the impact of aspirin and clopidogrel on platelet activation and function in the early and late phases after TIA or ischaemic stroke] (Kotzailias et al, 2007). They also measured VWF:Ag with enzyme-linked immunosorbent assay (ELISA). 5 patients within 3 weeks of ischaemic stroke, and 11 patients > 3 weeks following ischaemic stroke were treated
with clopidogrel for a minimum of 1 week. VWF:Ag levels were elevated in 7 patients, although the authors did not specify how they established their laboratory normal range.

Dai et al investigated 53 patients with acute ischaemic stroke, 22 patients with acute myocardial infarction and 47 controls (Dai et al, 2001). The interval between onset of ischaemic stroke or myocardial infarction to blood sampling was not specified. Plasma VWF:Ag levels were determined by ELISA. The mean plasma VWF:Ag level did not differ significantly between patients with acute ischaemic stroke (0.584 U/ml) and controls (0.468 U/ml, p = 0.2), but was significantly higher in patients with acute myocardial infarction (0.783 U/ml) than controls (p = 0.001).

6.3. Von Willebrand factor in the Late Phase Following Ischaemic Stroke or TIA

Kain et al reported that VWF:Ag levels were significantly higher in 80 South Asian patients ≥2 months following ischaemic stroke than in 80 South Asian controls (p = 0.006) (Kain et al, 2002).

McCabe et al (McCabe et al, 2004a) reported that mean plasma VWF:Ag antigen levels were higher in the early (< 4 weeks, N = 79) and late (> 3 months, N = 70) phases after TIA or ischaemic stroke compared with controls of similar age and gender who did not have cerebrovascular disease (N = 27; P < 0.001). However, the study was not designed to compare results from matched samples from the same patients who were studied in both the early and late phases after symptom onset, and thus, these data were not reported.
6.4. Longitudinal studies of von Willebrand factor following ischaemic stroke or TIA

While case control series demonstrate elevation in VWF:Ag in the early and late phases following TIA and ischaemic stroke, as discussed above, and there is some suggestion that serum VWF:Ag levels may be elevated in some at risk groups, the time course of VWF:Ag levels can only be elucidated by longitudinal studies following patients from the acute phase into the late phase after symptom onset. Although one study of both haemorrhagic stroke and ischaemic stroke combined demonstrated a reduction in serum VWF:Ag in patients more than 3 months following stroke when compared with the acute phase (Catto et al, 1997), two further studies (Cherian et al, 2003; Yip et al, 2004) have demonstrated no difference in VWF:Ag levels between patients in the early and late phases following ischaemic stroke. This includes one study which investigated a cohort larger than that studied by Catto et al (Cherian et al, 2003). Furthermore, one study found no difference in VWF activity between patients in the early versus the late phase following TIA or ischaemic stroke (Kozuka et al, 2002). These longitudinal studies are summarised below.

Cherian et al investigated 200 patients ≤ 7 days, and subsequently between 3 – 6 months of first-ever ischaemic stroke and 205 controls (Cherian et al, 2003). In this large study, there were no significant differences in VWF:Ag levels between patients and controls, or between patients in the acute vs. late phases following ischaemic stroke.

Yip et al investigated VWF:Ag levels in patients with or without stroke in association with atrial fibrillation (Yip et al, 2004). 61 patients were assessed at baseline (within 48 hours of cardioembolic ischaemic stroke in association with atrial fibrillation), and on
days 7, 21 and 90 after the stroke. 50 patients with non-valvular atrial fibrillation without a history of ischaemic stroke were also assessed at 4 time points (days 1, 7, 21 and 90). These data were compared with those obtained from 30 healthy controls who were assessed once. There were no significant differences in VWF:Ag levels between patients with or without ischaemic stroke in association with non-valvular atrial fibrillation compared with controls. Furthermore, there were no significant changes in VWF:Ag levels over time following stroke onset (p = 0.8).

Catto et al performed a longitudinal study to assess VWF:Ag levels following acute stroke (185 ischaemic and 23 haemorrhagic) (Catto et al, 1997). The authors found elevated VWF:Ag levels in the early (<10 days) and late (> 3 months) phases following stroke compared with a “reference population” of age- and gender-matched controls (p = 0.0001). On ‘paired analysis’ of data in patients from the acute and convalescent phases (n = 169) they found a lower level of VWF:Ag in the late versus early phase after symptom onset (p < 0.0001).

Kozuka et al compared VWF activity (not VWF:Ag levels) in 52 patients within 48 hours of acute ischaemic stroke, and again in 48 of those patients within 1 month of acute ischaemic stroke; the data were also compared with those derived from 86 controls (Kozuka et al, 2002). Plasma VWF activity was significantly higher in stroke patients within 48 hours (p < 0.001) or within 1 month of stroke onset (p < 0.001) compared with controls. There was no significant difference in VWF activity between stroke patients within 48 hours (p < 0.001) or within 1 month of stroke onset.
6.5. Conclusion

There is strong evidence that both VWF:Ag levels and corresponding VWF activity are elevated in the early and late phases following TIA and ischaemic stroke, most likely indicating ongoing endothelial activation because the majority of VWF:Ag is endothelial-derived (McGrath et al, 2010). There is conflicting evidence regarding the time course of VWF:Ag following TIA and ischaemic stroke in individual patients, and whether elevations in VWF:Ag levels or activity precede TIA and ischaemic stroke. There are no studies investigating the ratio of VWF:Ag to VWF:Ag II following TIA and ischaemic stroke. Furthermore, there are no well designed longitudinal studies investigating the impact of commonly prescribed antiplatelet regimens for secondary prevention following TIA and ischaemic stroke on VWF:Ag and VWF:Ag II, or the impact of VWF:Ag II levels on ex vivo responsiveness to antiplatelet therapy in this setting. Assessment of endothelial activation status should improve our understanding of the potential mechanisms mediating antiplatelet non-responsiveness in the laboratory and clinical setting in individual patients.

To answer some unresolved questions, we performed a pilot study to assess VWF:Ag and VWF:Ag II levels in a well characterised cohort of patients in the acute, subacute and late phases following TIA and ischaemic stroke, both before and after alterations in antiplatelet therapy. The results of this study are described in Chapter 14 [Assessment of von Willebrand factor and von Willebrand factor propeptide in patients following TIA and ischaemic stroke].
7. Endogenous Thrombin Potential

7.1. Introduction

The process of platelet activation may contribute to platelet hyper-reactivity, platelet-platelet aggregate formation and subsequent thrombus formation following TIA or ischaemic stroke (McCabe et al., 2005a; Monroe & Hoffman, 2006), but it is also intricately linked to activation of the coagulation system (Monroe et al., 2002). Activation of platelets and the coagulation system may occur simultaneously, and may be initiated by the same agonists in some instances (Monroe et al., 2002).

As outlined in Chapter 4 [Is Platelet Activation Increased Following TIA or Ischaemic Stroke?], increased platelet activation has been identified in patients in both the early and late phases after TIA or ischaemic stroke (McCabe et al., 2004a). Elevation in cytosolic calcium during platelet activation induces exposure of procoagulant phosphatidylserine (PS) on the platelet surface, thus promoting assembly of tenase and prothrombin complexes, and subsequent accelerated thrombin generation (van der Meijden et al., 2005). Thrombin is a potent platelet agonist and during thrombus formation activates platelets by binding to the GpIb-IX-V complex (Berndt et al., 2001). However ADP-induced activation of the P2Y_{12} ADP receptor on platelets actually increases the time taken to achieve peak thrombin formation (van der Meijden et al., 2005).

There have only been two studies investigating endogenous thrombin potential in the late phase after TIA or ischaemic stroke (Faber et al., 2003; van der Meijden et al., 2005).
7.2. Published literature on thrombin generation potential in ischaemic cerebrovascular disease

The first of these studies compared endogenous thrombin potential (ETP), platelet-derived procoagulant activity, VWF:Ag levels, plasma fibrinogen levels, and antithrombin activity in 41 patients under 50 years of age, who were at least 3 months following non-cardioembolic ischaemic stroke with 70 age- and gender-matched healthy controls. (Faber et al, 2003) Antiplatelet medication at the time of study entry was not reported. ETP data were reported as a percentage of ‘pooled normal plasma’ for the platelet poor plasma (PPP) samples, but as a percentage of the age- and gender-matched control’ samples for results in platelet rich plasma (PRP). There was no significant increase in ETP in non-cardioembolic stroke patients compared with ‘pooled normal’ PPP samples, but stroke patients had a higher median ETP than age-and gender-matched controls in PRP (p < 0.01). The authors suggested the finding of increased coagulation system potential (i.e. ETP) in this patient cohort might have been mediated by platelet surface-derived procoagulant factors in PRP.

Subsequently, Van der Meijden et al. investigated peak height, time-to-peak and ETP in PRP from 11 young (32 - 51 years) patients and 9 older stroke patients (60- 74 years) within 3-12 months of ischaemic stroke, and also in 12 patients with type-II diabetes mellitus (26 - 73 years) and 11 healthy controls, whose age was not outlined in the paper (van der Meijden et al, 2005). The 9 older stroke patients were also re-assessed 2 weeks following the introduction of clopidogrel monotherapy (75mg daily). It was not specified how many patients were on aspirin, or the prescribed aspirin dose at study entry. The peak thrombin generation rate was higher in PRP from the older stroke patients and diabetic patients (p ≤ 0.05) compared with healthy controls. There were no
significant differences in any thrombin generation markers between young stroke patients and controls. However, the numbers of subjects in each subgroup was too small to make any definite conclusions.

Therefore, the limited data available to date suggest that endogenous thrombin potential in PRP may be elevated in young patients (Faber et al, 2003) and peak thrombin generation rate higher in older patients (van der Meijden et al, 2005) following ischaemic stroke, when compared to controls, although the findings in young patients were not replicated in the second smaller study. However, thrombin generation potential has not been assessed in the same patients in the both the early and late phases following TIA or ischaemic stroke, and the impact of altering antiplatelet therapy on thrombin generation potential, or the potential impact of increased thrombin generation potential on ex vivo responsiveness to antiplatelet therapy following TIA or stroke have not been assessed.
8. Optimal therapeutic targets in ischaemic stroke

Stroke is a heterogeneous disease, both in terms of its aetiology and in terms of the pathways involved in the pathogenesis of thrombosis and thromboembolism. This is reflected by the varied mechanisms of action of the currently prescribed antiplatelet drugs, and by the fact that combination therapy with, for example, aspirin and dipyridamole, has been shown to be superior to treatment with either monotherapy with aspirin alone in two large clinical trials.

An ideal agent for the secondary prevention of TIA and ischaemic stroke would be one which adequately prevents thrombosis without any associated decrease in haemostatic ability. Against this background, there are many potential future targets for primary and secondary prevention of TIA and ischaemic stroke. These can broadly be divided up into those targets associated with Virchow’s triad of flow, endothelium and humeral factors (Bagot & Arya, 2008), and will be briefly alluded to below.

Flow-related targets include improved recognition and treatment of atrial fibrillation, a very important risk factor because of its high age-specific attributable risk (Bejot et al, 2009); improved techniques for identifying and managing ‘at risk’ large vessel atheroma; and improved techniques for identifying and managing patients with low cerebral perfusion pressures who may be at risk of larger cerebral infarctions.
Endothelial targets include pathways involved in endothelial activation or nitric oxide delivery, either by enhancement of current drugs or development of novel agents; increasing endothelial PGI$_2$ production; and stabilisation and/or reversal of atheroma, e.g. with statins for which we have no good evidence in the acute phase after TIA or ischaemic stroke.

Humeral targets probably represent the greatest array of targets, purely due to the number of components involved, and can be broadly subdivided into platelet-related targets, coagulation system targets and leucocyte targets.

Platelet serotonin receptor antagonists have recently been shown to be disappointing when compared with aspirin (Shinohara et al, 2008), but other platelet surface receptors and components of the second messenger system might be more promising targets. For example, one could consider targeting the CD62P-PSGL1 interaction to reduce platelet-leucocyte interactions, or target the adenosine receptor to increase intraplatelet c-AMP and stabilise platelet granules. Anti-collagen receptor antiplatelet agents could potentially reduce platelet adhesion without interfering significantly with aggregation, and might not be associated with such a risk of bleeding. Modification of existing antiplatelet agents and clinical trials of antiplatelet drugs shown to be relatively safe and effective in patients with ischaemic heart disease need to be explored in patients with cerebrovascular disease in whom bleeding has potentially more catastrophic consequences than in patients with an acute coronary syndrome.
Coagulation system targets include e.g. VWF antagonists or factor XII antagonists. Murine models have shown that factor XII-knockout mice are protected against collagen- and epinephrine-induced thromboembolism, without spontaneous or excessive injury-related bleeding (Renne et al, 2005).

Leucocyte-related targets include pathways which facilitate leucocyte recruitment, cholesterol metabolism, inflammatory activity and oxidative stress (Saha et al, 2009).

The heterogeneous nature of ischaemic cerebrovascular disease calls for an improvement in our understanding of particular pharmacokinetic, pharmacodynamic or pharmacogenomic risk factors to allow one to move towards individualised clinically effective therapy for TIA or ischaemic stroke.
9. General Methods

9.1. Study Subjects
The specific inclusion and exclusion criteria for subjects involved in each study described in this thesis are outlined in the respective results chapters, but the general inclusion / exclusion criteria are outlined below.

9.1.1. Ethical approval
The St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee approved the study (REC Ref: 2007/07/MA).

All study subjects were given a study information sheet outlining the details of the study, and written informed consent (or written assent by a relative or next of kin of the patient, where appropriate) was obtained in all cases.

9.1.2. Inclusion criteria

Patients with ischaemic stroke or transient ischaemic attack (TIA)
Patients older than 16 years of age, who had experienced an ischaemic stroke or TIA within the preceding four weeks, and who were about to undergo a change in antiplatelet therapy following advice from their treating physician.

Controls
Control subjects of similar age and gender to the stroke / TIA patient population who had no prior history of cerebrovascular disease were recruited.
9.1.3. Exclusion Criteria

Patients with transient ischaemic attack (TIA) or ischaemic stroke

- Myocardial infarction, PE or DVT within the preceding 3 months
- Platelet count < 120 x 10^9/L or > 450 x 10^9/L
- Urea > 10 mmol/l or GFR < 30 ml/min
- Major surgery within the preceding three months
- Systemic haemorrhage within the preceding three months (Haemoglobin drop of > 1 g/dl in one day, or requiring transfusion)
- Ongoing unstable angina or unstable symptomatic peripheral vascular disease.
- Prior history of primary intracerebral haemorrhage
- Known bleeding/ clotting diathesis, including known platelet disorders
- Active vasculitis or other inflammatory conditions
- Active neoplasia
- Current infection (Clinical signs of infection, white cell count > 11 x 10^9/L)
- NSAID intake within the previous 10 days

Control Subjects

The exclusion criteria for control subjects were the same as those for patients, as outlined above, 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 carotid or vertebral artery stenosis after study screening, or if they were on antiplatelet therapy.
9.1.4. Recruitment Source

Patients were recruited from the One-Stop Rapid Access Stroke Prevention Clinic, and from the inpatient population of the Neurology and Age-Related Health Care and Stroke service at AMNCH. Control subjects were recruited from amongst the staff at AMNCH and from the local population. Spouses of patients and control subjects were also recruited.

9.1.5. Baseline Assessment

Control subjects were clinically assessed by one of three examiners (WOT, DJHM, JAK) using a standardised protocol prior to recruitment. Colour Doppler ultrasound of carotid and vertebral arteries was performed to rule out moderate or severe carotid or vertebral stenosis.

TIA or stroke work up was performed in all patients by their primary physician according to ESO recommendations (European Stroke Organisation (ESO) Executive Committee & ESO Writing Committee, 2008), along with their research assessment by one of three examiners (WOT, DJHM, JAK) using a standardised protocol. The diagnosis of TIA or ischaemic stroke was confirmed in all cases by Dr Tobin and/or Dr McCabe, and patients were not included if Dr Tobin or Dr McCabe had any doubt about the working diagnosis.
9.2. Sample collection and separation

9.2.1. Sample collection

All subjects were rested for at least 20 to 30 minutes before venepuncture to standardise conditions and to minimise platelet activation in vivo. A tourniquet was applied to the arm and careful venepuncture was performed in all cases. Blood was collected from a free-flowing vein using a sterile 21G Butterfly® needle (Venisystems™, Abbott, Ireland) and a Vacutainer® system with a luer adaptor (Becton Dickinson Vacutainer® Systems, U.K.).

For all studies, the tourniquet was released during collection of the first 4 ml of blood which was drawn into two 2 ml sterile Vacutainer® tubes containing K\textsubscript{2} EDTA. The first 2ml EDTA sample was used for measurement of a full blood count (FBC), including measurement of the mean platelet volume (MPV) and platelet distribution width (PDW), and the second 2ml EDTA tube containing whole blood was stored at −70°C for future planned genetic studies (see later).

Eight further 3 ml samples were collected into sterile Vacutainer® tubes containing 0.105 M (3.2%) buffered sodium citrate. All samples were gently inverted five to eight times to ensure thorough mixing of the anticoagulant with the blood sample. The first 3 ml blood sample was used for whole blood flow cytometric analysis and for measurement of platelet function in whole blood using the platelet function analyser (PFA-100®, Dade-Behring, Germany; see below). The next four citrate-anticoagulated samples were used to prepare platelet poor plasma (PPP). The remaining sample was used for measurement of the platelet count, MPV and PDW in citrate-anticoagulated whole blood.
Three 6ml K₂ EDTA tubes were also taken: the first two 6 ml K₂ EDTA tubes were used to prepare PPP for assessment of antiplatelet drug levels in a collaborative high performance liquid chromatography (HPLC) study, and the last K₂ EDTA-containing tube was used for ABO blood grouping.

9.2.2. Sample separation

Separation of plasma samples was performed in all subjects within 60 minutes of venepuncture, and the samples were frozen at -70°C within 90 minutes of venepuncture, unless stated otherwise.

Platelet poor plasma (PPP)

PPP was prepared from three 3.2% (0.105 M) buffered sodium citrate anticoagulated blood samples within one hour of venepuncture. The samples were centrifuged at 2250g for 15 minutes at room temperature. The upper two thirds of each sample was carefully aspirated using a plastic Pasteur pipette and pipetted into a 12 x 75 mm polypropylene sample tube. The sample was centrifuged again at 2250g for 15 min. Double-spun PPP was then recovered from the upper two thirds of this sample, aliquoted into three polypropylene tubes (Sarstedt®, Germany) and immediately frozen at −70°C. The remaining PPP was removed from the lower third of the centrifuge tube and also stored in a polypropylene tube at −70°C; this sample was not considered to be double-spun.
Serum for Thromboxane $B_2$ Analysis

Serum was prepared for thromboxane $B_2$ analysis from three 4ml serum tubes that did not contain any anticoagulant. The samples were incubated at 37°C for 30 minutes, within 30 minutes of venepuncture. The samples were then centrifuged at 4200g for 10 minutes at room temperature. The upper two thirds of each sample was aspirated using a plastic Pasteur pipette and stored in a polypropylene tube at -70°C.
9.3. Flow Cytometry

9.3.1. General principle

The flow cytometer used in this study was a Coulter® EPICS® XL-MCL (Beckman Coulter United Kingdom Ltd.), and 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 & Idziorek, 1994). The signals that are ultimately produced by the flow cytometer provide information about the cell. The process begins with the insertion of a cell suspension into the flow cell, through which sheath fluid (ISOTON II®, Beckman Coulter, U.K.) also flows. The sheath fluid is filtered with a 0.2 μm filter. It contains bacteriostatic and fungistatic agents and is transparent and non-fluorescent in response to 488 nm laser light. The sheath fluid exerts a constant pressure on the cell suspension and, using a low flow rate of 10μl/min, the cells are aligned in single file by a process known as 'hydrodynamic focusing'.

A 488 nm argon ion laser is used in the Coulter® EPICS® XL-MCL (see Figure 9-1). When a cell passes through the laser beam, light is scattered in different directions. The degree of light scatter in the forward direction (forward scatter (FS)) is proportional to the size of the cell, with larger cells producing more FS. Light from the laser beam also enters the cell and is reflected and refracted by the internal structures and granules within the cell. This produces side scatter (SS) light, and the degree of SS is proportional to the granularity of the cell. The cells may also be labelled with fluorochrome-linked antibodies, or stained with fluorescent dyes. Fluorochromes or
fluorescent dyes absorb laser light energy and emit fluorescence at different colour wavelengths.

The FS sensor is positioned behind the sample stream, whereas the SS and other fluorescence sensors are positioned at 90° to the laser beam and sample stream. A series of dichroic mirrors (beam splitters) direct the different components of the transmitted light to appropriate sensors, with filters removing unwanted wavelengths of light. The sensors are called photomultiplier tubes (PMTs). They serve as detectors and amplifiers that convert the transmitted light to a voltage pulse that rises and falls depending on the amount of light entering the PMT. Smaller cells or particles generate smaller voltage pulses, whereas larger cells yield larger pulses. The process of logarithmic amplification may amplify the pulses. This makes smaller pulses much larger, but amplifies larger pulses to a lesser degree, thus accentuating differences between two small pulses (Macey & Idziorek, 1994).
Figure 9-1 Diagrammatic Representation of a Flow Cell
(Redrawn from reference: Beckman Coulter United Kingdom Ltd, 1999)
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 & Shattil, 1991). Whole blood flow cytometry was used in this thesis because this method has the advantages of allowing analysis of platelets in the physiological milieu of whole blood, and is less susceptible to artefactual in vitro platelet activation and potential loss of platelet subpopulations than methods which use PRP or washed platelets (Michelson, 1996).

Table 9-1: Fluorochromes / Fluorescent Dye Used In Flow Cytometry
*RPE-Cy5 is a combination of two fluorochromes [R-phycoerthrin [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

<table>
<thead>
<tr>
<th>Fluorochrome / Fluorescent Dye</th>
<th>Emission Wavelength (nm)</th>
<th>Fluorescence</th>
<th>Fluorescence detector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td></td>
<td>Green</td>
<td>FL1</td>
</tr>
<tr>
<td>Isothiocyanate (FITC)</td>
<td>525</td>
<td>Green</td>
<td>FL1</td>
</tr>
<tr>
<td>Thiazole Orange (TO)</td>
<td>533</td>
<td>Green</td>
<td>FL1</td>
</tr>
<tr>
<td>Phycoerythrin (PE)</td>
<td>575</td>
<td>Orange</td>
<td>FL2</td>
</tr>
<tr>
<td>R-phycoerthrin-Cy5 (RPE-Cy5)*</td>
<td>670</td>
<td>Red</td>
<td>FL3</td>
</tr>
</tbody>
</table>

The fluorochromes and fluorescent dye (Thiazole Orange [TO]) used for whole blood flow cytometric analyses in this thesis are listed in Table 9-1. The fluorochromes are conjugated to monoclonal antibodies to facilitate 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 & Shattil, 1996). All of the monoclonal antibodies used in this thesis were purchased in conjugated form from the manufacturers.

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 & Shattil, 1996). All blood samples will exhibit some degree of non-specific fluorescence because of (i) autofluorescence of the sample and (ii) non-specific staining of the cell with a given monoclonal antibody or fluorochrome (Schmitz et al, 1998).

To account for this non-specific fluorescence when monoclonal antibodies were used, matched ‘isotype control’ monoclonal antibodies that were conjugated to the same fluorochrome, but that did not recognise target antigens on platelets were employed (Schmitz 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 each assay, the ‘antibody positive population’ was determined by using an analysis marker placed to the right of a histogram of ‘fluorescence versus intensity’ from a matched control sample (Michelson & Shattil, 1996). Expressing results as percent positive platelets is simpler and, unlike MFI, is independent of variation in signal amplification caused by changes in PMT voltage or gain over time, because the isotype control signal changes in proportion with the test sample (Michelson & Shattil, 1996). This method has the advantage of being very sensitive at detecting an increase in antigen expression by a small subpopulation of cells with a heterogeneous staining pattern (Schmitz et al, 1998). However, one must
remember 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. Therefore, MFI is the preferred method of data presentation if the goal is to determine the total amount of platelet surface antigen expression (Michelson & Shattil, 1996), or when minor decreases in the expression of a ubiquitous platelet marker are being investigated (Schmitz et al, 1998).

In this thesis, the percent positive platelets was calculated for each flow cytometric assay because we predicted small changes in the expression of the different platelet activation markers between groups. The voltage and gain settings on the flow cytometer were not changed during the study.
9.3.2. Quality control

To ensure day-to-day sample reproducibility, the flow cytometer was calibrated using commercially available fluorescent beads (Flow-Check® Fluorospheres, Beckman Coulter, U.K.) to verify the optical alignment of the laser and the fluidics of the system. These 10 μm fluorescent beads are excited by the 488 nm argon ion laser and emit within the wavelength range of 525 nm to 700 nm. 5,000 events were collected for each sample tested at a rate of < 200 events per second, and a half peak coefficient of variation of ≤2.0 on the relevant histograms was deemed acceptable.

9.3.3. Platelet surface markers

**GpIb (CD42b) - Principle**

Using flow cytometry, platelets can be distinguished from red and white blood cells by their characteristic pattern of forward and side scatter, which 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 (Ruggeri, 1997; Clemetson, 1997; Escolar & 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 & Shattil, 1991). The method used in our laboratory for identifying GpIb-positive platelets was adapted from the whole blood flow cytometric protocol described by Shattil et al (Shattil 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 redistribution from the platelet surface membrane to the membranes of the surface connected open canalicular system upon platelet activation (Abrams & Shattil, 1991; Schmitz et al,
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 platelet surface expression of GpIb-IX-V is time-dependent, decreasing within 30 seconds of platelet activation, reaching a nadir at approximately 5 minutes and returning to normal over the next 45 minutes. Because immediate fixation of samples was not performed before labelling with monoclonal antibodies, the reduction in Gplb 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 saline (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 – 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 debris.

**Method**

Because the concentration of the anti-IgG₁-PE isotype control antibody was 6.25 μg/ml, and the concentration of the anti-CD42b-PE monoclonal antibody was 1.56 μg/ml, 5 μl of the anti-IgG₁-PE control antibody was diluted 1:4 with 15 μl of HBS before use in the assay. 5 μl of citrate anticoagulated whole blood was aliquoted into the control and
test polypropylene tube 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. 40 µl of HBS was then added 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 and flow cytometric analysis began within 45 minutes of fixation.

A 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. 10,000 platelet events were analysed 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 using a histogram that plotted the platelet count versus log fluorescence detected in FL2. The ‘positive analysis function’ on the flow cytometer was set at 0.5% for this and all other assays performed as part of the panel set-up i.e. the percentage of ‘antibody-positive’ platelets in the test sample was determined by measuring those platelets with a fluorescence intensity exceeding that of 99.5% of the control sample. Therefore, 0.5% of the matched control sample histogram was included in the calculation of percent positive cells in the test sample to avoid excluding any weakly positive cells in the analysis. 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 $\geq 95\%$. If the % Gplb binding was $< 95\%$, the process was repeated until $\geq 95\%$

Gplb positivity was obtained for the test sample.

Because the other markers of platelet activation described below were analysed using a

‘panel’ set-up on the flow cytometer, the gating settings around the platelet cloud were

saved and not repositioned after gating on Gplb positive cells. This facilitated single

labelling of platelets with the fluorochrome-conjugated monoclonal antibody of interest.
Figure 9-2 Scatterplot and Histogram from a GpIb Control Sample

Figure 9-2-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 9-2-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 nonspecific background fluorescence of the control sample. The positive analysis function includes 0.5% of the control sample in region E.
Figure 9-3 Scatterplot and Histogram from a GpIb Test Sample

Figure 9-3-III: Scatterplot of log FS versus log SS from a GpIb test sample. The position of gate 'A' is identical to that in the control sample in Figure 9-2-I.

Figure 9-3-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
**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 & Shattil, 1991). CD62P is only expressed on the platelet surface membrane after alpha (α)-or dense-granule secretion, and the CD63 antigen is expressed on the platelet surface after its release from lysosomes or dense granules (Grau et al, 1998); (Israels et al, 1992). By adding 'activation-dependent' monoclonal antibodies that are conjugated to a fluorochrome and specific for CD62P or CD63, whole blood flow cytometry can be used to quantify the sample fluorescence, and hence, the expression of these activation markers on the platelet surface.

**CD62P**

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

**Reagents**

- Anti-IgG₁-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25ug/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 initial methodology used for the CD62P assay was identical to that used for the GpIb assay with the exception that 5 µl of anti-IgG\textsubscript{i}-PE antibody was added to the isotype control sample and 5 µl of anti-CD62P-PE antibody 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 9-4 Scatterplot and Histogram from a CD62P Test Sample

Figure 9-4-I: Scatterplot of log FS versus log SS from a CD62P test sample. Figure 9-4-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 sample expressed CD62P.
CD63

Reagents

- Anti-IgG<sub>1</sub>-FITC isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 50 ug/ml)
- Anti-CD63-FITC mouse monoclonal antibody (IgG<sub>1</sub>) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 50 ug/ml)
- HBS
- Fixative: 0.2% formalin saline solution

Method

The initial methodology used for the CD63 assay was also identical to that used for the GpIb assay with the exception that 5 μl of anti-IgG<sub>1</sub>-FITC antibody was added to the isotype control sample and 5 μl of anti-CD63-FITC antibody 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 9-5 Scatterplot and Histogram from a CD63 Test Sample

Figure 9-5-I: Scatterplot of log FS versus log SS from a CD63 test sample. Figure 9-5-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.
9.3.4. Leucocyte-platelet Complexes (LPCs)

Principle

CD62P expressed on the platelet surface membrane mediates the adhesion of platelets to leucocytes, including neutrophils, monocytes and lymphocytes (de Bruijne-Admiraal et al, 1992). The predominant receptor for CD62P on leucocytes is P-selectin glycoprotein ligand-1 (PSGL-1) (Furie et al, 2001), and the percentage of leucocytes that are bound to platelets can be measured by flow cytometry (Li et al, 1997; Furman et al, 1998). Because CD62P is rapidly shed from the surface of circulating degranulated platelets (Michelson, 1996), 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). Recent studies in patients with acute myocardial infarction or in those undergoing percutaneous coronary intervention 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 et al, 2001; Michelson, 2006). For these reasons, flow cytometry was used to measure the percentage of leucocyte-platelet aggregates in addition to measurement of the other surface markers of platelet activation.
Reagents

- Anti-IgG₁-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25 μg/ml)
- Anti-CD42b-PE mouse monoclonal antibody (IgG₁) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 1.56 μg/ml)
- Anti-CD45-RPE-Cy5 (Dako, Glostrup, Denmark. Concentration: 200 μg/ml)
- HBS
- 10 x Hanks Balanced Saline Solution (HBSS) without calcium, magnesium or phenol red (Gibco BRL, Life Technologies, Paisley, UK)
- Distilled H₂O
- 10% formaldehyde solution – 0.25 ml of 40% formaldehyde solution diluted 1:4 with 0.75ml of distilled H₂O
- 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 H₂O.

Method

Because the concentration of the anti-IgG₁-PE isotype control antibody was 6.25 μg/ml, and the concentration of the anti-CD42b-PE monoclonal antibody was 1.56 μg/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 that had been established by Joseph et al (Joseph et al, 2001). Initially, 5 μl 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. 5 μl 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. Then, the samples were fixed with 84 µl of diluent fixative, and after a further 10 minute incubation period, 840 µl of distilled water was added to each tube in order to induce erythrocyte lysis. The samples were 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 listmode 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 identify three distinct subpopulations of leucocytes (i.e. neutrophils, monocytes, and lymphocytes); these were separated by manually drawing a gate around each of the leucocyte subpopulations. Cells that were dual stained with anti-CD42b-PE 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 stopped after 1,000 monocyte events were detected.
Figure 9-6 Scatterplot from an LPC Test Sample

Figure 9-6-I: Only anti-CD45-RPE-Cy5-positive cells (leucocytes) are identified in region 'B' with RPE-Cy5 fluorescence detected in FL4.

Figure 9-6-II: Three distinct leucocyte subpopulations can be identified by their light scatter profiles, and gates drawn around each subpopulation: C = neutrophils, D = monocytes, E = lymphocytes.
Figure 9-7 Histogram from an LPC Test Sample

Figure 1-9-III, IV & V: Histograms of typical fluorescence profiles for neutrophil-platelet (III), monocyte-platelet (IV) and lymphocyte-platelet (V) complexes in a patient with ischaemic stroke. Platelets complexed to leucocytes are stained with an anti-CD42b-PE monoclonal antibody.
9.3.5. Reticulated platelets

Principle

Platelets are released into the peripheral blood following megakaryocyte fragmentation within the bone marrow and/or pulmonary circulation (Harrison et al., 1997; Lunetta & Penttila, 1997). Young platelets that have been recently released into the circulation contain a residual amount of megakaryocyte-derived mRNA and were first identified by Ingram and Coopersmith in 1969 (Ingram & Coopersmith, 1969). They were termed ‘reticulated platelets’ because of the analogy with red cell reticulocytes. Reticulated platelets were reported to be larger in size and have an increased mean density compared with normal platelets (Ingram & Coopersmith, 1969). Because reticulated platelets have been shown to be unstable and to undergo degradation within 24 hours in the circulation in animal studies (Ault & Knowles, 1995), measurement of the percentage of reticulated platelets in humans has the potential to be a useful marker of increased platelet production and/or turnover that could occur in patients with increased platelet activation.

Thiazole orange (TO) is a fluorescent dye originally synthesised for erythrocyte reticulocyte analysis (Michelson, 1996). It readily permeates live cell membranes without the need for a permeabilisation step, and fluoresces at 533 nm on binding to nucleic acids, especially RNA more than DNA (Ault et al., 1992; Michelson, 1996). Therefore, TO can be used to label reticulated platelets within the circulation. Whole blood flow cytometric methods have been developed to identify reticulated platelets using TO, but there is no ‘gold standard’ reference method available and no standardised control against which the results obtained can be compared (Harrison et al., 1997; Robinson et al., 2000b). 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 et al, 1998; Robinson et al, 2000a; Robinson et al, 2000b). A low concentration of TO was therefore used in this assay.

Reagents

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

Method

Experiments in our laboratory 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. Subsequent experiments that were performed in the laboratory also showed 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. However, analyses were performed on non-refrigerated samples in this thesis. 1 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, incubated for exactly 30 minutes and then centrifuged at 1200 g for 2.5 minutes. The supernatant was discarded to prevent further incubation of the test sample with Retic-COUNT™, and the remaining pellet was resuspended in 1 ml of Isoton® II before being analysed on the flow cytometer within an hour of resuspension.
The platelet cloud in the control sample tube was identified on a scatterplot of log FS versus log SS that had been saved on the flow cytometer. 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 9-8 Scatterplot of Log FS vs. SS from TO Control and Test Sample

Figure 9-8-II: 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 9-8-III: 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 9-9 Scatterplot of Log FS vs. SS from TO Control and Test Sample

Figure 9-9-IV: Scatterplot of log fluorescence in FL1 versus log FS from a TO test sample confirms that the TO-positive platelets (blue cells) are amongst the largest of the platelet cloud.

Figure 9-9-V: Histogram of cell count versus log fluorescence detected in FL1 from the same sample; flow cytometric analysis showed that 16.8% of platelets in the region of interest in this patient sample were TO-positive.
9.4. Platelet Function Analyser - PFA-100®

9.4.1. Background

Reliable evaluation of platelet function is critically important in the diagnosis and management of patients with platelet-related bleeding disorders, and has the potential to be very useful in both monitoring and predicting the response to antiplatelet therapy. Until recently, the *in vivo* bleeding time was the only widely available global screening test of platelet function. However, the test is invasive, time consuming, poorly reproducible, insensitive (Harrison, 2000), and cannot be used to serially monitor the response to therapy (Kerényi *et al.*, 1999). Platelet aggregometry has been considered to be the ‘gold standard’ test of platelet function over the past five decades, and is based on the principle that platelets aggregate in response to exogenous agonists added to the system (Born G.V.R. & Cross M.J., 1963; Harrison, 2000). However, because aggregometry is often performed using platelet rich plasma (PRP), platelet function is not usually studied in the physiological milieu of whole blood. The test also requires a considerable amount of sample preparation, and it is labour intensive and reasonably expensive.

9.4.2. General Principle

The PFA-100® (Dade-Behring, Germany) is a device that was designed to test platelet function in whole blood by simulating the *in vivo* haemostatic process at moderately high shear stress rates (Kundu *et al.*, 1995). During the test, 800 μl of citrated anticoagulated whole blood is aliquoted into two disposable test cartridges that are placed in a carousel.
**Legend for Figure 9-10:**

**In Vivo Haemostasis,** If there is a defect in the endothelial lining of an artery, as may occur if there is rupture of a stenosing atherosclerotic plaque, blood flow at and distal to the stenosis will become turbulent, thus increasing the shear stress that platelets are exposed to. This will in turn, activate the platelets, and if there is exposure of subendothelial collagen, the platelets will adhere to the plaque and subsequently aggregate to one another. A platelet-rich thrombus forms that will help to stabilise the plaque, but may also lead to subsequent platelet thromboembolism.

**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 (see text) (Figure redrawn from slide kindly donated by Dade-Behring, Germany). 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 9-11).
Figure 9-11 PFA-100® Cup-capillary system

A: The 800 µl blood sample is loaded into the sample loading port at the start of the test and is 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 (see text) (redrawn from slides kindly donated by Dade-Behring, Germany)

The shear rate that the blood sample is exposed to is equivalent to that seen in a moderately stenosed artery (Kroll et al, 1996). The membrane is coated with collagen (2 µg) in combination with either ADP (50 µg in the C-ADP cartridge) or epinephrine bitartrate (10 µg in the C-EPI cartridge). At the beginning of the test, a predetermined volume of saline trigger solution is dispensed onto the membrane to solubilise the ADP or epinephrine. The combination of high shear stress and biochemical stimulation activates the platelets, they adhere to the membrane and aggregate to one another, thus forming a platelet plug that ultimately occludes the aperture.
The time taken to occlude the aperture is called the ‘closure time’ and this provides a measure of platelet function in the sample. The maximum closure time recorded by the device is 300 s, and results greater than 300 s are recorded as “test time exceeded”. We arbitrarily defined these closure times as 301 s. The procedure is fully automated and the results are printed out when the test is completed. The cartridges are disposed of at the end of the test and cannot be reused. Because the high concentration of ADP in the C-ADP cartridge provides a stronger stimulus to platelet activation than the epinephrine in the C-EPI test cartridge (Heilmann et al, 1997), and because ADP can mediate platelet aggregation independent of the arachidonic acid pathway, the C-EPI cartridge should be more sensitive at identifying aspirin-induced platelet dysfunction than the C-ADP cartridge. The test results are influenced by the levels of functional von Willebrand Factor (VWF) in the circulating blood (Kundu et al, 1995; Fressinaud et al, 1998; Harrison et al, 1999), and by the platelet count and haematocrit (Harrison et al, 1999).

9.4.3. Quality control

The PFA-100® test system utilises a highly integrated microcontroller chip to manage and monitor the functions of the instrument (Kundu et al, 1995). Each day, an automated self-test was performed that assessed the function of all of the major components and subsystems within the instrument. The microcontroller chip detected any problems with the function of the instrument, and this information was displayed on the liquid crystal display (LCD) on the front of the instrument. The test could not proceed until the problem had been rectified and a repeat self-test performed to validate this. In our laboratory, the coefficients of variation (CVs) for the C-ADP and C-EPI assays in normal control subjects were 7% and 7.5%, respectively.
9.4.4. Methodological issues

Initially, it was reported that the results obtained with the PFA-100® did not vary significantly in normal controls when the samples were analysed repeatedly during a five to six hour period following venepuncture (Mammen et al., 1995; Harrison et al., 1999). 0.129 M (3.8%) or 0.105 M (3.2%) buffered trisodium citrate Becton Dickinson Vacutainer® tubes were used in these studies, respectively. Subsequently, von Pape et al. carried out a study on 24 subjects who had recently discontinued long term aspirin therapy (100 mg daily) prior to epidural anaesthesia (von Pape et al., 2000). As part of this study, venepuncture was performed using 0.106 M and 0.129 M buffered sodium citrate Sarstedt Monovette tubes, and the blood samples were analysed at ≤ 1 minute, and subsequently at 10 and 60 minutes after venepuncture. They reported that the median C-EPI closure time was significantly prolonged at ≤ 1 minute after venepuncture, but significantly shortened relative to this baseline level at 10 and 60 minutes after venepuncture using 0.106 M buffered sodium citrate. In contrast, the median C-EPI closure time was persistently prolonged throughout this time period using 0.129 M buffered sodium citrate. The authors suggested that PFA-100® testing for aspirin-induced platelet dysfunction should only be performed using 0.129 M buffered sodium citrate. However, the results obtained from this small subgroup were not consistent with those from a larger group of 80 patients who had discontinued aspirin therapy within the preceding 24 hours, and in whom C-EPI closure times were measured between 10 and 20 minutes after venepuncture. In the larger study, the median C-EPI closure time was prolonged in comparison with normal controls when 0.106 M buffered sodium citrate was used as an anticoagulant, despite the interval to sample analysis of 10 to 20 minutes.
Although these studies were not directly comparable because of the different blood collection systems and concentrations of sodium citrate used, the disparity in results did raise some uncertainty about the effects of the time interval between venepuncture and sample processing on the results obtained. For this reason, all tests in this thesis were performed between 120 and 150 minutes after venepuncture to standardise the time interval between venepuncture and PFA-100® analysis. In addition, because closure times tend to be longer with 0.129 M compared with those seen with 0.105 M or 0.106 M buffered sodium citrate blood collection systems, only 0.105 M (3.2%) buffered sodium citrate Vacutainer® tubes were used in our laboratory (Mammen et al, 1995; Heilmann et al, 1997; von Pape et al, 2000).

9.5. Routine Haematology Investigations

9.5.1. Full Blood Count

For each study subject, 4 ml of blood was drawn into two 2 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, as described previously. A full blood count was performed in all subjects using a Sysmex XE-2100 haematology analyser (Sysmex U.K. Ltd., Milton Keynes, U.K.). Furthermore, because EDTA causes more platelet swelling over time compared with citrate, and because the sodium citrate solution in the Vacutainer® tube dilutes the sample and reduces the platelet count more than EDTA (Bath, 1993), measurements of the total platelet count and MPV were performed using both anticoagulants. In addition, because the MPV increases and the platelet count decreases over time with both anticoagulants (Bath, 1993), it was planned that the FBC measurements would be performed between 2 and 4 hours after venepuncture to standardise the effect of the delay between venepuncture and sample analysis on the results obtained.
9.5.2. Coagulation Assays

Patients who were or had been receiving warfarin or heparin therapy had their INR and APTT ratios checked in the routine haematology laboratory at AMNCH using standard techniques to determine whether they were adequately anticoagulated on treatment or not.
9.6. Endothelial Activation

9.6.1. General principle

As discussed earlier, activated platelets play a key role in arterial thrombus formation, and because platelets interact with both the endothelium and coagulation system, endothelial or coagulation system activation has the potential to cause or exacerbate an ischaemic insult in patients with cerebrovascular disease (CVD).

In this thesis, I assessed von Willebrand factor antigen (VWF:Ag) and von Willebrand factor propeptide (VWF:Ag II) levels as markers of endothelial activation in patients following ischaemic stroke or TIA, and to assess the impact of changing antiplatelet regimens on these parameters over time. The nature and functions of these proteins has been discussed in Chapter 6 [Review of the literature on von Willebrand factor and von Willebrand factor propeptide in TIA and ischaemic stroke].

9.6.2. Subject Groups

(a) Case-control study: Patients were assessed within 4 weeks of onset of a TIA or ischaemic stroke, regardless of their prescribed antiplatelet regimen.

Control subjects, of similar age and gender, who did not have a history of vascular disease, were assessed once.

(b) Longitudinal study in ischaemic CVD patients following changes in antiplatelet therapy: Patients who were enrolled in the platelet activation and platelet function studies, outlined in Section 9.3 [Flow Cytometry] and Section 9.4 [Platelet Function Analyser - PFA-100®], were assessed at each time point: i.e. before (baseline), and 14
days after changing (14d) from one antiplatelet regimen to another, and again at >90 days (90d) following symptom onset. Three groups of patients undergoing alteration in antiplatelet therapy were studied:

- Patients initially assessed on no medication and subsequently assessed on aspirin.
- Patients initially assessed on aspirin and subsequently assessed on the combination of aspirin and dipyridamole MR.
- Patients initially assessed on aspirin and subsequently assessed on clopidogrel monotherapy.

9.6.3. Laboratory Methods

Platelet Poor Plasma (PPP)

PPP was obtained from fresh citrate-anticoagulated whole blood by centrifugation as described earlier, and stored at -70°C. Samples were thawed once, in a water bath at 37°C for 20 minutes before analysis in the VWF:Ag ELISA assay. Samples were then refrozen and stored at -70°C until they were thawed again for the VWF:Ag II ELISA assay.
Enzyme Linked Immunosorbant Assay (ELISA)

Reagents

- Polyclonal rabbit Anti-Human VWF antibody (DAKO, Denmark)
- Polyclonal rabbit Anti-Human VWF/HRP antibody (DAKO, Denmark)
- Anti-human VWF propeptide M193902 CLB-Pro 35 coating antibody and M103904HRP CLB-Pro 14.3 detection antibody (Sanquin Reagents, Amsterdam, The Netherlands).

Method

The concentration of VWF:Ag and VWF:Ag II in each PPP sample was quantified, as previously described (O'Donnell et al, 2005; Preston et al, 2009). Double spun PPP that had been collected and prepared as outlined in section 9.2 was warmed to 37°C. Polyclonal Rabbit Anti-Human VWF antibody (DAKO) was used as coating antibody, and polyclonal rabbit Anti-Human VWF/HRP antibody (DAKO) as a detection antibody for the VWF:Ag ELISA. M193902 CLB-Pro 35 coating antibody (Plesmanlaan 125) and M103904HRP CLB-Pro 14.3 detection antibody (Plesmanlaan 125) were used for VWF:Ag II quantification. The ELISA data were quantified by spectrophotometry at 490nm, using a VERSA Max Tuneable Microplate Reader. VWF:Ag and VWF:Ag II levels were recorded as μg/mL.
9.7. Coagulation System Potential

9.7.1. General principle

Platelet activation is also intricately linked to activation of the coagulation system (Monroe et al., 2002). Increased thrombin production has been demonstrated in platelet rich plasma from patients > 3 months following an acute stroke or TIA (van der Meijden et al., 2005). The contribution of platelet activation to overall thrombin generation in the both the early and late phases after ischaemic stroke is undetermined. Whether increased thrombin production is 'platelet-dependant' or 'platelet-independent' is uncertain. In this thesis, thrombin generation was assessed as a marker of coagulation system potential.

9.7.2. Subject Groups

We performed (a) a case-control study and (b) a longitudinal study in the subject groups described in Section 9.6 [Endothelial Activation] above.

9.7.3. Laboratory Methods

Platelet Poor Plasma (PPP)

PPP was obtained from fresh citrate-anticoagulated whole blood by centrifugation, as described earlier, and stored at -70°C. Samples were thawed once in a water bath at 37°C for 20 min before analysis in the thrombin generation assay.
Thrombin Generation Assay:

Reagents

- 5pM PPP-Reagent, containing a mixture of phospholipids and tissue factor (Thrombinoscope BV, Netherlands)
- Thrombin Calibrator (Thrombinoscope BV, Netherlands)
- Fluo-Substrate, containing the fluorogenic substrate solubilised in dimethyl sulfoxide (DMSO) (Thrombinoscope BV, Netherlands).
- Fluo-Buffer, containing Hapes (pH 7.35) and Calcium Chloride. (Thrombinoscope BV, Netherlands)
- Fluroskan Ascent® microplate fluorometer with Thrombinoscope™ software (Thrombinoscope BV, Netherlands)

Method

The method is briefly described here, but described in more detail in Chapter 15 [Coagulation system potential in TIA and ischaemic stroke]. Coagulation system potential was measured, as previously described, in a 96-well polystyrene plate (Faber et al, 2003). Fluo-Substrate was added to the warmed Fluo-Buffer shortly before the experiment to prepare FluCa, and warmed in a water bath at 37°C prior to the experiment.

20 uL of 5pM PPP-Reagent was added to 80 uL of platelet poor plasma in triplicate. Thrombin Calibrator was suspended in 1 ml of deionized water. 20 uL of Thrombin Calibrator was added to 80 uL of platelet poor plasma from the same patient in a fourth well to act as an internal control. The plate was then pre-heated to 37°C for 5 min in the Fluroskan Ascent® microplate fluorometer. The device automatically dispensed 20 µL of
premixed warmed FluCa into each of the wells, so that the final reaction mixture contained 5 pM tissue factor and 4 μM phospholipids.

The Fluroskan Ascent® microplate fluorometer automatically measured the fluorescent signal that corresponded to thrombin generation every 20 seconds. Corrected thrombin generation curves were calculated by the Thrombinscope™ software, after accounting for the fluorogenic signal the signal from the internal control.
10. Assessment of the impact of aspirin and clopidogrel on platelet activation and function in the early and late phases after TIA or ischaemic stroke

10.1. Introduction
Antiplatelet agents have the potential to play a key role in the secondary prevention of vascular events in patients with ischaemic cerebrovascular disease (CVD), but there are no routinely employed reliable laboratory data to guide optimal antiplatelet treatment in individual patients, akin to the INR in patients on warfarin. In addition to the importance of investigating inhibition of platelet function \textit{ex vivo} after the commencement of dipyridamole (Chapter 11 – Assessment of the impact of dipyridamole on platelet activation and function in the early and late phases after TIA or ischaemic stroke), one needs to longitudinally quantify the effects of adding other commonly prescribed antiplatelet regimens (aspirin or clopidogrel monotherapy) on platelet activation and function in patients following TIA or ischaemic stroke.

Previous studies investigating the effects of aspirin and clopidogrel on platelet activation have been discussed in Chapter 4 [Is Platelet Activation Increased Following TIA or Ischaemic Stroke?]. In brief, there is no convincing evidence that platelet activation status is altered by commonly prescribed antiplatelet regimens, although studies to date have been small, or have not been specifically designed to longitudinally detect alterations in platelet activation after the addition of antiplatelet agents.
In one longitudinal study on platelet activation in patients with ischemic CVD, platelet CD62P and CD63 expression in fixed whole blood was found to be similar in patients on treatment with aspirin, clopidogrel, or anticoagulants 90 days following onset of ischaemic stroke (n = 50, p = NS) (Marquardt et al, 2002). In a further study, the expression of CD62P and CD63 in fixed whole blood was not significantly different on treatment with aspirin (100 to 300 mg daily), clopidogrel (75 mg daily), or aspirin and clopidogrel combination therapy in patients in the convalescent phase after atherothrombotic (n = 20) or lacunar ischaemic stroke (n = 11) (Grau et al, 2003). The results were similar in patients on 300 mg of aspirin daily (n = 26) compared with those on treatment with < 300 mg daily (n = 5) (p > 0.3)

Data on the assessment of inhibition of platelet function ex vivo at high shear stress on the PFA-100® are also quite limited in ischaemic CVD. Aspirin has been shown to prolong C-EPI closure times in patients with TIA or ischaemic stroke either as monotherapy (Serebruany et al, 2004a; McCabe et al, 2005b; von Lewinski et al, 2009) or in combination with clopidogrel (Grau et al, 2003; von Lewinski et al, 2009).

Treatment with aspirin monotherapy resulted in significantly prolonged C-EPI closure times compared with the patients’ baseline on no medication (p < 0.05) in Japanese patients 2-6 months after TIA or lacunar ischaemic stroke (Serebruany et al, 2004a). Serebruany et al. also reported that C-ADP closure times were not prolonged at 15 days, but were significantly prolonged after 30 days of treatment with clopidogrel (p < 0.05) in type II diabetic patients in the late stage after TIA. Treatment with clopidogrel monotherapy in patients with ischaemic stroke or TIA has been shown to prolong C-ADP closure times by some authors (Raman & Jilma, 2004), but not by others (Grau et
Interestingly, combination therapy with aspirin and clopidogrel monotherapy has been shown to prolong C-EPI closure times to a greater degree than treatment with either agent alone (Grau et al., 2003), suggesting a synergistic effect of aspirin and clopidogrel on C-EPI closure times.

Therefore, few well-designed, longitudinal studies have assessed the impact of starting aspirin, or changing from aspirin to clopidogrel monotherapy in patients following TIA or ischaemic stroke. Most prior studies have used a ‘cross-sectional definition’ of antiplatelet nonresponsiveness, where patients were only deemed responsive to an antiplatelet medication if their test results exceeded a normal ‘control range’. This definition of *ex vivo* antiplatelet non-responsiveness has several limitations. From a statistical viewpoint, it does not account for inter-individual differences in platelet function, and the number of patients required to detect an effect is therefore higher, as random variation in both intra-individual and inter-individual platelet function needs to be accounted for before determining that an effect is significant. Secondly, patients who have had an ischaemic stroke or TIA may benefit from some degree of inhibition of platelet function, and do not necessarily require profound inhibition of platelet function which might be associated with a significantly increased risk of bleeding. Physicians routinely employ such a strategy in patients with TIA or stroke of cardioembolic aetiology who are treated with warfarin to achieve an INR of 2.5. Although a similar paradigm may be appropriate for treatment with antiplatelet agents, a suitable, user-friendly, clinically-relevant test has yet to be validated in patients with CVD.
To address these issues, we designed a longitudinal, case-crossover study to simultaneously investigate platelet activation and function in ischaemic CVD patients who were commencing aspirin, or changing from aspirin to clopidogrel monotherapy.
10.2. Aims and Hypotheses

Aims: We designed a pilot, longitudinal, case-crossover study in ischaemic CVD patients to thoroughly assess whether (a) commencing aspirin therapy in patients not on antiplatelet therapy or (b) changing from aspirin to clopidogrel monotherapy significantly inhibited ex vivo platelet activation or platelet function. We also examined the relationship between platelet activation and function in whole blood to investigate whether platelet activation status interfered with the inhibitory effects of antiplatelet agents on platelet function.

We hypothesised that inhibition of platelet function would be greater, and platelet activation would be less marked in some patients after commencing aspirin, and in some patients on clopidogrel monotherapy versus aspirin monotherapy. We also hypothesised that a novel definition of antiplatelet non-responsiveness to aspirin and clopidogrel could be established with these longitudinal data on an existing test of platelet function, called the PFA-100®. We anticipated that the prevalence of antiplatelet non-responsiveness, according to these longitudinal definitions, would be lower than previously reported in studies using ‘cross-sectional, case-control’ definitions of antiplatelet non-responsiveness on the PFA-100®.
10.3. Methods

10.3.1. Recruitment

*Inclusion criteria:*

Consecutive eligible patients older than 18 years of age, who had experienced a TIA or ischaemic stroke within the preceding four weeks, and whose treating physician opted to change their antiplatelet treatment regimen from either no antiplatelet therapy to aspirin monotherapy, or aspirin to clopidogrel monotherapy were recruited. Patients were recruited from the Rapid Access Stroke Prevention Clinic, and from the inpatient population of the Neurology and Age-Related Health Care and Stroke Service at AMNCH.

*Exclusion criteria:*

Patients were excluded if they had a history of primary intracerebral haemorrhage, myocardial infarction within the preceding 3 months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding 3 months (haemoglobin decrease of >1g/dl in one day, or requiring transfusion), systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis, platelet count <120 x 10^9/L or >450 x 10^9/L, urea >10mmol/l or GFR <30ml/min, known platelet disorder, current infection (clinical signs of infection or white cell count >11 x 10^9/L), or non-steroidal anti-inflammatory use within 14 days of recruitment.

Written informed consent, or assent where appropriate, was obtained from all subjects, and this study was approved by the St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2007/07/MA).
10.3.2. Clinical assessment

Clinical assessment was performed as outlined in Chapter 9 [General Methods]. In brief, all subjects underwent a detailed clinical assessment by at least one of three examiners, and had neurovascular work-up performed according to ESO guidelines (European Stroke Organisation (ESO) Executive Committee & ESO Writing Committee, 2008). The underlying mechanism responsible for the TIA or ischaemic stroke was categorized according to the TOAST classification (Adams, Jr. et al, 1993).

All patients underwent clinical and laboratory assessment before (baseline), 14 days after (14d), and ≥ 90 days (90d) after a change in their antiplatelet therapy. In the “large artery atherosclerosis / extracranial carotid stenosis subgroup, the 90d follow-up was performed at least 3 months following carotid surgery or endovascular treatment, unless this treatment had been delayed for at least 3 months after the initial presenting TIA or stroke.

Because most patients at baseline and 14d were assessed during their inpatient stay, adherence to antithrombotic therapy in inpatients was confirmed by checking the inpatient prescription chart. Adherence in outpatients was assessed by history taking alone, but all were phoned to stress 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 regimen.

10.3.3. Blood sampling and laboratory tests

Blood sampling and laboratory testing was performed as described in Chapter 9 [General Methods]. In brief, venepuncture was performed in a standardised manner, as described previously (Chapter 9 [General Methods]) (McCabe et al, 2004b). Full blood
counts were performed between 2 and 4 hours following venepuncture in EDTA- and citrate-anticoagulated whole blood. Platelet activation status in unstimulated whole blood was assessed with flow cytometry. The whole blood flow cytometry technique employed in this study was adapted from a previously described (Shattil et al, 1987) and validated protocol (McCabe et al, 2004b) as outlined in Chapter 9 [General Methods] to quantify platelet surface expression of CD62P and CD63, and leucocyte-platelet complex formation.

Inhibition of platelet function was assessed with the PFA-100® C-EPI and C-ADP cartridges in citrate-anticoagulated whole blood between 2 and 2.5 hours after venepuncture, as described in Chapter 9 [General Methods]. The maximum closure time recorded by the device is 300s, and we arbitrarily defined closure times above 300 s as 301 s for non-parametric statistical analysis.

Previous studies have used arbitrary ‘cut-points’ derived from normal ranges in healthy control populations to define patients as having *ex vivo* antiplatelet ‘responsiveness’ or ‘non-responsiveness’ on the PFA-100®. The potential limitations of such a ‘cross-sectional’ definition of non-responsiveness have been outlined previously and could adversely influence the potential value of the PFA-100® in predicting the risk of recurrent vascular events in patients with CVD. We therefore also established novel definitions of antiplatelet ‘non-responsiveness’ on the PFA-100® in the two patient groups in this study using patients as their own baseline controls. The coefficient of variation (CV) of the C-EPI assay was 7.5% and that of the C-ADP assay was 7% in our laboratory. Because the C-EPI cartridge is sensitive at detecting inhibition of platelet function with aspirin in 83-100% of healthy controls (Kundu et al, 1995; Mammen et al,
1995; Harrison et al, 1999), aspirin non-responsiveness was defined as failure to prolong the C-EPI closure time compared with the patient’s own baseline on no antiplatelet therapy by more than twice the CV of the C-EPI assay i.e. failure to prolong C-EPI closure times by >15% of the patient’s baseline C-EPI closure time.

At the time when this study was being performed, prior to the development of the INNOVANCE® PFA P2Y* cartridge (Linnemann et al, 2010), there was some controversy over whether one could detect inhibition of platelet function on clopidogrel with the PFA-100® C-ADP cartridge (Grau et al, 2003; Raman & Jilma, 2004; Kotzailias et al, 2007). Because clopidogrel is a P2Y\(_{12}\) ADP-receptor antagonist, and because aspirin prolongs C-ADP closure times in only a minority (0 - 24.2%) of controls (Mammen et al, 1998; Grundmann et al, 2003; Serebruany et al, 2004b; McCabe et al, 2005a), we investigated whether one could detect inhibition of ADP-induced platelet reactivity with the C-ADP cartridge following a change from aspirin to clopidogrel in this longitudinal study. Clopidogrel non-responsiveness was therefore defined as failure to prolong the C-ADP closure time after treatment with clopidogrel compared with the patient’s own baseline on aspirin monotherapy by more than twice the CV of the C-ADP assay i.e. failure to prolong C-ADP closure times by >14% of the patient’s baseline C-ADP closure time.
10.3.4. Statistical Methods

Chi squared tests were used to compare proportions between groups. Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively. The Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. Spearman rank-order correlation was used to assess the relationship between platelet function and platelet activation. $P < 0.05$ was considered to be statistically significant. All statistical calculations were performed using R, version 2.10.1 (R Development Core Team, 2009).
10.4. Results

Twenty six patients were assessed at baseline on no medication. Twenty five of those patients were reassessed 14d after commencing aspirin monotherapy, and 10 patients had serial data at baseline, 14d and 90d after commencing aspirin monotherapy. One patient was only assessed at baseline and at 90d after commencing aspirin therapy, because of a wrist fracture during the early follow-up phase. The remaining patients were changed to an alternate anti-thrombotic regimen (usually combination antiplatelet therapy or anticoagulation by their treating physician prior to the 90d assessment, and this explains why only 10 (38%) patients had 90d outcome data in the aspirin monotherapy group. In this patient group, the median daily aspirin dose was 300 mg at 14d and 75 mg at 90d.

Twenty two patients were assessed at baseline on aspirin monotherapy and subsequently 14 days after changing to clopidogrel monotherapy, and 20 of these patients had 90d follow up data, and thus had data at all 3 time points. The 2 patients who did not have 90d outcome data had undergone a change in antiplatelet therapy prior to this assessment: one for medication intolerance, and one following renal artery stent insertion. In this group of patients who changed from aspirin to clopidogrel, the median daily dose of aspirin at baseline was 75mg; all patients were on 75 mg of clopidogrel daily at both 14d and 90d.

One patient had a prolonged collagen ephinephrine closure time (> 300 seconds). This patient confirmed that he had not taken aspirin or NSAIDS over preceding 14 days. It is possible that this patient had an undiagnosed platelet-related bleeding disorder, although his collagen ADP closure time was not prolonged (103 seconds).
Figure 10-1 Flowchart of patients assessed initially on no antiplatelet medication and subsequently on aspirin monotherapy

Baseline  No antiplatelets  (N = 26)

Day 14  Aspirin monotherapy  (N = 25)

Day 90  Aspirin monotherapy  (N = 10)

16 patients not reassessed  (Not on aspirin monotherapy)

1 patient not reassessed  (wrist fracture)

Figure 10-2 Flowchart of patients assessed initially on aspirin monotherapy and subsequently on clopidogrel monotherapy

Baseline  Aspirin monotherapy  (N = 22)

Day 14  Clopidogrel monotherapy  (N = 22)

Day 90  Clopidogrel monotherapy  (N = 20)

2 patients not reassessed  (Not on clopidogrel)
The clinical details of the study subjects (Table 10-1), and their stroke subtypes (Table 10-2) are tabulated below. No patient had a recurrent vascular event during follow-up.

### Table 10-1: Demographic Data of Patients at Enrolment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No Medication – Aspirin (n = 26)</th>
<th>Aspirin – Clopidogrel (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age (years)</td>
<td>57 (±14)</td>
<td>70 (±9)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>18 / 8</td>
<td>14 / 8</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>IHD</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Hypertension</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>A Fib/Flutter at Enrolment</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Family History of Stroke</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Migraine</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Never smoker</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Statin Therapy*</td>
<td>5</td>
<td>17</td>
</tr>
</tbody>
</table>

TIA – Transient Ischaemic Attack, IHD – Ischaemic Heart Disease, A Fib – Atrial Fibrillation, DVT – Deep Venous Thrombosis, PE – Pulmonary Embolism  
*Refers to number of patients on statin therapy at enrolment

<table>
<thead>
<tr>
<th>TIA / Stroke Subtype</th>
<th>No Meds – Aspirin (N = 26)</th>
<th>Aspirin – Clopidogrel (N = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Artery Atherosclerotic</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>7 (27%)</td>
<td>3 (13%)</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>6 (23%)</td>
<td>3 (13%)</td>
</tr>
<tr>
<td>Undetermined Aetiology</td>
<td>9 (35%)</td>
<td>15 (68%)</td>
</tr>
<tr>
<td>Other Determined</td>
<td>4 (15%)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>
10.4.1. Effect of commencing aspirin or clopidogrel on platelet activation and function

*Platelet activation after changing from no medication to aspirin*

The commencement of aspirin monotherapy in antiplatelet-naive patients did not significantly influence platelet surface CD62P or CD63 expression, or the percentage of any circulating leucocyte-platelet complexes during follow-up at 14d or 90d (Table 10-3 and Figure 10-3).
### Table 10-3: Platelet activation and platelet function in response to commencement of aspirin monotherapy in 'antiplatelet naïve' ischaemic CVD patients

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n =26)</th>
<th>14d (n =25)</th>
<th>90d (n =11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet Surface</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD62P %</td>
<td>2.02 (1.35-3.07)</td>
<td>2.54 (1.54-2.68)</td>
<td>2.57 (1.11-3.36)</td>
</tr>
<tr>
<td>p value</td>
<td>0.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CD63 %</td>
<td>10.10 (7.29-15.10)</td>
<td>11.10 (6.82-14.30)</td>
<td>10.40 (6.68-11.90)</td>
</tr>
<tr>
<td>p value</td>
<td>0.7</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td><strong>Leucocyte-Platelet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Complexes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil – Platelet</td>
<td>2.77 (2.40-3.08)</td>
<td>2.61 (2.33-2.99)</td>
<td>2.42 (2.00-2.63)</td>
</tr>
<tr>
<td>Complexes</td>
<td>p value</td>
<td>0.09</td>
<td>0.2</td>
</tr>
<tr>
<td>Monocyte – Platelet</td>
<td>4.90 (3.50-5.60)</td>
<td>4.70 (3.98-5.88)</td>
<td>4.50 (3.75-5.20)</td>
</tr>
<tr>
<td>Complexes</td>
<td>p value</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Lymphocyte - Platelet</td>
<td>2.40 (2.08-2.66)</td>
<td>2.49 (2.25-2.66)</td>
<td>2.10 (1.94-2.34)</td>
</tr>
<tr>
<td>Complexes</td>
<td>p value</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>PFA-100</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-EPI</td>
<td>102 (81-116)</td>
<td>288 (120-301)</td>
<td>139 (117-174)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.0001</strong></td>
<td></td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>C-ADP</td>
<td>84 (73-99)</td>
<td>91 (74-103)</td>
<td>80 (71-94)</td>
</tr>
<tr>
<td>p value</td>
<td>0.2</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

P values refer to comparison between data at 14d and 90d follow-up versus baseline
Values are medians (25-75% inter quartile range)
Figure 10-3 Percentage of circulating leucocyte platelet complexes in response to commencement of aspirin.

Each point represents a single patient, red lines represent medians; p values refer to comparisons of median values at 14d and 90d compared with the median value at baseline on no antiplatelet therapy.
Platelet activation after changing from aspirin to clopidogrel monotherapy

There were no significant changes in platelet surface CD62P or CD63 expression after changing from aspirin to clopidogrel monotherapy (Table 10-4). There was an initial significant reduction in the percentage of circulating neutrophil-platelet complexes 14d after changing from aspirin to clopidogrel monotherapy (p = 0.02), but this difference did not remain statistically significant at the 90d follow-up timepoint compared with baseline (p = 0.3, Table 10-4). There was no significant reduction in the percentage of monocyte-platelet or lymphocyte-platelet complexes at any time point during follow-up (Table 10-4).
Table 10-4: Platelet activation and platelet function in response to changing from aspirin to clopidogrel monotherapy following TIA or ischaemic stroke

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=22)</th>
<th>14d (n=20)</th>
<th>90d (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet Surface Markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD62P %</td>
<td>1.80 (1.61-2.22)</td>
<td>1.65 (1.04-2.06)</td>
<td>1.91 (1.34-2.33)</td>
</tr>
<tr>
<td>p value</td>
<td>0.2</td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>CD63 %</td>
<td>11.30 (6.73-16.58)</td>
<td>8.48 (6.56-14.30)</td>
<td>11.90 (6.75-15.85)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Leucocyte - Platelet Complexes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil - Platelet Complexes</td>
<td>2.81 (2.37-3.08)</td>
<td>2.30 (2.25-2.61)</td>
<td>2.56 (2.24-3.00)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.02</strong></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Monocyte - Platelet Complexes</td>
<td>4.90 (4.18-6.00)</td>
<td>4.50 (4.15-5.10)</td>
<td>5.10 (4.40-5.85)</td>
</tr>
<tr>
<td>p value</td>
<td>0.1</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Lymphocyte - Platelet Complexes</td>
<td>2.65 (±0.61)</td>
<td>2.36 (±0.48)</td>
<td>2.34 (±0.52)</td>
</tr>
<tr>
<td>p value</td>
<td>0.1</td>
<td></td>
<td>0.1</td>
</tr>
</tbody>
</table>

**PFA-100**

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=22)</th>
<th>14d (n=20)</th>
<th>90d (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-EPI</td>
<td>150 (127-301)</td>
<td>114 (90-129)</td>
<td>109 (96-123)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>&lt;0.001</strong></td>
<td></td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>C-ADP</td>
<td>82 (74-96)</td>
<td>101 (86-108)</td>
<td>98 (82-112)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.001</strong></td>
<td></td>
<td>0.09</td>
</tr>
</tbody>
</table>

P values refer to comparison between data at 14d and 90d follow-up vs. baseline
Values are means (±standard deviation) or medians (25-75% inter quartile range)
Figure 10-4 Percentage of circulating leucocyte platelet complexes in response to changing from aspirin to clopidogrel.
Each point represents a single patient, red lines represent medians and p values refer to comparisons of median values at 14d and 90d compared with the median value at baseline on aspirin
Platelet function inhibition after changing from no medication to aspirin

The commencement of aspirin monotherapy significantly prolonged median C-EPI closure times at 14d (288s vs. 102s, \( p = 0.0001; \ n = 25 \)) and 90d (139s vs. 87s, \( p = 0.002; \ n = 11 \)), but not median C-ADP closure times (\( p > 0.2 \)) during follow-up at 14d or 90d (Table 10-3 and Figure 10-3).

In the subgroup of 10 patients who had serial data at all three time points after changing from no medication to aspirin, there was a trend toward prolongation of the median C-EPI closure time from 87s at baseline to 120s at 14d (\( p = 0.07 \)), and a significant increase from to 143s at 90d (\( p = 0.008 \)). Using our novel definition of aspirin non-responsiveness on the PFA-100, \( \frac{6}{25} \) (24%) patients had \textit{ex vivo} non-responsiveness to aspirin on the PFA-100 at 14d, and \( \frac{2}{11} \) (18%) patients were aspirin non-responsive at 90d. The two non-responders at 90d had both previously been aspirin responsive at the 14d follow-up visit. Conversely, two patients who were deemed non-responsive at 14d were responsive at the 90d follow-up visit. In the subgroup of 10 patients with serial data at all three time points after commencing aspirin, 6 (60 %) patients retained the same 'aspirin responder status' at both 14d and 90d.
Platelet function inhibition after changing from aspirin to clopidogrel

In the group of patients who were changed from aspirin to clopidogrel monotherapy, the median C-EPI closure time significantly decreased at both 14d and 90d compared with baseline (\(p \leq 0.007\)), consistent with discontinuation of aspirin therapy (Table 10-4). Conversely, compared with baseline values on aspirin, the median C-ADP closure time significantly increased at 14d after changing from aspirin to clopidogrel monotherapy (\(p = 0.001\)), but this effect did not remain statistically significant at 90d (\(p = 0.09\), Table 10-4).

Using our novel definition of clopidogrel non-responsiveness on the C-ADP cartridge, 9/22 (41%) patients were non-responsive to clopidogrel at 14d, and 7/20 (35%) were non-responsive at 90d. One patient who was non-responsive to clopidogrel at 14d became responsive at 90d, and one patient who was responsive at 14d was deemed non-responsive at 90d. In the subgroup of 20 patients with serial data at all three time points after changing from aspirin to clopidogrel, 18 (90 %) patients retained the same ‘clopidogrel responder status’ at both 14d and 90d.
Figure 10-5: Collagen epinephrine closure time in response to changing antiplatelet therapy.
Each point represents a single patient; red lines represent medians; p values refer to comparisons of median values at 14d and 90 d compared with the median value at baseline on each treatment regimen. See Table 10-3 and Table 10-4.
Figure 10-6: Collagen ADP closure times in response to changes in antiplatelet therapy.
Each point represents a single patient; red lines represent medians; \( p \) values refer to comparisons of median values at 14d and 90d compared with the median value at baseline on each treatment regimen. See Table 10-3 and Table 10-4.
Potential relationship between platelet activation and function

There was no significant difference in the platelet surface expression of CD62P or CD63, or the percentage of leucocyte-platelet complexes, between aspirin non-responsive and aspirin responsive patients on the PFA-100® at either 14d (p ≥ 0.1) or 90d (p ≥ 0.4). Similarly, there was no significant difference in the expression of CD62P or CD63 or the percentage of leucocyte-platelet complexes between clopidogrel non-responsive and clopidogrel responsive patients on the PFA-100® at 14d (p ≥ 0.1) or 90d (p ≥ 0.1). However, these assessments of the relationship between antiplatelet responsiveness on the PFA-100® and platelet activation status on whole blood flow cytometry must be interpreted with extreme caution because of the very small number of subjects in each of these subgroups.
Platelet count, platelet volume and variability in platelet size after (A) commencing aspirin or (B) changing from aspirin to clopidogrel

A. There was no change in platelet count, mean platelet volume or platelet distribution width at 14d or 90d on aspirin monotherapy compared with baseline values before starting aspirin (p ≥ 0.1, Table 10-5).

B. The mean platelet count in citrate anticoagulated blood did not significantly decrease at any stage during follow-up after changing from aspirin to clopidogrel monotherapy (p ≥ 0.2). In contrast, the mean platelet count in EDTA-anticoagulated blood transiently decreased 14 days after changing from aspirin to clopidogrel monotherapy (p = 0.01), although this reduction was not sustained at 90d (p = 0.5, Table 10-6). The MPV did not significantly change in citrate, but decreased in EDTA-anticoagulated blood at 14d and 90d compared with baseline (p ≤ 0.04). The mean PDW did not change in citrate-anticoagulated blood at any stage during follow-up after changing from aspirin to clopidogrel (p = 0.3). However, the mean PDW in EDTA-anticoagulated blood initially decreased 14 days after changing to clopidogrel monotherapy (p = 0.02), but this reduction was not sustained at 90d (p = 0.08).
Table 10-5: FBC parameters in response to commencement of aspirin monotherapy following TIA or ischaemic stroke

<table>
<thead>
<tr>
<th>FBC Parameter</th>
<th>Baseline (n = 26)</th>
<th>14d (n = 25)</th>
<th>90d (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count x $10^9$/L EDTA</td>
<td>236 (±52)</td>
<td>230 (±43)</td>
<td>221 (±54)</td>
</tr>
<tr>
<td>p value</td>
<td>0.5</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Platelet Count x $10^9$/L Citrate</td>
<td>176 (±49)</td>
<td>172 (±35)</td>
<td>159 (±32)</td>
</tr>
<tr>
<td>p value</td>
<td>0.7</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution % EDTA</td>
<td>13.4 (±1.9)</td>
<td>13.7 (±2.3)</td>
<td>12.8 (±1.2)</td>
</tr>
<tr>
<td>p value</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution % Citrate</td>
<td>11.5 (±1.8)</td>
<td>11.3 (±1.7)</td>
<td>11.1 (±1.3)</td>
</tr>
<tr>
<td>p value</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) EDTA</td>
<td>10.9 (±0.9)</td>
<td>11.0 (±1.1)</td>
<td>10.7 (±0.8)</td>
</tr>
<tr>
<td>p value</td>
<td>0.5</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) Citrate</td>
<td>9.8 (±0.8)</td>
<td>9.8 (±0.9)</td>
<td>9.7 (±0.7)</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WCC x $10^9$/L EDTA</td>
<td>7.42 (±2.10)</td>
<td>7.04 (±2.26)</td>
<td>7.55 (±2.17)</td>
</tr>
<tr>
<td>p value</td>
<td>0.07</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Neutrophils x $10^9$/L EDTA</td>
<td>4.24 (3.01-5.74)</td>
<td>3.68 (2.78-5.07)</td>
<td>4.79 (3.49-5.73)</td>
</tr>
<tr>
<td>p value</td>
<td>0.06</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Monocytes x $10^9$/L EDTA</td>
<td>0.59 (0.51-0.78)</td>
<td>0.60 (0.47-0.71)</td>
<td>0.62 (0.57-0.78)</td>
</tr>
<tr>
<td>p value</td>
<td>1.0</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Lymphocytes x $10^9$/L EDTA</td>
<td>1.88 (1.55-2.34)</td>
<td>1.70 (1.42-2.35)</td>
<td>1.63 (1.45-2.29)</td>
</tr>
<tr>
<td>p value</td>
<td>0.05</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Haemoglobin (g/dl) EDTA</td>
<td>15.3 (±1.6)</td>
<td>14.4 (±1.0)</td>
<td>15.1 (±0.9)</td>
</tr>
<tr>
<td>p value</td>
<td>0.0004</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>HCT (L/L) EDTA</td>
<td>0.45 (±0.04)</td>
<td>0.42 (±0.03)</td>
<td>0.44 (±0.03)</td>
</tr>
<tr>
<td>p value</td>
<td>0.0004</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

P values refer to comparison between follow-up data at 14d and 90d on aspirin versus baseline values on no antiplatelet therapy. Values are Means (±SD) or medians (25-75% Inter-quartile range).
Table 10-6: FBC parameters in response to changing from aspirin to clopidogrel monotherapy following TIA or ischaemic stroke

<table>
<thead>
<tr>
<th>FBC Parameter</th>
<th>Baseline (n =22)</th>
<th>14d (n =20)</th>
<th>90d (n =20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count x 10^9/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>215 (±45)</td>
<td>200 (±38)</td>
<td>211 (±41)</td>
</tr>
<tr>
<td>p value</td>
<td>0.01</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Platelet Count x 10^9/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>158 (±39)</td>
<td>151 (±28)</td>
<td>161 (±32)</td>
</tr>
<tr>
<td>p value</td>
<td>0.2</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Width % EDTA</td>
<td>13.2 (±1.9)</td>
<td>12.7 (±1.9)</td>
<td>12.9 (±1.9)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.02</strong></td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Width % Citrate</td>
<td>11.1 (9.9-12.3)</td>
<td>11.150 (9.7-11.9)</td>
<td>12.6</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Mean Platelet Volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fl) EDTA</td>
<td>11.0 (10.0-11.4)</td>
<td>10.8 (9.8-11.3)</td>
<td>11.2</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.02</strong></td>
<td><strong>0.04</strong></td>
<td></td>
</tr>
<tr>
<td>Mean Platelet Volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fl) Citrate</td>
<td>9.8 (9.2-10.1)</td>
<td>9.9 (9.0-10.3)</td>
<td>10.1 (9.3-10.5)</td>
</tr>
<tr>
<td>p value</td>
<td>0.9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>WCC x 10^9/L EDTA</td>
<td>6.84 (±1.84)</td>
<td>6.17 (±1.54)</td>
<td>6.54 (±1.50)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.02</strong></td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Neutrophils EDTA</td>
<td>3.8 (3.0-5.2)</td>
<td>3.7 (2.9-4.5)</td>
<td>3.6 (3.1-4.4)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Monocytes x 10^9/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>0.66 (±0.21)</td>
<td>0.55 (±0.15)</td>
<td>0.59 (±0.16)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.009</strong></td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes x 10^9/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>1.92 (±0.72)</td>
<td>1.70 (±0.65)</td>
<td>1.82 (±0.67)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.003</strong></td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>14.1 (±1.6)</td>
<td>13.8 (±1.6)</td>
<td>14.0 (±1.5)</td>
</tr>
<tr>
<td>p value</td>
<td><strong>0.01</strong></td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>HCT (L/L) EDTA</td>
<td>3.8 (3.0-5.2)</td>
<td>3.7 (2.9-4.5)</td>
<td>3.6 (3.1-4.4)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

P values refer to comparison between follow-up data at 14d and 90d on clopidogrel versus baseline values on aspirin

Values are Means (±SD) or medians (25-75% Inter-quartile range)
Red Cell Parameters after (A) commencing aspirin or (B) changing from aspirin to clopidogrel

A. Following the commencement of aspirin monotherapy, the mean haemoglobin concentration and haematocrit initially decreased compared with baseline (p = 0.0004), but these reductions were not sustained at 90 days (p = 0.2, Table 10-5).

B. In patients who changed from aspirin to clopidogrel monotherapy, there was an initial reduction in the mean haemoglobin concentration at 14d days (p = 0.01), but this reduction was not sustained at 90 d (Table 10-6; p = 0.09). Changing from aspirin to clopidogrel did not affect the haematocrit during follow up (p ≥ 0.3).

Leucocyte parameters and subsets after (A) commencing aspirin or (B) changing from aspirin to clopidogrel

A. There was no significant change in the total white cell count or white cell subsets in response to commencing aspirin monotherapy (p ≥ 0.06, Table 10-5).

B. There was a transient reduction in the mean total white cell count, monocyte count and lymphocyte count 14 days after changing to clopidogrel therapy (p ≤ 0.02), but these reductions were not sustained at 90d (p = 0.09, Table 10-6).

Because none of the patients had recurrent vascular events during follow up, one cannot comment about the potential value of any of the platelet function tests, or measurement of the changes in FBC parameters in predicting the response to antiplatelet therapy in the clinical setting in ischaemic CVD patients in this study.
10.5. Discussion

Although the efficacy of both aspirin and clopidogrel has been proven in clinical trials, the impact of commencing these agents on platelet activation and function, and red and white cell parameters, has not been extensively studied in individual patients with ischaemic cerebrovascular disease (CVD).

In agreement with previous pilot studies, we did not find any change in unstimulated platelet surface CD62P or CD63 expression in patients who changed from no medication to aspirin (Meiklejohn et al., 2001; Grau et al., 2003) or from aspirin to clopidogrel (Grau et al., 2003) following TIA or ischaemic stroke. We have also shown that measurement of unstimulated levels of circulating leucocyte-platelet complexes is not a sensitive test for detecting the antiplatelet effects of aspirin monotherapy.

Although there was a transient reduction in the percentage of circulating neutrophil-platelet complexes 14d after changing from aspirin to clopidogrel, this was not sustained during longer-term follow up. The results of this and other pilot studies indicate that measurement of changes in platelet activation status in unstimulated samples with whole blood flow cytometry, as a measure of laboratory responsiveness to antiplatelet therapy, is not likely to be informative at predicting the response to aspirin or clopidogrel in the clinical setting in patients following TIA or ischaemic stroke. However, preliminary data from control subjects on clopidogrel (Frelinger, III et al., 2010), or in patients with ischaemic heart disease on aspirin and clopidogrel (Fox et al., 2009) indicate that whole blood flow cytometry protocols that assess ‘inducible platelet activation’ as a measure of platelet inhibition with antiplatelet agents have the potential to be informative. At the time of writing, there were no recent data assessing inducible
platelet activation/reactivity with whole blood flow cytometry in patients with ischaemic CVD on commonly prescribed antiplatelet regimens.

Our platelet functional data clearly indicate that the C-EPI cartridge of the PFA-100® is sensitive at detecting the *ex vivo* antiplatelet effects of aspirin in a large proportion of patients with TIA or ischaemic stroke. This finding concurs with previous published studies using this device (Grau et al, 2003; Grundmann et al, 2003; Alberts et al, 2004; Harrison et al, 2005; McCabe et al, 2005a; Zytkiewicz et al, 2008; Harrison et al, 2008; Boncoraglio et al, 2009; von Lewinski et al, 2009). As anticipated, withdrawal of aspirin was also detected by shortening of C-EPI closure times when patients changed from aspirin to clopidogrel, confirming that clopidogrel monotherapy does not significantly prolong C-EPI closure times.

If one uses a ‘cross sectional, case-control definition’, data on platelet function inhibition with aspirin indicate that 16-62% of TIA or ischaemic stroke patients exhibit ‘aspirin non-responsiveness’ on the PFA-100® (Grau et al, 2003; Grundmann et al, 2003; Alberts et al, 2004; Harrison et al, 2005; McCabe et al, 2005a; Zytkiewicz et al, 2008; Harrison et al, 2008; Boncoraglio et al, 2009; von Lewinski et al, 2009). Our study indicates that if one uses a longitudinal definition, the prevalence of aspirin non-responsiveness on the PFA-100® is lower than that anticipated from studies employing cross-sectional definitions in the early (24% vs. 60%) or late phases (18% vs. 43%) after TIA or stroke (McCabe et al, 2005a). Several mechanisms may influence *ex vivo* inhibition of platelet function with aspirin on the PFA-100® including medication dosage, body weight, the extent of intestinal drug absorption, potential interaction with concomitant medications, very recent vascular events, vascular risk factor profile,
differences in platelet surface receptor expression caused by possibly functionally
important platelet polymorphisms, and von Willebrand factor antigen levels (McCabe et al, 2005a). It is important that one tries to establish an accurate measure of inhibition of platelet function with aspirin therapy because a recent meta-analysis, that predominantly included patients with ischaemic heart disease, indicated that patients with ex vivo aspirin non-responsiveness may have a higher rate of recurrent vascular events overall than responders (Krasopoulos et al, 2008). The clinical utility of the PFA-100® in predicting recurrent vascular events in patients with ischaemic stroke or TIA on aspirin has not been demonstrated, but is the subject of future investigation by our group. Further analysis of stored plasma and serum samples is awaited to assess salicylate and thromboxane B$_2$ levels in a proportion of our study population, as further measures of medication compliance, drug absorption and inhibition of cyclo-oxygenase 1 function.

Consistent with prior data on this topic, aspirin did not significantly prolong C-ADP closure times in ischaemic CVD in this study (Serebruany et al, 2004b) (Grau et al, 2003;Grundmann et al, 2003;McCabe et al, 2005a). Clopidogrel monotherapy prolonged C-ADP closure times at 14 days, but this effect was not significant in the late phase after symptom onset (p = 0.09). Our 14 day data are in keeping with a small, longitudinal pilot study in nine acute ischaemic stroke patients which suggested a time lag of at least 12 days before one sees an initial ex vivo antiplatelet effect with 75 mg of clopidogrel monotherapy daily, and that the maximal ex vivo antiplatelet effects of clopidogrel on the C-ADP cartridge are seen after 4 weeks of therapy (Raman & Jilma, 2004). Our negative findings in the late phase after starting clopidogrel could relate to a type II error because only 20 or 22 patients had 90d follow-up data, but our data concur with another case-crossover study (Grau et al, 2003) which did not demonstrate
significant prolongation of C-ADP closure times after 4 weeks of clopidogrel monotherapy. We have no evidence that non-compliance with clopidogrel significantly influenced our results because all patients were phoned the week before follow-up testing to ensure they were adhering to their treatment regimen. Although it is possible that some of our study subjects may not have been fully compliant for a full 4 weeks prior to testing, when the maximum antiplatelet effects of clopidogrel have been reported to be seen (Raman & Jilma, 2004), this does not explain why there was a significant prolongation of C-ADP closure times with clopidogrel at 14d compared with baseline. Our data also agree with data from patients with coronary artery disease where the value of the PFA-100 in predicting the risk of vascular events in patients on clopidogrel undergoing elective coronary percutaneous intervention was found to be poor (Breet et al, 2010).

Using our novel longitudinal definition of non-responsiveness, 41% of patients at 14d and 35% at 90d were non-responsive to clopidogrel on the C-ADP cartridge. These figures are lower than those reported in pilot studies that employed cross-sectional definitions of clopidogrel non-responsiveness in patients with ischaemic CVD on 75 mg of clopidogrel monotherapy daily (75%-93.5%) (Grau et al, 2003; Kotzailias et al, 2007). If we had categorised our patients according to a cross-sectional definition of clopidogrel non-responsiveness as failure to prolong C-ADP closure times above the normal range in controls, 82% at 14 d and 84% at 90d would have been deemed non-responsive in our laboratory. This is higher than previously reported in ischaemic heart disease (IHD) patients on clopidogrel (24.4%), (Paniccia et al, 2007) but in keeping with reported figures for clopidogrel non-responsiveness in pilot studies in patients with prior ischaemic CVD outlined above (75%-93.5%) (Grau et al, 2003; Kotzailias et al, 2007).
Unpublished cross-sectional data in patients in the late stable phase after TIA or ischaemic stroke from our lab have shown that six of 25 (24%) patients on clopidogrel monotherapy had \textit{ex vivo} non-responsiveness to clopidogrel on a low shear stress platelet function test (VerifyNow P2Y12 cartridge based analyser; Kinsella JA, Tobin WO et al., in preparation). Our research group did not have access to the VerifyNow for the duration of this study, so one cannot comment on its potentially utility in assessing clopidogrel non-responsiveness in longitudinal studies in ischaemic CVD.

The failure of clopidogrel to significantly prolong C-ADP closure times in all patients with ischaemic CVD may relate to a variety of factors outlined above in the discussion on aspirin non-responsiveness. In addition, specific pharmacogenomic factors that lead to clopidogrel non-responsiveness (hepatic enzyme CYP2C19 loss of function polymorphisms (Mega \textit{et al}, 2009)) may account for variable inhibition of platelet function in the laboratory and clinical setting. The concentration of ADP in the C-ADP cartridge might also stimulate platelets to an excessive non-physiological degree, allowing P2Y\textsubscript{12} receptor blockade to be bypassed in the laboratory when it might be sufficient in the clinical setting. Furthermore, ADP in the C-ADP cartridge may activate both P2Y\textsubscript{1} and P2Y\textsubscript{12} ADP platelet receptors in patients on clopidogrel, and has been felt to be responsible for the high prevalence of apparent clopidogrel non-responsiveness on the PFA-100® (Gachet, 2006). A new INNOVANCE® PFA P2Y cartridge has since been designed to address some of the potential limitations of the C-ADP cartridge in detecting the antiplatelet effects of clopidogrel, but this cartridge was not available at the time of this study (Linnemann \textit{et al}, 2010). However, the product literature from the company indicates that 35.8% of patients with cardiovascular disease
are non-responsive to 75 mg of clopidogrel daily on the INNOVANCE® PFA P2Y cartridge (defined in a cross sectional manner as failure to prolong closure times beyond 106 s \textit{ex vivo} in patients on clopidogrel). This cartridge is reported to be more sensitive and specific at detecting inhibition of platelet function with P2Y\textsubscript{12} receptor antagonists than the C-ADP cartridge. There are no published longitudinal data with this cartridge in patients with ischaemic CVD.

Previous studies have shown that mean platelet volume (O'Malley \textit{et al}, 1995) and monocyte counts are elevated within 48 hours of ischaemic stroke (Marquardt \textit{et al}, 2009). This study has shown that starting aspirin, or changing from aspirin to clopidogrel is associated with a transient reduction in haemoglobin concentration or haematocrit at 14 days which is not sustained at the 90d follow up timepoint; this change in haemoglobin concentration was not deemed to be clinically significant in either patient group. In addition, changing from aspirin to clopidogrel was associated with (i) a transient reduction in the mean platelet count, PDW (as a measure of variability in platelet size), total white cell count, monocyte and lymphocyte counts at 14 days only, and (ii) a persistent reduction in the MPV. Clopidogrel is well known to cause a reduction in the white cell count and platelet count in whole blood, but to our knowledge, has not been shown to impact on the PDW or MPV in patients with ischaemic CVD. Although these parameters could also have increased initially in response to, and resolved in association with resolution of an acute phase response, if these changes are replicated in further studies, they may explain some of the potential beneficial antithrombotic effects of clopidogrel that may be independent of direct platelet P2Y\textsubscript{12} ADP receptor antagonism.
One must acknowledge that this pilot study included a relatively small number of patients in each treatment group at baseline and 14d, and we have limited follow-up data in the group of patients changing from no medication to aspirin. As most international guidelines advise treatment of patients with aspirin and dipyridamole combination therapy in preference to aspirin monotherapy, and because some physicians who contributed patients to this study opted to use clopidogrel monotherapy instead of aspirin monotherapy for long-term secondary prevention following TIA or stroke, only 10 patients had 90 d follow up data on aspirin alone. Therefore, we may not have identified significant changes in platelet activation status due to underpowering of this aspect of the study (type II error). Even allowing for this fact, a small but statistically significant change in unstimulated CD62P or CD63 surface expression is unlikely to be clinically useful in monitoring CVD patients undergoing changes in antiplatelet therapy. As stated above, our PFA-100® data in the group of patients changing from aspirin to clopidogrel may also have been subject to a type II error at the 90d timepoint, and we did not have access to the recently licensed INNOVANCE® PFA P2Y cartridge. We cannot comment on the clinical value of the PFA-100® at predicting the risk of recurrent vascular events on aspirin or clopidogrel following TIA or ischaemic stroke because no patients recruited to this study had recurrent events on their respective treatment regimen during follow up.
10.6. Conclusion

Measurement of changes in platelet activation status in unstimulated samples with whole blood flow cytometry, as a measure of laboratory responsiveness to antiplatelet therapy, is not likely to be informative at predicting the response to aspirin or clopidogrel in the clinical setting in patients following TIA or ischaemic stroke. The potential usefulness of whole blood flow cytometry assays of inducible platelet reactivity deserves further study in ischaemic CVD. The inhibitory effect of aspirin on platelet function in whole blood can be detected with the PFA-100® in patients with ischaemic CVD, with a prevalence of \textit{ex vivo} aspirin non-responsiveness of 18-24\%. The C-ADP cartridge does not reliably detect \textit{ex vivo} inhibition of platelet function in CVD patients on long-term clopidogrel monotherapy (35-41\% non-responsive), but further longitudinal studies with the INNOVANCE\textsuperscript{c} PFA P2Y cartridge are warranted. The clinical utility and predictive value of these novel definitions of antiplatelet non-responsiveness need to be evaluated in a long term clinical follow-up study.
11. Assessment of the impact of dipyridamole on platelet activation and function in the early and late phases after TIA or ischaemic stroke

11.1. Introduction

As discussed in Chapter 4 [Is Platelet Activation Increased Following TIA or Ischaemic Stroke?], platelets are activated in the early, (Grau et al, 1998; Meiklejohn et al, 2001; Marquardt et al, 2002; Garlichs et al, 2003; McCabe et al, 2004a) subacute, (Marquardt et al, 2002) or late phases (Grau et al, 1998; Meiklejohn et al, 2001; Garlichs et al, 2003; McCabe et al, 2004a) after TIA or ischaemic stroke. Consequently, antiplatelet agents have the potential to play a key role in the secondary prevention of vascular events in patients with ischaemic cerebrovascular disease (CVD). However, there are no routinely-employed, reliable laboratory data to guide optimal antiplatelet treatment in individual CVD patients, akin to the INR in patients on warfarin.

There is extensive interest in the phenomenon of ‘resistance’ or ‘non-responsiveness’ to aspirin or clopidogrel in ischaemic heart disease and ischaemic CVD. However, relatively little attention has been paid to the potential phenomenon of ‘dipyridamole non-responsiveness’.
Two pilot randomised trials have indicated that 30 days of treatment with aspirin and dipyridamole combination therapy may lead to enhanced inhibition of platelet function compared with treatment with aspirin alone in healthy Japanese control subjects (Serebruany et al, 2008a) or in Japanese patients with ischaemic CVD (Serebruany et al, 2004a). A further pilot randomised study comprehensively assessed platelet activation and function in type II diabetic patients in the late stages after TIA, who were allocated to receive aspirin and dipyridamole combination therapy, clopidogrel monotherapy, or aspirin and clopidogrel combination therapy (Serebruany et al, 2008b). There were no significant differences in platelet function, as assessed by a platelet function analyser called the PFA-100®, between the three treatment groups, but the pattern of inhibition of platelet activation or function varied according to the allocated treatment regimen. No studies have longitudinally assessed the ability of dipyridamole to cause additional inhibition of platelet function ex vivo in the same patients when dipyridamole is added to aspirin. A longitudinal study design allows individual patients to act as their own controls, thus avoiding the potential influence of confounding inter­group variables that could hinder interpretation of results.

To address these issues, we designed a longitudinal, case-crossover study in ischaemic CVD patients to assess whether adding dipyridamole to aspirin therapy inhibited platelet function or platelet activation to a greater degree compared with baseline values on aspirin alone. We examined the relationship between platelet function and activation in whole blood. We hypothesised that inhibition of platelet function would be greater, and platelet activation would be less marked in some patients on aspirin and dipyridamole compared with aspirin alone, and that a novel definition of antiplatelet non-responsiveness could be established with an existing test of platelet function.
11.2. Patients and methods

Consecutive eligible patients with TIA or ischaemic stroke within the preceding 4 weeks were recruited from the inpatient population, and from the Rapid Access Stroke Prevention (RASP) service at our secondary and tertiary referral university teaching hospital. Patients were >18 years old, were on aspirin monotherapy, and were going to be initiated on dipyridamole modified-release (MR) in conjunction with aspirin by their treating physician. One patient was changed from aspirin monotherapy to the commercially available combination preparation of asasantin retard® twice daily (containing 25 mg of aspirin and 200 mg of dipyridamole MR per tablet); another patient was changed from 75 mg of aspirin monotherapy daily to 75 mg of aspirin in combination with asasantin retard® twice daily. All other patients in this study had 200 mg of dipyridamole MR twice daily added to their once daily dose of aspirin. Patients were enrolled up to 4 weeks following ischaemic stroke or TIA, to allow recruitment of patients who presented late, and patients who were commenced on aspirin monotherapy out of hours, who would subsequently be eligible to commence on dipyridamole MR.

Patients were excluded if they had a history of primary intracerebral haemorrhage, myocardial infarction within the preceding 3 months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding 3 months, systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis, platelet count <120 x 10^9/L or >450 x 10^9/L, urea >10 mmol/L or GFR <30 ml/min, known platelet disorder, current infection (clinical signs of infection or white cell count >11 x 10^9/L), or non-steroidal anti-inflammatory use within 14 days of recruitment.
All subjects underwent detailed clinical assessment, and information regarding vascular risk factors, smoking status and medication use was collected prospectively. Results of routine haematological, coagulation, biochemical and blood glucose testing were collected prospectively. CT and/or MRI of brain, and colour Doppler ultrasound of carotid and vertebral arteries were performed in all patients. Magnetic resonance or computed tomography angiography was performed when deemed appropriate by the treating physician, as described previously (McCabe et al, 2004a). A chest radiograph, electrocardiograph (ECG), 24-hour ECG recording and transthoracic or transoesophageal echocardiography were obtained in all patients. The underlying mechanism responsible for the TIA or ischaemic stroke was categorized according to TOAST classification (Adams, Jr. et al, 1993).

All patients underwent clinical and laboratory assessment before (baseline), 14 days after (14d), and at least 90 days (90d) after adding dipyridamole to aspirin monotherapy. In the atherothrombotic subgroup, 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.

As most patients at baseline and 14d were assessed during their inpatient stay, adherence to antithrombotic therapy was confirmed by checking the prescription chart. Adherence in outpatients was assessed by history taking alone, but all were phoned to stress 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 regimen.
11.2.1. Blood sampling and laboratory tests

All subjects were rested for at least 20 min, and venepuncture performed from a free-flowing vein using a 21G butterfly needle and a Vacutainer® system with a luer adaptor, as previously described (McCabe et al, 2004a).

Full blood count was measured in EDTA Vacutainer® tubes between 2 and 4 hours after venepuncture on a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd). Seven further 3 ml samples were collected into 3.2% sodium citrate-anticoagulated Vacutainer tubes.

The first 3 ml citrate-anticoagulated sample was used for whole blood flow cytometric analysis on a Beckman Coulter XL MCL flow cytometer. Platelets were distinguished from white and red cells by their characteristic forward and side scatter pattern, and by ensuring that >95% of cells expressed GpIbα (CD42b) (McCabe et al, 2004a). Platelet activation was assessed by quantifying platelet surface CD62P and CD63 expression within 90 minutes of venepuncture, (McCabe et al, 2004a) and the percentages of circulating neutrophil–platelet, monocyte–platelet, and lymphocyte–platelet complexes within 3 hours of venepuncture (Joseph et al, 2001; McCabe et al, 2004a; McCabe et al, 2005b) using previously described (Shattil et al, 1987) and validated methodology (McCabe et al, 2004a; McCabe et al, 2005b)[Chapter 9, General Methods].

The degree of inhibition of platelet function in whole blood was assessed with the PFA-100® platelet function analyser (Dade-Behring, Germany) between 2-2.5 hours after venepuncture (McCabe et al, 2005a)[Chapter 9, General Methods]. The PFA-100® activates platelets by exposure to moderately high shear stress (5000-6000 s⁻¹) and biochemical stimulation with collagen and either ADP (C-ADP cartridge) or
epinephrine (C-EPI cartridge) (Kundu et al, 1995). The PFA-100® was used to assess platelet function, because, in contrast to some other available platelet function assays, it is a whole blood assay which assesses platelet function in response to stimulation by both collagen and ADP or collagen and adrenaline as compared to single agonists in other commercially available tests. It is an assay of both adhesion and aggregation under conditions of high shear stress.

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, and we arbitrarily defined closure times >300s as 301s (McCabe et al, 2005a). Aspirin prolongs C-EPI closure times in 83-100% of healthy controls, (Kundu et al, 1994; Mammen et al, 1995; Mammen et al, 1998; Harrison et al, 1999) with minimal prolongation of C-ADP closure times in 0-24.2% of healthy controls (Mammen et al, 1995; Mammen et al, 1998; Harrison et al, 1999). To our knowledge, longitudinal studies assessing the impact of adding dipyridamole to aspirin in individual patients following TIA or ischaemic stroke have not been performed on the PFA-100®. Initial experiments in our laboratory revealed that C-ADP, but not C-EPI closure times, were prolonged ex vivo in a proportion of patients following the addition of dipyridamole to aspirin. The coefficient of variation (CV) of the C-ADP assay was 7%, and that of the C-EPI assay was 7.5%. Because baseline PFA-100® measurements were available on aspirin, ‘dipyridamole non-responsiveness’ was defined as failure to prolong the C-ADP closure time compared with the patient’s own baseline on aspirin by more than twice the CV of the C-ADP assay i.e. failure to prolong C-ADP closure times by >14% of the patient’s baseline C-ADP closure time.
The next four tubes were used to prepare platelet poor plasma that was stored at -70°C for further prospective studies. The seventh citrate-anticoagulated sample was processed to measure the platelet count, MPV and PDW in citrate-anticoagulated whole blood.

The study was approved by the local research ethics committee. Written informed consent (or assent, where appropriate) was obtained in all cases.
11.2.2. Statistical Methods

Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively, the Wilcoxon signed rank test and the Wilcoxon rank sum test for comparison of paired and unpaired non-parametric variables, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. McNemar's Chi-squared test with continuity correction was used to compare changes in proportions over time. Spearman rank-order correlation analysis assessed the relationship between platelet activation markers, leucocyte-platelet complexes and PFA-100® closure times. Of note, PFA-100® closure times are reported by the device as a continuous scale of seconds up to 300 seconds. All results above 300 seconds are reported as $>300$ seconds. Therefore only non-parametric tests can be used to investigate this device, which excludes the use of linear regression. $P < 0.05$ was considered statistically significant. All statistical calculations were performed with R version 2.10.1 (R Development Core Team, 2009).
11.3. Results

52 patients were assessed at baseline, 14d and 90d (Table 11-1). One patient had an elevated white cell count at 14d, so data from this assessment were excluded. One patient at 14d, and two at 90d did not have successful PFA-100® testing due to 'device errors'. The TIA/stroke subtypes are outlined in Table 11-2. Of the 9 patients with cardioembolic stroke, 1 had paroxysmal atrial fibrillation following repeated cardiac rhythm monitors, 8 had a patent foramen ovale, 3 of those with associated atrial septal aneurysm. 17 patients were on aspirin at the time of their qualifying stroke or TIA. 4 patients had a modified Rankin scale score of ≥2 at enrolment. The median daily aspirin dose was 150mg at baseline and 75mg at 14d and 90d. No patient had a recurrent vascular event during follow-up.

Figure 11-1 Flowchart for patients assessed initially on aspirin monotherapy and subsequently on aspirin and dipyridamole combination therapy

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Day 14</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin monotherapy</td>
<td>Aspirin + Dipyridamole</td>
<td>Aspirin + Dipyridamole</td>
</tr>
<tr>
<td>52 Patients assessed</td>
<td>52 Patients assessed</td>
<td>52 Patients assessed</td>
</tr>
<tr>
<td>1 patient - ↑WCC</td>
<td>1 patient - PFA-100 'device error'. Both excluded from 14d analysis</td>
<td>2 patients had PFA-100 device errors. Both excluded from 90d analysis</td>
</tr>
</tbody>
</table>

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### Table 11-1: Demographic Data in Patients at Enrolment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Stroke (24) or TIA (28) (n = 52)</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>57 (SD 12.9)</td>
<td>35</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>IHD</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Hypertension</td>
<td>25</td>
<td>48</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Family History Stroke</td>
<td>25</td>
<td>48</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Migraine (with and without aura)</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>Current smokers (at enrolment)</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>Never Smoked</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>Statin use</td>
<td>20</td>
<td>39</td>
</tr>
</tbody>
</table>

### Table 11-2: Etiological supotyping by TOAST classification

<table>
<thead>
<tr>
<th>Stroke/TIA subtype (n=52)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherothrombotic</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>16 (31%)</td>
</tr>
<tr>
<td>Cardiogenic Embolic</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>Undetermined aetiology</td>
<td>22 (42%)</td>
</tr>
<tr>
<td>Other determined</td>
<td>3 (6%)</td>
</tr>
</tbody>
</table>
Median C-ADP closure time increased from 91s at baseline on aspirin to 98s at 14d (p = 0.02) and 95s at 90d (p = 0.03) on aspirin and dipyridamole. There was no relationship between C-ADP closure time and aspirin dose (p ≥ 0.12). 30/51 patients (59%) at 14d and 28/50 (56%) at 90d were 'dipyridamole non-responders' on the PFA-100®. There was no difference in the proportion of dipyridamole non-responders on the PFA-100® between 14d and 90d (p = 0.8). 4 patients (8%) were initially dipyridamole responsive at 14d, but subsequently non-responsive at 90d; 6 (12%) were initially non-responsive on the PFA-100® at 14d, but subsequently became responsive at 90d. There was no significant difference in gender (p = 0.07), age (p = 0.2), or the prevalence of statin therapy use (p = 0.051) between dipyridamole non-responders on the PFA-100® and responders.

Median C-EPI closure time was 239s at baseline, 207s at 14d (p = 0.7), and decreased to 189s at 90d (p = 0.02). Patients on higher doses of aspirin had higher C-EPI closure times at baseline (r = 0.33, p = 0.02). All except 3 patients were on 75mg of aspirin daily at 14d and 90d, so correlation analysis was not performed at these timepoints. C-EPI closure times were similar in dipyridamole non-responders on the PFA-100® and responders (p = 0.96). There was no correlation between the total platelet count and C-ADP or C-EPI closure times (p ≥ 0.2).
Table 11-3: Changes in Platelet Activation Status at 14d and 90d in Response to Dipyridamole

<table>
<thead>
<tr>
<th>Marker</th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CD62P</td>
<td>1.68 (1.23-2.44)</td>
<td>1.95 (1.39-2.68)</td>
<td>1.71 (1.26-2.67)</td>
</tr>
<tr>
<td>P value</td>
<td>0.19</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>%CD63</td>
<td>11.4 (8.52-15.8)</td>
<td>11.6 (9.03-18.90)</td>
<td>12.9 (9.17-17.48)</td>
</tr>
<tr>
<td>P value</td>
<td>0.59</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>%Monocyte-Platelet Complexes</td>
<td>4.6 (3.4-5.4)</td>
<td>5.0 (4.1-6.2)</td>
<td>4.9 (4-6.2)</td>
</tr>
<tr>
<td>P value</td>
<td><strong>0.03</strong></td>
<td><strong>0.03</strong></td>
<td></td>
</tr>
<tr>
<td>%Neutrophil-Platelet Complexes</td>
<td>2.68 (2.32-2.99)</td>
<td>2.71 (2.42-3.09)</td>
<td>2.97 (2.49-3.24)</td>
</tr>
<tr>
<td>P value</td>
<td>0.42</td>
<td><strong>0.005</strong></td>
<td></td>
</tr>
<tr>
<td>%Lymphocyte-Platelet Complexes</td>
<td>2.33 (2.09-2.58)</td>
<td>2.49 (2.14-2.89)</td>
<td>2.63 (2.27-3.07)</td>
</tr>
<tr>
<td>P value</td>
<td>0.24</td>
<td><strong>0.016</strong></td>
<td></td>
</tr>
</tbody>
</table>

P value refers to comparison between baseline and follow-up at 14d and 90d. Values are medians (25-75th percentile).

CD62P and CD63 expression did not change after adding dipyridamole to aspirin (Table 11-3). There was no significant correlation between CD62P or CD63 and C-ADP closure times (p ≥ 0.3). Median CD62P expression was significantly higher in dipyridamole non-responders on the PFA-100® than responders at 14 days (2.06% vs. 1.62%, p = 0.04), but not at 90 days (p = 0.5). There was no difference in CD63 expression between non-responders on the PFA-100® and responders (p ≥ 0.7).
Compared with baseline, the % monocyte-platelet complexes increased at 14d (p = 0.03), and the % monocyte-platelet, neutrophil-platelet and lymphocyte-platelet complexes increased at 90d (p ≤ 0.04; Table 11-3). C-ADP closure times inversely correlated with % monocyte-platelet complexes at 14d (r=-0.32, p = 0.02) and 90d (r = -0.33, P=0.02; Figure 11-2 and Figure 11-3). The % monocyte-platelet complexes serially increased in the subgroup of dipyridamole non-responders on the PFA-100® (p ≤ 0.045), but not in dipyridamole responders (p ≥ 0.5) at 14d and 90d compared with baseline. Patients who had a lower percentage change in C-ADP closure time between baseline and 14 days and between baseline and 90 days had higher monocyte platelet complexes (p ≤ 0.04). There were no significant correlations between C-ADP closure times and % neutrophil-platelet or lymphocyte-platelet complexes (p ≥ 0.32).
Table 11-4: Differences in Platelet Activation Status Between Responders and Non-responders to Dipyridamole on the PFA-100®

<table>
<thead>
<tr>
<th>Marker</th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responder</td>
<td>Non-responder</td>
<td>P value</td>
</tr>
<tr>
<td>%CD62P</td>
<td>1.96 (1.29-2.81)</td>
<td>1.55 (1.25-1.99)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1.62 (1.24-2.00)</td>
<td>2.06 (1.59-2.79)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>1.89 (1.51-2.97)</td>
<td>1.71 (1.23-2.29)</td>
<td>0.5</td>
</tr>
<tr>
<td>%CD63</td>
<td>11.85 (9.44-18.03)</td>
<td>12.9 (8.48-20.45)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>11.35 (8.19-15.20)</td>
<td>11.4 (9.30-16.93)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>12.1 (9.55-17.15)</td>
<td>13.1 (8.60-15.83)</td>
<td>0.9</td>
</tr>
<tr>
<td>%Monocyte-Platelet Complexes</td>
<td>4.75 (3.25-5.53)</td>
<td>4.2 (3.28-5.45)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>4.60 (3.60-5.40)</td>
<td>5.7 (4.50-6.50)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>4.9 (4.0-5.90)</td>
<td>4.9 (4.1-6.45)</td>
<td>0.5</td>
</tr>
<tr>
<td>%Neutrophil-Platelet Complexes</td>
<td>2.79 (2.39-3.20)</td>
<td>2.60 (2.31-2.81)</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>2.68 (2.32-2.80)</td>
<td>2.86 (2.54-3.23)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>3.02 (2.67-3.23)</td>
<td>2.88 (2.37-3.35)</td>
<td>0.9</td>
</tr>
<tr>
<td>%Lymphocyte-Platelet Complexes</td>
<td>2.43 (2.14-2.74)</td>
<td>2.38 (2.17-2.67)</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2.31 (2.13-2.53)</td>
<td>2.49 (2.14-2.92)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>2.84 (2.29-2.97)</td>
<td>2.47 (2.20-3.13)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

P value refers to comparison between responders and non-responders to dipyridamole on the PFA-100®

Values are medians (25-75th percentile)
Figure 11-2 Relationship Between % Monocyte-Platelet Complexes and C-ADP Closure Times at 14d
(Spearman's rho = -0.32, p = 0.02)
Figure 11-3 Relationship Between % Monocyte-Platelet Complexes and C-ADP Closure Times at 90d
(Spearman's rho = -0.33, p = 0.02).
11.4. Discussion

This longitudinal study has revealed persistent, additional \textit{ex vivo} inhibition of platelet adhesion/aggregation with the PFA-100\textsuperscript{©} Collagen-ADP cartridge in a large proportion of patients when dipyridamole is added to aspirin following TIA or ischaemic stroke. However, 59-56\% of patients were dipyridamole non-responders on the PFA-100\textsuperscript{©} in the early and late phases after symptom onset. The data were consistent over time, with the majority (88-92\%) of patients retaining the same dipyridamole ‘responder status’ during follow-up between 14 and 90 days. The C-ADP cartridge appears specific to detect the effect of dipyridamole, and is not affected by concurrently administered aspirin, which exerts an effect on the C-EPI cartridge. We have detected an effect of dipyridamole, even though this assay does not assess all of the potential inhibitory effects of dipyridamole on platelet activation and function. These findings illustrate the potential to monitor the additional \textit{ex vivo} antiplatelet effects of dipyridamole with the C-ADP cartridge, when baseline values on aspirin are available in individual patients.

Two large clinical trials have shown a significant, but modest, additional benefit of aspirin and dipyridamole combination therapy over aspirin alone for secondary prevention following TIA or ischaemic stroke (Diener et al, 1996; Halkes et al, 2006), with a 23\% relative reduction in the 2-year risk of stroke with combination therapy compared with aspirin alone in ESPS-2 (Diener et al, 1996). Therefore, it is not surprising that a large proportion of patients would be deemed ‘dipyridamole non-responsive’ on an \textit{ex vivo} test of platelet function in whole blood. No patient had a recurrent vascular event during our follow-up period, so one cannot make any conclusions about the long-term usefulness of the PFA-100\textsuperscript{©} in predicting the response to dipyridamole in the clinical setting.
The lack of ability of the C-EPI cartridge to identify additional inhibition of platelet function with dipyridamole is likely to have been confounded by the reduction in aspirin dose over time. This and prior studies have shown that there is a significant positive relationship between aspirin dose and C-EPI closure times in ischaemic CVD (Alberts et al, 2004).

The antiplatelet effects of dipyridamole may be mediated via several different cellular pathways (McCabe D.J.H. et al, 2004; Weyrich et al, 2005; Cao et al, 2009) and these data suggest that it may ultimately inhibit ADP-induced platelet reactivity, perhaps through intraplatelet inhibition of phosphodiesterase E5 and phosphodiesterase 3A, thereby increasing intraplatelet cGMP and cAMP (Kim & Liao, 2008). Alternatively, it may act indirectly by reducing adenosine uptake by erythrocytes or endothelial cells, thus increasing adenosine available to bind to platelet adenosine receptors which could reduce the propensity for platelet degranulation (Haslam et al, 1999; Kim & Liao, 2008).

This study indicates that measurement of ‘unstimulated’ platelet surface CD62P or CD63 expression with whole blood flow cytometry is not a sensitive test for detecting additional inhibition of platelet activation with aspirin and dipyridamole compared with aspirin alone. However, we did not study inducible-platelet reactivity with flow cytometry in response to in vitro agonist-stimulation (Tarnow et al, 2009).

Elevated leucocyte-platelet complexes have been previously reported in the early and late phases after TIA or ischaemic stroke compared with controls, (Garличs et al, 2003; McCabe et al, 2004a) although most patients were studied on aspirin monotherapy. Marquardt et al reported increased neutrophil-platelet and monocyte-
platelet complexes in the first 10 days following ischaemic stroke, with levels returning to baseline at 90 days (Marquardt et al, 2009); but no data regarding antiplatelet treatment were reported in that study. It was initially somewhat unexpected that the percentage of circulating monocyte-platelet complexes increased at 14 days in our study, and all subtypes of leucocyte-platelet complexes increased in the overall patient population at 90 days, and as such may represent a type I error. However, the novel finding of an inverse correlation between the percentage of monocyte-platelet complexes and C-ADP closure times indicates that patients with excessive platelet activation are less likely to exhibit an *ex vivo* response to dipyridamole. It is also likely from our subgroup analysis that the dipyridamole non-responsive patients are predominantly responsible for the observed increase in the percentage of circulating monocyte-platelet complexes in ischaemic CVD, because there are no data to suggest that adding dipyridamole to aspirin activates platelets or leucocytes, or enhances leucocyte-platelet interactions. There was no significant correlation between CD62P or CD63 and C-ADP closure times, indicating that these markers do not influence PFA-100® closure times. However, in keeping with our data on monocyte-platelet complexes, CD62P expression was higher at 14d in dipyridamole non-responders on the PFA-100® than responders, indicating that platelet degranulation/activation status in the early phase after TIA or ischaemic stroke may influence dipyridamole responsiveness on the PFA-100®. This deserves further study, because if this relationship were proven, it should also extend to the late phase after symptom onset.
The mean age of patients enrolled in this study was low. This may reflect the age distribution of the population served by the recruiting centre, and the fact that it is a tertiary referral centre. Age did not appear to confound the results, so this should not affect the validity of the findings. There were a small number of patients with large vessel atheroembolism as qualifying events enrolled into the study. This reflects the practicality of assessing patients in 14 days following commencement of antiplatelet therapy. At this stage, most patients with stroke or TIA of large vessel atheroembolic aetiology would have had their offending vessel operated upon, excluding them from the study.

Adequately-sized, multi-centre, prospective studies, assessing the clinical predictive value of \textit{ex vivo} dipyridamole responsiveness on the long-term risk of recurrent vascular events, are required to optimise antiplatelet therapy in individual ischaemic CVD patients. Such data could allow one to enhance secondary prevention strategies, and save costs, by potentially changing to an alternative antiplatelet regimen in selected non-responsive subjects.
12. Reticulated platelets in patients on antiplatelet therapy in the early and late phases after TIA or ischaemic stroke

12.1. Introduction

Platelets are released into the peripheral blood following megakaryocyte fragmentation within the bone marrow and/or pulmonary circulation (Harrison et al, 1997; Lunetta & Penttila, 1997). Young platelets that have been recently released into the circulation contain a residual amount of megakaryocyte-derived mRNA and were first identified by Ingram and Coopersmith in 1969 (Ingram & Coopersmith, 1969). They were termed 'reticulated platelets' because of the analogy with red cell reticulocytes. The percentage of reticulated platelets has been shown to be increased in conditions associated with high platelet turnover, and may be useful in monitoring the response to treatment in patients with idiopathic thrombocytopenic purpura (Thomas-Kaskel et al, 2007). Reticulated platelets were reported to be larger in size and have an increased mean density compared with normal platelets (Ingram & Coopersmith, 1969). Because reticulated platelets have been shown to be unstable and to undergo degradation within 24 hours in the circulation in animal studies (Ault & Knowles, 1995), measurement of the percentage of reticulated platelets in humans has the potential to be a useful marker of increased platelet production and/or turnover that could occur in patients with increased platelet activation. However, the reticulated platelet fraction may not be increased unless the stimulus to platelet activation also promotes thrombopoiesis. Studies using washed, fixed platelets, obtained from PRP, have shown that circulating reticulated platelets may increase when thrombopoiesis is increased, and may be normal or decreased when platelet production is suppressed (Ault et al, 1992).
Measurement of circulating reticulated platelets may also facilitate a better understanding of the mechanisms that mediate antiplatelet non-responsiveness, including aspirin non-responsiveness in healthy controls (Guthikonda et al, 2007) and CVD patients. It has been suggested that COX-2 expression may be upregulated in newly released, reticulated platelets (Rocca et al, 2002), and that functionally active COX-2 could contribute to thromboxane A2 formation in platelets, despite inhibition of COX-1 by aspirin therapy (Guthikonda et al, 2007).

There is some evidence of increased circulating reticulated platelets, indicating increased platelet turnover, following ischaemic stroke or TIA. One study included a small, unspecified number of patients with a recent ischaemic cerebrovascular event (Rinder et al, 1998) and 3 studies focused on the identification of reticulated platelets by flow cytometry in patients with recent ischaemic or haemorrhagic stroke (Smith et al, 2002), or in the early or late phases after TIA (McCabe et al, 2004b) or ischaemic stroke (Nakamura et al, 2002; McCabe et al, 2004b). Rinder et al measured the percentage of reticulated platelets (% RP) by flow cytometry in a small number of patients in the acute phase after an ‘arterial thrombosis’ (Rinder et al, 1998). Patients with thrombotic events in association with transient (n = 6) or chronic thrombocytosis (n = 14) had an increase in the % RP compared with patients with asymptomatic thrombocytosis (n = 23) or normal controls (n = 83) (Rinder et al, 1998). In contrast, the % RP was not increased in a group of 25 patients with ‘arterial thrombosis’ compared with normal controls. This group included patients who were studied within 12 hours of developing a myocardial infarction (n = 10), or thrombosis of the middle cerebral, iliac, femoral or popliteal arteries (n = 15), although the exact number of stroke patients was not specified. The authors suggested that the reticulated platelet count decreased after treatment with
aspirin in patients with chronic thrombocytosis, but did not specify whether or not these patients had been recently symptomatic at the time of initial sampling. If this had been the case, the change in the % RP could have been secondary to resolution of the acute stimulus to reticulated platelet formation rather than an effect of aspirin itself.

In a small pilot case-control study, Smith et al assessed 24 consecutive patients (18 with ischaemic stroke and 6 with haemorrhagic stroke) within 3 days of symptom onset and 14 demographic- and risk factor-matched controls (Smith et al, 2002). Mean platelet volume (MPV), platelet count, and the percentage of circulating reticulated platelets in fixed whole blood were measured. The authors found an increased mean percentage of reticulated platelets in the 18 patients with ischaemic stroke compared with 14 controls (12.8 vs. 8.2%; p = 0.044). All patients were treated with aspirin, but they were not reassessed in the late phase after symptom onset. The findings from this pilot study indicated that platelet turnover may be increased in acute ischaemic stroke.

In a larger case-control study, Nakamura et al compared the % RP in washed and fixed platelets obtained from PRP in 68 patients with ischaemic stroke with 140 'non-neurovascular' neurological controls (Nakamura et al, 2002). Ischaemic stroke subtypes were categorized as lacunar (n = 25), atherothrombotic (n = 26) or cardioembolic (n = 17). Venepuncture was performed in the acute (days 1 - 7), subacute (days 8 - 30) or chronic phases (≥ day 31) after stroke onset, with most samples taken in the chronic phase, but the same patients were not assessed at each time point in this study. Mean % RP was higher in patients with cardioembolic stroke than in controls (6.36 vs. 3.85%; p < 0.05). Mean % RP was also higher in patients with cardioembolic stroke compared with those with atherothrombotic (4.62%) or lacunar stroke (4.36%; p < 0.05). It was
postulated that the higher % RP represented enhanced thrombopoiesis following excessive thrombin-mediated platelet activation and excessive platelet turnover in the cardioembolic subgroup. In addition, the % RP was lower in patients on antiplatelet therapy (n = 50) and anticoagulant therapy (n = 16) compared with those not on antithrombotic therapy (n = 8). One must assume that some patients were included twice on different antithrombotic drug regimens, because the number of patients included in this analysis (n = 74) was greater than the total number of patients involved in the study. However, the authors clearly acknowledged that further studies are needed before drawing any firm conclusions about the effects of antithrombotic therapy on the circulating pool of reticulated platelets.

McCabe et al performed a prospective, case-control study in 79 patients in the early (1 – 27 days) phase after TIA or ischaemic stroke, and followed up 70 of these patients in the late phase (79 – 725 days after the event) (McCabe et al, 2004b). These data were compared with those obtained from 27 controls without cerebrovascular disease (CVD). The impact of aspirin dose escalation from 75mg to 150mg to 300mg daily on the percentage of reticulated platelets (% RP) in 10 patients in the late phase after TIA or ischaemic stroke was also investigated. The unadjusted % RP was not significantly higher in early or late phase CVD patients overall, or individual ischaemic CVD subtypes, compared with controls (p ≤ 0.3). However, after adjustment for age, the % RP was higher in early (p = 0.047) and late phase ischaemic CVD patients compared with controls (p = 0.01). There was a positive correlation between the % RP and MPV in EDTA- and citrate-anticoagulated whole blood in both the early and late phase after TIA or ischaemic stroke onset (p ≤ 0.01). The pilot data indicated that the % RP was not significantly influenced by aspirin dose. The authors concluded that their findings did
not convincingly support an excessive stimulus to platelet production in the early or late phases after or TIA or ischaemic stroke, but were consistent with the hypothesis that reticulated platelets are larger than more mature ‘non-riticulated’ platelets in ischaemic CVD.

Therefore, data pertaining to reticulated platelets in ischaemic CVD were limited prior to commencement of this thesis, and none of the published studies systematically assessed circulating reticulated platelets before and after commencing commonly prescribed antiplatelet regimens in TIA or ischaemic stroke patients compared with healthy controls.
12.2. Aims and Hypotheses

The aims of the case-control component of this study were:

(a) To compare the circulating reticulated platelet fraction in patients with TIA or ischaemic stroke (regardless of their antiplatelet treatment regimen) with healthy controls

(b) To assess demographic and laboratory variables influencing the circulating reticulated platelet fraction in ischaemic CVD.

We hypothesised that:

(a) Circulating reticulated platelets would be elevated in the early or late phase after TIA or ischaemic stroke compared with controls;

(b) There would be a positive correlation between the percentage of 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 ischaemic CVD.

A separate chapter has been dedicated to a longitudinal study that assessed the impact of changing antiplatelet therapy on circulating reticulated platelets in patients with ischaemic CVD [Chapter 13, Impact of antiplatelet agents on circulating reticulated platelets in the early and late phases after TIA or ischaemic stroke], and these data will not be discussed in this chapter.
12.3. Methods
12.3.1. Recruitment

Consecutive eligible patients older than 18 years of age, who had experienced an ischaemic stroke or TIA within the preceding four weeks, and who were going to undergo a change in antiplatelet therapy, were recruited to this component of the 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. We excluded patients with a history of primary intracerebral haemorrhage, myocardial infarction within the preceding 3 months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding 3 months (haemoglobin decrease of >1g/dl in one day, or requiring transfusion), if they had systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis including known platelet disorders, platelet count < 120 x 10^9/L or > 450 x 10^9/L, urea >10mmol/l or GFR < 30ml/min, current infection (clinical signs of infection, white cell count > 12 x 10^9/L), non-steroidal anti-inflammatory use within 14 days of recruitment.

Control subjects of similar age and gender were recruited from amongst the staff at AMNCH 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 assent 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: 2007/07/MA).

12.3.2. Clinical assessment

All subjects underwent a detailed clinical assessment by at least one of three examiners (WOT, JAK, DJHM), and information regarding vascular risk factors, smoking status and medication use was collected prospectively. Results of routine haematological, coagulation, biochemical and blood glucose testing were collected in the patient population. Brain computed tomography scans and/or magnetic resonance imaging, and imaging examination of carotid and vertebral arteries were performed in all CVD patients. MRA or CTA were performed when deemed appropriate by the treating physician, as described previously (McCabe et al, 2004a). A chest radiograph, an electrocardiograph (ECG), a 24-hour holter recording and transthoracic or transoesophageal echocardiography was obtained in all patients.

The underlying mechanism responsible for the TIA or ischaemic stroke was categorised according to the TOAST classification (Adams, Jr. et al, 1993). The degree of functional impairment following ischaemic stroke was quantified by using the modified Rankin scale. A score of 0 on this scale indicates full recovery with no residual symptoms or disability, and a score of 5 indicates severe disability (Farrell et al, 1991).
All patients underwent clinical and laboratory assessment at study entry before changing antiplatelet therapy (baseline), 14 days after changing antiplatelet therapy (14d), and at least 90 days (90d) following recruitment. In the ‘large artery atherosclerosis’ subgroup, the 90d follow-up was performed at least 3 months following carotid surgery or endovascular treatment, unless this treatment had been delayed for at least 3 months after the initial event.

12.3.3. Blood sampling and laboratory tests

All subjects were rested for at least 20 min, and careful venepuncture was performed from a free-flowing vein using a sterile 21G Butterfly needle (VenisystemsTM, Abbott, Ireland) and a Vacutainer® system with a luer adaptor (Becton Dickinson Vacutainer Systems, UK).

Blood sampling was performed as outlined in the General Methods section, and as described previously (McCabe et al, 2004b). Full blood count was performed in EDTA- and citrate-anticoagulated whole blood Vacutainer® tubes, including measurement of the mean platelet volume and platelet distribution width, between 2 and 4 hours after venepuncture using a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd, Milton Keynes, UK). This was to standardize the effect of the delay between venepuncture and sample analysis on the results obtained, because the MPV increases and the platelet count decreases over time with both anticoagulants (Bath, 1993).

The whole blood flow cytometry method used in this study was adapted from a previously described (Shattil et al, 1987) and validated protocol (McCabe et al, 2004b). In brief, a manual gate was positioned around the cloud of platelets, identified by their
characteristic pattern of forward scatter (FS) and side scatter (SS) on a scatterplot of log FS versus log SS that had been saved on the flow cytometer. To confirm that the cells within the gate in the test sample were platelets, the non-specific fluorescence of the control sample was calculated, and 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%. Thereafter, the gating settings around the platelet cloud were saved and not repositioned after gating on GpIb positive cells using the ‘panel’ set-up on the flow cytometer. The reticulated platelet assays were performed as part of a panel set-up on the flow cytometer, and this facilitated single labelling of platelets with Thiazole orange (Retic-COUNT™, Becton Dickinson, San Jose, USA). The % RP was calculated by measuring the % of TO-positive (reticulated) platelets in the test sample in FL1 (Figure 9-8 and Figure 9-9).

12.3.4. Statistical Methods

Paired and unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively, and the Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, respectively. Spearman rank-order correlation was used to assess the relationship between reticulated platelets and FBC components. Multiple linear regression analysis was performed to examine the potential influence of different independent predictor variables on the percentage of circulating reticulated platelets, in turn. P < 0.05 was considered to be statistically significant. All statistical calculations were performed with R version 2.10.1 (R Development Core Team, 2009).
12.4. Results
Eighty six eligible patients with TIA or ischaemic stroke were assessed within 4 weeks of onset of an acute TIA or ischaemic stroke, and subsequently at 14d and 90d after recruitment. The same patients were assessed at each time point. The median time from index TIA or stroke to assessment was 8 days (range 0-50 days); two patients were initially assessed at 37 and 50 days because of delays in recruitment, but all other patients were recruited within 4 weeks of symptom onset, as planned. No patient had a recurrent vascular event during follow-up. The clinical details of the study subjects, and their TIA or stroke subtypes are outlined in Table 12-1 and Table 12-2. 63 patients (73%) were taking aspirin monotherapy at baseline, 3 patients were taking clopidogrel, 2 were taking aspirin and dipyridamole combination therapy, and 21 patients (24%) were taking no antiplatelet medication. All flow cytometric analyses were performed within 120 min of venepuncture.
Table 12-1: Demographic Data in Patients at Enrolment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Stroke or TIA (n =86)</th>
<th>Controls (n=24)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years</td>
<td>60 [±16]</td>
<td>54 [±13]</td>
<td>0.04</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>55/31</td>
<td>15/9</td>
<td>1.0</td>
</tr>
<tr>
<td>Prior Stroke/TIA (%)</td>
<td>19 (22%)</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>IHD* (%)</td>
<td>15 (17%)</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>52 (60%)</td>
<td>4 (17%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes Mellitus (%)</td>
<td>13 (15%)</td>
<td>0</td>
<td>0.06</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment (%)</td>
<td>1 (1%)</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Family History Stroke (%)</td>
<td>37 (43%)</td>
<td>7 (29%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Prior DVT/PE (%)</td>
<td>4 (5%)</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>Peripheral Vascular Disease (%)</td>
<td>7 (8%)</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Migraine (%)</td>
<td>18 (21%)</td>
<td>2 (8%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>22 (26%)</td>
<td>2 (8%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Ex-smoker (%)</td>
<td>11 (1%)</td>
<td>6 (25%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Never Smoked (%)</td>
<td>33 (38%)</td>
<td>16 (67%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>39 (45%)</td>
<td>3 (13%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Index event occurred on antiplatelet agent</td>
<td>15 (17%)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

p values relate to comparisons between patients and controls (Pearson's Chi-squared test or Fisher's Exact Test where appropriate)
Values are Means [±SD] or absolute values with percentages in parentheses (%)
IHD* = History of ischaemic heart disease
A Fib/Flutter = Atrial fibrillation or flutter
DVT/PE = Deep venous thrombosis or pulmonary embolism

Table 12-2: Aetiological TIA and stroke patient subtyping by TOAST classification

<table>
<thead>
<tr>
<th>TIA / Stroke subtype</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Artery Atherosclerotic</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Lacunar</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Undetermined aetiology</td>
<td>41</td>
<td>48</td>
</tr>
<tr>
<td>Other determined</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>
12.4.1. Reticulated platelets in ischaemic CVD patients vs. controls

The median % Gplb expression was similar in CVD patients (99.1%) and controls (98.9%), confirming that the majority of cells analysed on the flow cytometer in each group were platelets. There were no significant differences in the % RP between CVD patients at baseline or 14d versus controls (p ≥0.1), but the % RP was significantly increased in patients at 90d compared with controls (p = 0.008) (Table 12-3 and Figure 12-1).

Because there were some differences in demographic and vascular risk factor profiles between CVD patients and controls, multiple linear regression analysis was performed to investigate the impact of these variables on the comparison of % RP between patients and controls. Because a normal distribution could not be obtained with conventional data log transformation, the central limit theorem was used to perform multiple linear regression to investigate the impact of age, prior history of stroke or TIA, hypertension, ischaemic heart disease, statin use and smoking on % RP. None of these variables independently influenced the results at any stage during follow up (p ≥ 0.2).
Table 12-3 Comparison of % reticulated platelets (% RP) in patients at different stages of follow-up compared with controls

<table>
<thead>
<tr>
<th>Patients at baseline</th>
<th>14d</th>
<th>90d</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>% RP</td>
<td>13.95</td>
<td>16.35</td>
<td>19.25</td>
</tr>
<tr>
<td>p-value</td>
<td>0.1</td>
<td>0.2</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Values are medians (25-75% inter quartile range)
p values refer to comparisons between patients and controls
Figure 12-1 Scatterplot of circulating reticulated platelet levels in CVD patients at baseline, 14 days, > 90 days, and in control subjects. Each point represents a single patient. Red lines and numbers above indicate median values. p values refer to comparison of median % RP between CVD patients at different time points with controls.
12.4.2. FBC parameters in ischaemic CVD patients vs. controls

The total white cell count was higher in patients than in controls at each time point during the study (p ≤ 0.004, Table 12-4). This was mainly driven by the neutrophil and monocyte counts. There were no significant differences in haemoglobin concentrations (p ≥ 0.06) or platelet counts (p ≥ 0.4) between patients and controls at any time point during the study. The haematocrit was higher in patients than controls at baseline (p = 0.02) only, but not at any other time point during follow-up.

There was no significant difference in MPV or PDW in either EDTA or citrate between patients and controls at any time point (p ≥ 0.05). There was no significant correlation between the platelet count in either EDTA or citrate and the % RP at baseline or at 14d in ischaemic CVD patients (p ≥ 0.1). However, there was a positive correlation between the mean platelet volume and % RP at baseline and 90d in both citrate and EDTA (rho ≥ 0.22, p ≤ 0.04), but not at 14d (p ≥ 0.2). There was also a significant positive correlation between the PDW and the percentage of circulating reticulated platelets at baseline (rho ≥ 0.23, p ≤ 0.03) and 90d (rho ≥ 0.29, p ≤ 0.006), but not at 14 days (p ≥ 0.1) in both citrate and EDTA.
Table 12-4: Haematological parameters in patients at baseline, 14 days and > 90 days compared with controls

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count</td>
<td>227</td>
<td>226</td>
<td>229</td>
<td>221</td>
</tr>
<tr>
<td>x 10^9/L EDTA</td>
<td>(196-257)</td>
<td>(198-358)</td>
<td>(201-267)</td>
<td>(196-257)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.5</td>
<td>0.7</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Platelet Count</td>
<td>174</td>
<td>173</td>
<td>175</td>
<td>171</td>
</tr>
<tr>
<td>x 10^9/L Citrate</td>
<td>(143-205)</td>
<td>(153-198)</td>
<td>(150-200)</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.9</td>
<td>0.9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution Width</td>
<td>13.2 (12.1-14.2)</td>
<td>12.9 (11.5-14.3)</td>
<td>12.6 (11.4-14.0)</td>
<td>12.4 (11.8-13.1)</td>
</tr>
<tr>
<td>% EDTA</td>
<td>0.05</td>
<td>0.3</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution Width</td>
<td>11.2 (10.3-12.3)</td>
<td>11.1 (9.8-11.8)</td>
<td>11 (9.9-11.8)</td>
<td>10.8 (10.3-11.6)</td>
</tr>
<tr>
<td>% Citrate</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) EDTA</td>
<td>9.8 (0.8)</td>
<td>9.8 (0.8)</td>
<td>9.7 (0.8)</td>
<td>9.7 (0.7)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.3</td>
<td>0.7</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl) EDTA</td>
<td>14.3 (13.2-15.6)</td>
<td>13.9 (12.9-14.7)</td>
<td>13.9 (12.9-15.0)</td>
<td>13.9 (13.3-14.4)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.06</td>
<td>0.1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>MCH (fl) EDTA</td>
<td>30.8 (29.6-31.9)</td>
<td>30.9 (29.5-31.6)</td>
<td>30.7 (29.6-31.9)</td>
<td>31.3 (30.2-32.2)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>MCV (pg) EDTA</td>
<td>90.2 (86.5-92.5)</td>
<td>89.8 (86.9-91.7)</td>
<td>90.3 (87.6-92.7)</td>
<td>91.1 (88.1-93.5)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.3</td>
<td>0.2</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>HCT (L/L) EDTA</td>
<td>0.42 (±0.04)</td>
<td>0.40 (±0.03)</td>
<td>0.41 (±0.04)</td>
<td>0.40 (±0.02)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.02</td>
<td>0.9</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>WCC</td>
<td>7.26 (6.16-8.99)</td>
<td>6.48 (5.69-7.99)</td>
<td>6.79 (5.71-8.05)</td>
<td>5.33 (4.82-6.76)</td>
</tr>
<tr>
<td>x 10^9/L EDTA</td>
<td>&lt;0.0001</td>
<td>0.004</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.35 (3.12-5.65)</td>
<td>3.94 (2.85-4.76)</td>
<td>3.96 (3.14-4.81)</td>
<td>2.98 (2.52-3.33)</td>
</tr>
<tr>
<td>x 10^9/L EDTA</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.64 (0.51-0.77)</td>
<td>0.58 (0.50-0.71)</td>
<td>0.44 (0.37-0.54)</td>
<td>0.44 (0.37-0.54)</td>
</tr>
<tr>
<td>x 10^9/L EDTA</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.99 (1.54-2.54)</td>
<td>1.91 (1.39-2.43)</td>
<td>1.90 (1.49-2.42)</td>
<td>1.88 (1.53-2.31)</td>
</tr>
<tr>
<td>x 10^9/L EDTA</td>
<td>0.7</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.16 (0.10-0.24)</td>
<td>0.17 (0.12-0.23)</td>
<td>0.16 (0.1-0.25)</td>
<td>0.17 (0.08-0.22)</td>
</tr>
<tr>
<td>x 10^9/L EDTA</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

P values indicate results of unpaired two-sample t-test or Wilcoxon rank sum test comparing data from patients with controls

Values are Means (±SD) or medians (25-75% Inter-quartile range)
12.4.3. Subgroup comparisons between ischaemic CVD patients and controls

Subgroup analysis revealed that patients with stroke or TIA of small vessel aetiology had a higher percentage circulating reticulated platelets at baseline than controls (p = 0.03, Figure 12-2). Patients with stroke or TIA of small vessel aetiology, undetermined aetiology and cardioembolic aetiology had higher percentage circulating reticulated platelets at 90d compared with controls (p ≤ 0.0496, Figure 12-4).

The impact of altering antiplatelet therapy on the circulating reticulated platelet pool in the early and late phases after TIA or stroke will be dealt with in a separate chapter [Chapter 13, Impact of antiplatelet agents on circulating reticulated platelets in the early and late phases after TIA or ischaemic stroke].
Figure 12-2: Comparison of median circulating reticulated platelet levels at baseline in patients with stroke from large artery atherosclerosis (Athero), cardioembolism (Cardio), small vessel occlusion (Lac), other determined causes (Other), undetermined causes (Undet) and controls (Con). Red lines and values above indicate median values. P values refer to pairwise comparison with controls.
Figure 12-3 Comparison of median circulating reticulated platelet levels at 14 days in patients with stroke from large artery atherosclerosis (Athero), cardioembolism (Cardio), small vessel occlusion (Lac), other determined causes (Other), undetermined causes (Undet) and controls (Con). Red lines and values above indicate median values. P values ≥ 0.1 for pairwise comparison with controls.
Figure 12-4 Comparison of median circulating reticulated platelet levels at > 90 days in patients with stroke from large artery atherosclerosis (Athero), cardioembolism (Cardio), small vessel occlusion (Lac), other determined causes (Other), undetermined causes (Undet) and controls (Con).

Red lines and values above indicate median values. P values refer to pairwise comparison with controls.
12.5. Discussion

This is the largest study of reticulated platelets in a well-characterised cohort of patients with TIA or ischaemic stroke, and the first study to investigate circulating reticulated platelets in the same patients in the early, subacute and late phases following symptom onset.

In contrast to some, (Smith et al, 2002; Nakamura et al, 2002) and in keeping with other published data (McCabe et al, 2004b) this adequately-sized study did not find an increase in the unadjusted percentage of circulating reticulated platelets in the early phase (median 8 days, range: 0-50 days) after TIA or ischaemic stroke compared with controls. However, somewhat unexpectedly, we did demonstrate elevated levels of circulating reticulated platelets in CVD patients in the late phase after symptom onset compared with controls.

Previous studies have demonstrated elevated platelet activation in similar populations in the early (Grau et al, 1998; Meiklejohn et al, 2001; Markel et al, 2002; Marquardt et al, 2002; Cha et al, 2003; Karakantza et al, 2003; McCabe et al, 2004a) and late (Grau et al, 1998; Yamazaki et al, 2001; Meiklejohn et al, 2001; Cha et al, 2004; McCabe et al, 2004a) phases after TIA or ischaemic stroke. However, excessive platelet production appears to occur only in the late phase, as evidenced by an elevated percentage of circulating reticulated platelets. Our data could be interpreted as indicating that increased platelet production does not precede the onset of a TIA or ischaemic stroke, but is a consequence of the ischaemic event. However, larger studies are needed to confirm this hypothesis in ischaemic CVD patients because several subgroups of patients at risk of experiencing vascular events, particularly those associated with
increased platelet activation, have been shown to have elevated levels of circulating reticulated platelets in the absence of a recent thrombotic event. For example, an increased % RP has been identified in patients with metabolic syndrome (Vaduganathan et al, 2008), essential thrombocytosis (Arellano-Rodrigo et al, 2009), post-renal transplantation (Cesari et al, 2010), peripheral vascular disease (Esposito et al, 2003) or coronary artery disease (Cesari et al, 2008). On the contrary, patients with hypercholesterolaemia (Pathansali et al, 2001) and the antiphospholipid syndrome (Joseph et al, 1998) have not been shown to have elevated circulating reticulated platelets compared with controls. These data suggest that certain risk factors may promote increased platelet production, whereas others do not, but larger studies in individual ‘at risk groups’ need to be performed to investigate this further. We found elevated circulating reticulated platelets in lacunar, cardioembolic, and undetermined TIA or stroke subtypes compared with controls at 90d, although we accept that these subgroup analyses are prone to both type I and type II errors due to the limited number of subjects in each CVD subgroup.

Furthermore, although subgroup analysis of prior studies (Nakamura et al, 2002) raised the possibility that reticulated platelets in washed and fixed platelet preparations obtained from PRP were significantly higher in patients in the very early (< 7 days, n=4) compared with the later phase (≥ 31 days, n = 10) after cardioembolic stroke, the same patients were not studied at each time point. Our case-control and longitudinal study data do not support the hypothesis that the % RP increases early, and decreases later after symptom onset because the % RP actually increased in our overall patient population during follow up (p < 0.008).
The previous finding that circulating reticulated platelets are larger than more mature ‘non-reticulated’ platelets in ischaemic CVD (McCabe et al, 2004b) is again supported by our findings that there was a positive correlation between both the MPV and PDW and % RP at baseline and 90d. The absence of a significant positive correlation between the MPV, PDW and reticulated platelet fraction at 14 days is unexplained at present. However, because all patients in this study were undergoing a change in antiplatelet therapy during this time interval, the effect of altering antiplatelet therapy on reticulated platelet formation was formally explored to see whether these changes could have influenced some of the results outlined above [Chapter 13, Impact of antiplatelet agents on circulating reticulated platelets in the early and late phases after TIA or ischaemic stroke].

In keeping with prior smaller studies (Smith et al, 2002; McCabe et al, 2004b), but in contrast to other larger studies (O'Malley et al, 1995; Butterworth & Bath, 1998), we did not find an increase in the MPV in ischaemic CVD patients compared with controls in either EDTA- or citrate-anticoagulated blood. These findings do not support the concept that the MPV remains elevated in the early or late phases after TIA or stroke onset, but because the positive studies on this topic were larger and presumably more adequately powered, our negative MPV data could also reflect a type II error. Larger studies in patients with ischaemic cerebrovascular disease, treated with modern secondary preventative treatment, are warranted to determine whether the MPV is significantly increased in the hyperacute, subacute or late phases after symptom onset, and whether the MPV is still of prognostic significance in ischaemic CVD. To our knowledge, the PDW has not previously been systematically studied in ischaemic CVD patients, and in
view of the positive correlation between the PDW and the % RP in this study, this also
deserves further prospective assessment.

As reported previously (Smith et al, 2002; McCabe et al, 2004b), there was no
significant increase in the platelet count in ischaemic CVD patients at any time point
during follow-up compared with controls. These combined data on the reticulated
platelet fraction and total platelet count indicate that there may an ongoing stimulus to
reticulated platelet formation, but that the overall platelet count remains stable in the
late phase after TIA or stroke onset.

Although none of the patients had any signs of infection during the study period, the
total white cell count, neutrophil, monocyte and lymphocyte counts were significantly
elevated at 14d and at > 90 days in patients compared with controls. The median total
white cell, neutrophil, monocyte and lymphocyte counts remained within the ‘normal
laboratory range’ of 4-11 x 10^9/L, but the duration of the increase in leucocyte fractions
was more prolonged than the 3 days reported previously (Marquardt et al, 2009).
Because of these findings, the impact of changing antiplatelet therapy on circulating
white cells was also investigated in the relevant chapters [Chapter 10, Assessment of the
impact of aspirin and clopidogrel on platelet activation and function in the early and
late phases after TIA or ischaemic stroke and Chapter 11, Assessment of the impact of
dipyridamole on platelet activation and function in the early and late phases after TIA or
ischaemic stroke].
12.6. Conclusion
This study indicates that the percentage of circulating reticulated platelets is significantly increased in the late phase (> 90 days) but not in the early phase (≤ 4 weeks) after TIA or ischaemic stroke compared with controls. It is unclear whether the stimulus to reticulated platelet formation precedes or follows the ischaemic cerebrovascular event, but these data suggest that it may follow the event because the reticulated platelet fraction was not significantly increased in the early phase after presentation. We have confirmed prior findings that reticulated platelets are larger than more mature, non-reticulated platelets in ischaemic CVD, but have not shown any evidence that the MPV or PDW are significantly elevated in patients with ischaemic CVD compared with controls. The mechanisms responsible for increased circulating reticulated platelets following TIA and ischaemic stroke remain to be elucidated, as does the impact of changing antiplatelet therapy on reticulated platelet formation. If higher levels of circulating reticulated platelets are shown to influence \textit{ex vivo} non-responsiveness to antiplatelet therapy in the laboratory in patients with ischaemic CVD, this may improve our understanding of the mechanisms responsible for recurrent vascular events in patients in the clinical setting.
13. Impact of antiplatelet agents on circulating reticulated platelets in the early and late phases after TIA or ischaemic stroke

13.1. Introduction

As discussed in detail in Chapter 12, measurement of the percentage of circulating reticulated platelets has the potential to be a useful marker of increased platelet production and/or turnover that could occur in patients with increased platelet activation e.g. following TIA or ischaemic stroke. We have shown in Chapter 12 that the percentage of circulating reticulated platelets is significantly increased in the late phase (> 90 days) but not in the early phase (≤ 4 weeks) after TIA or ischaemic stroke compared with controls. Prior to commencement of this thesis, none of the published studies had longitudinally assessed the impact of commencing commonly prescribed antiplatelet regimens on the circulating reticulated platelet fraction in TIA or ischaemic stroke patients. Furthermore, to our knowledge, no studies had assessed the potential relationship between circulating reticulated platelets and platelet reactivity or inhibition of platelet function \textit{ex vivo} in ischaemic CVD. Measurement of circulating reticulated platelets could facilitate a better understanding of the mechanisms that mediate antiplatelet non-responsiveness, including aspirin non-responsiveness in healthy controls (Guthikonda \textit{et al}, 2007) and CVD patients, because it has been suggested that COX-2 expression is upregulated in newly released, reticulated platelets (Rocca \textit{et al}, 2002), and that functionally active COX-2 could contribute to thromboxane A\textsubscript{2} formation in platelets, despite inhibition of COX-1 by aspirin therapy (Guthikonda \textit{et al}, 2007). Therefore, elucidation of the impact of changing antiplatelet regimens on
circulating reticulated platelets, and the relationship between circulating reticulated platelets and platelet function deserved investigation.

13.2. Aims and Hypotheses

The aims of this longitudinal observational study were:

(a) To assess the impact of altering antiplatelet therapy on the circulating reticulated platelet fraction during follow-up in patients recruited within 4 weeks of onset of a TIA or ischaemic stroke;

(b) To assess whether patients with ex vivo 'non-responsiveness' to antiplatelet therapy on platelet function testing in whole blood had higher levels of circulating reticulated platelets than those who were deemed 'responsive'.

We hypothesised that:

(a) The percentage of circulating reticulated platelets would reduce following the introduction of aspirin monotherapy, after changing from aspirin monotherapy to aspirin and dipyridamole combination therapy, or after changing from aspirin to clopidogrel monotherapy in ischaemic CVD patients;

(b) Patients with ex vivo 'non-responsiveness' to antiplatelet agents on platelet function testing in whole blood would have higher levels of circulating reticulated platelets than those who were deemed to be 'responsive'.
13.3. Methods

13.3.1. Recruitment

The methods of recruitment, and inclusion and exclusion criteria have already been outlined in detail in Chapter 12 [Reticulated platelets in patients on antiplatelet therapy in the early and late phases after TIA or ischaemic stroke]. In brief, consecutive eligible patients older than 18 years of age, who had experienced an ischaemic stroke or TIA within the preceding four weeks, and whose treating physician decided to change antiplatelet therapy were recruited to this component of the study.

Written informed consent, or assent 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: 2007/07/MA).

13.3.2. Clinical assessment

As outlined in Chapter 9, General Methods, all subjects underwent a detailed clinical assessment by at least one of three examiners (WOT, JAK, DJHM), and information regarding vascular risk factors, smoking status and medication use was collected prospectively. All investigations, stroke subtyping and assessment of stroke severity were performed as outlined in Chapter 9 (Farrell et al, 1991; Adams, Jr. et al, 1993; McCabe et al, 2004a). All patients underwent detailed clinical and laboratory assessment before (baseline), 14 days after (14d), and at least 90 days (90d) after a change in their antiplatelet therapy. In the 'large artery atherosclerotic subgroup', the 90d follow-up was performed at least 3 months following carotid surgery or endovascular treatment, unless this treatment had been delayed for at least 3 months after the initial event.
As the majority of patients at baseline and 14d were assessed during their inpatient stay, adherence to antithrombotic therapy was confirmed by checking the inpatient drug chart. All outpatients were phoned before their appointment to stress the importance of medication adherence in the week prior to assessment. Adherence in this group was assessed by history taking alone. Patients who were considered to be non-adherent to their antithrombotic regimen were invited back for reassessment after a further 14 days.

### 13.3.3. Blood sampling and laboratory tests:

All subjects were rested for at least 20 min, and careful venepuncture was performed as outlined in Chapter 9, General Methods, and in the Chapter 12 [Reticulated platelets in patients on antiplatelet therapy in the early and late phases after TIA or ischaemic stroke]. Full blood count was performed in EDTA- and 3.2% citrate-anticoagulated whole blood Vacutainer® tubes, including measurement of the mean platelet volume and platelet distribution width, between 2 and 4 hours after venepuncture using a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd, Milton Keynes, UK). This was to standardize the effect of the delay between venepuncture and sample analysis on the results obtained, because the MPV increases and the platelet count decreases over time with both anticoagulants (Bath, 1993).

The whole blood flow cytometry method used in this study was adapted from a previously described (Shattil et al, 1987) and validated protocol (McCabe et al, 2004b). The reticulated platelet fraction (%RP) was quantified as described in detail in Chapter 9 and Chapter 12, and was calculated by measuring the test sample fluorescence in FL1.
Platelet function was assessed with the PFA-100® to measure C-ADP and C-EPI closure times in citrate-anticoagulated whole blood between 2 and 2.5 hours after venepuncture, as described in Chapter 9 and also Chapter 11. The maximum closure time recorded by the device is 300s, and we arbitrarily defined closure times above 300s as 301s to allow for statistical analysis with non-parametric tests. As discussed in Chapter 11, most previous studies have used arbitrary ‘cut-points’ to define patients as being either non-responsive or responsive to antiplatelet therapy ex vivo on the PFA-100® in a ‘cross-sectional, case-control’ fashion. Because we hypothesised, and have shown in Chapters 10 and 11, that there may be a large inter-individual variation in the degree of inhibition of platelet function with antiplatelet therapy on the PFA-100®, we used our novel ‘longitudinal definitions’ for the purpose of this study assessing the impact of reticulated platelets on ex vivo antiplatelet non-responsiveness. Antiplatelet non-responsiveness was defined as failure to prolong the relevant PFA-100® closure time, compared with the patient’s own baseline value, by more than twice the coefficient of variation (CV) of the assay (see Chapters 10 and 11). The CV of the C-EPI assay was 7.5%, and the CV of the C-ADP assay was 7% in our lab.

Therefore:

- Aspirin non-responsiveness’ was defined as failure to prolong C-EPI closure times with aspirin compared with the baseline on no antiplatelet treatment by > 15%.

- Dipyridamole non-responsiveness’ was defined as failure to prolong C-ADP closure times compared with the baseline on aspirin by > 14% when patients changed from aspirin monotherapy to aspirin and dipyridamole combination therapy.

- Clopidogrel non-responsiveness was defined as failure to prolong Collagen-ADP closure times by >14% in patients who were initially assessed on aspirin and subsequently assessed on clopidogrel.
13.3.4. Statistical Methods

We analysed the longitudinal data in each antiplatelet treatment subgroup individually at first i.e. at 14d versus baseline, and at 90d versus baseline. We then compared the longitudinal data in the overall ischaemic CVD patient population at 14d versus baseline, and at 90d versus baseline, to assess the impact of any change in antiplatelet therapy on the % RP over time. We also compared the % RP in 'antiplatelet non-responders' with 'antiplatelet responders' in the overall ischaemic CVD population, regardless of the antiplatelet treatment regimen they received. Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, and the Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, respectively. The Kruskal-Wallis rank sum test was used for comparison of multiple non-parametric variables, where appropriate. Chi squared or Fisher exact tests were used to compare proportions between unpaired groups, where appropriate. Spearman rank-order correlation was used to assess the relationship between reticulated platelets and FBC components. P < 0.05 was considered to be statistically significant. All statistical calculations were performed using R version 2.10.1 (R Development Core Team, 2009).
13.4. Results

Seventy-eight patients were assessed at baseline, 14d and 90d. The clinical details of the study subjects, and their stroke subtypes are outlined in Table 13-1 and Table 13-2.

No patient had a recurrent vascular event during follow-up. Some patients were initially included in one subgroup after starting aspirin monotherapy, and they then moved to another subgroup during follow up if their treating physician later changed them to aspirin and dipyridamole combination therapy or clopidogrel monotherapy.

Therefore, eighteen patients were studied at baseline on no antiplatelet agents and subsequently at 14d on aspirin monotherapy (median dose 300 mg, range: 75 mg-100 mg daily); only 8 of these patients were reassessed again at 90d on aspirin alone because they had undergone a change in antithrombotic therapy and were no longer on aspirin monotherapy. One of these patients did not have PFA-100® testing at the 14d follow-up visit because of device failure, but all 8 patients had PFA-100® testing at 90d.

Forty seven patients were studied at baseline on aspirin monotherapy (median dose 75 mg daily, range: 75mg-300 mg daily) and subsequently at 14d on combination therapy with 75mg of aspirin daily and 200mg of dipyridamole MR BD; 45 of these patients had follow up data at 90d. All assessed patients had PFA-100® testing at 14d. Two of these patients did not have PFA-100® testing at 90d because of device failure.
Twenty three patients were studied at baseline on aspirin monotherapy (75-300mg daily) and subsequently at 14d and 90d on clopidogrel monotherapy (75mg daily). All assessed patients had PFA-100® testing at 14d. One of these patients did not have PFA-100 testing at 90d because of device failure.

All analyses were performed on the flow cytometer within 129 minutes of venepuncture. The median % GpIb expression in our entire CVD patient population was 99.1% confirming that the majority of cells analysed on the flow cytometer were platelets.
### Table 13-1: Demographic Data in Patients at Enrolment

<table>
<thead>
<tr>
<th></th>
<th>Stroke or TIA (n = 78)</th>
<th>% Total</th>
<th>Responders (n = 38)</th>
<th>Non-Responders (n = 37)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (± SD)</td>
<td>61 (±13)</td>
<td>58 (±13)</td>
<td>63 (±14)</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Gender (Male)</td>
<td>51 65%</td>
<td>21</td>
<td>27</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>14 18%</td>
<td>7</td>
<td>7</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IHD</td>
<td>13 17%</td>
<td>5</td>
<td>7</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Hypertension</td>
<td>47 61%</td>
<td>20</td>
<td>25</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>10 13%</td>
<td>6</td>
<td>4</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment</td>
<td>0 0%</td>
<td>0</td>
<td>0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Family History Stroke</td>
<td>33 42%</td>
<td>19</td>
<td>14</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>4 5%</td>
<td>2</td>
<td>2</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>4 5%</td>
<td>2</td>
<td>2</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Current smokers (at enrolment)</td>
<td>18 23%</td>
<td>9</td>
<td>9</td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>20 26%</td>
<td>13</td>
<td>15</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Never Smoked</td>
<td>30 38%</td>
<td>16</td>
<td>13</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Statin use</td>
<td>35 45%</td>
<td>17</td>
<td>15</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>6 7%</td>
<td>4</td>
<td>2</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Index event occurred on antiplatelet agent</td>
<td>36 47%</td>
<td>15</td>
<td>17</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

P values relate to chi squared testing between antiplatelet responders and non-responders, or Fisher exact test when individual cell values were < 10.

Values are Means (±SD) or absolute values.

### Table 13-2: Aetiological subtyping by TOAST classification

<table>
<thead>
<tr>
<th>Stroke/TIA subtype</th>
<th>Total (78)</th>
<th>Responders (38)</th>
<th>Non-Responders (37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Artery Atherosclerotic</td>
<td>3 (4%)</td>
<td>0 (0%)</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>19 (24%)</td>
<td>6 (16%)</td>
<td>12 (32%)</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>11 (14%)</td>
<td>6 (16%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>Undetermined Aetiology</td>
<td>39 (50%)</td>
<td>22 (58%)</td>
<td>15 (41%)</td>
</tr>
<tr>
<td>Other Determined</td>
<td>6 (8%)</td>
<td>4 (11%)</td>
<td>2 (5%)</td>
</tr>
</tbody>
</table>

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Impact of changing from no medication to aspirin monotherapy

Commencing aspirin monotherapy did not significantly alter the percentage of circulating reticulated platelets at either 14d (mean % RP 18.24 ± 8.21%) or 90d (mean % RP 20.87 ± 9.15%) compared with baseline (mean % RP 20.16 ± 7.81%; p ≥ 0.1),

4/17 (24 %) patients at 14d, and 2/8 patients (25%) at 90d were non-responsive to aspirin. Aspirin non-responders had a higher percentage of circulating reticulated platelets than aspirin responders at 14d (28.85 % vs. 16.29 %, p = 0.01), but the differences between non-responders and responders was not significant at 90d (23.80 % vs. 19.90 %; p = 0.5). It is important to re-emphasise that only 8 patients in this subgroup had 90d follow-up. There were no significant changes in MPV or PDW in response to starting aspirin monotherapy (p ≥ 0.08).

Table 13-3: Summary of flow cytometry findings – Percentage circulating reticulated platelets in patients who were initially assessed on no medication and subsequently on aspirin

<table>
<thead>
<tr>
<th>No Medication - Aspirin</th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall % RP p value*</td>
<td>20.16 (± 7.81)</td>
<td>18.24 (± 8.21)</td>
<td>0.1</td>
</tr>
<tr>
<td>Responders p value†</td>
<td>16.29 (± 8.21)</td>
<td>28.85 (± 1.48)</td>
<td>0.01</td>
</tr>
<tr>
<td>Non-Responders p value†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means (± SD)

*P value refers to comparisons between values at 14d and 90d versus baseline

†P value refers to comparison of % RP between responders and non-responders at each time point
Impact of changing from aspirin monotherapy to aspirin and dipyridamole MR combination therapy

The addition of dipyridamole MR to aspirin did not significantly alter the % RP at either 14d (median % RP 13.70 %) or 90d (median % RP 18.20 %) compared with baseline (median % RP 13.40 %; p ≥ 0.3).

26/47 (55%) patients were non-responsive to dipyridamole at 14d, and 23/45 (51%) were dipyridamole non-responsive at 90d. There were no significant differences in % RP between dipyridamole non-responders and responders at either 14d or 90d (p ≥ 0.5). There was no correlation between the percentage of circulating reticulated platelets and the collagen-ADP closure times on the PFA-100 at 14 days or 90 days (p ≥ 0.4).

Mean MPV and PDW decreased at 90d compared with baseline in both EDTA and citrate-anticoagulated blood after changing from aspirin monotherapy to aspirin and dipyridamole combination therapy (p ≤ 0.004).

Table 13-4: Summary of flow cytometry findings – Percentage circulating reticulated platelets in patients who were initially assessed on aspirin and subsequently on aspirin and dipyridamole MR

<table>
<thead>
<tr>
<th>Aspirin – Aspirin + Dpy</th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td>p value*</td>
<td>1.0</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>11.85 (9.15-21.65)</td>
<td>17.45 (9.29-23.18)</td>
<td></td>
</tr>
<tr>
<td>Non-Responders</td>
<td>17.30 (7.43-21.65)</td>
<td>18.80 (11.08-23.40)</td>
<td></td>
</tr>
<tr>
<td>p value^</td>
<td>0.9</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

Values are medians (25-75% Inter-quartile range)
*P value refers to comparisons between values at 14d and 90d versus baseline
^P value refers to comparison of % RP between responders and non responders at each time point
Impact of changing from aspirin to clopidogrel monotherapy

Changing from aspirin monotherapy (median 12.60 %) to clopidogrel monotherapy did not initially affect the % RP at the 14d follow-up visit (median % RP 14.20 %, p = 0.5), but did lead to a significant rise in the % RP at 90d (median % RP 21.20 %, p = 0.0006).

14/23 (61%) patients were non-responsive to clopidogrel at 14d, and 12/22 patients (55%) were non-responsive to clopidogrel at 90 d. There were no significant differences in % RP between clopidogrel non-responders and responders at either 14d or 90 d (p ≥ 0.2).

There were no significant changes in MPV or PDW in response to changing from aspirin to clopidogrel monotherapy to mirror the changes in circulating reticulated platelets (p ≥ 0.1, Table 13-6). Mean platelet count transiently dropped from 223 x 10^9/L at baseline to 207 x 10^9/L at 14d (p = 0.004), but increased again at 90d (mean 214 x 10^9/L, p = 0.2). Monocyte and lymphocyte counts were lower at 14d than at baseline (p ≤ 0.03), and lymphocyte counts remained lower at 90d compared with baseline on aspirin (p = 0.04). Haemoglobin and haematocrit were both lower at 14d and 90d than at baseline (p ≤ 0.03).
<table>
<thead>
<tr>
<th>Aspirin - Clopidogrel</th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall % RP</td>
<td>12.60 (9.39-21.05)</td>
<td>14.20 (6.63-20.05)</td>
<td>21.20 (16.35-24.70)</td>
</tr>
<tr>
<td>p value*</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>9.79 (6.45-15.05)</td>
<td>17.20 (8.52-22.93)</td>
<td>22.7 (18.28-27.10)</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>17.20 (8.52-22.93)</td>
<td>22.7 (18.28-27.10)</td>
<td></td>
</tr>
<tr>
<td>p value^</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are medians (25-75% Inter-quartile range)

*P value refers to comparisons between values at 14d and 90d versus baseline

^P value refers to comparison of % RP between responders and non responders at each time point
Table 13-6: FBC in patients assessed on aspirin and subsequently on clopidogrel

<table>
<thead>
<tr>
<th>FBC Parameter</th>
<th>Baseline (n =78)</th>
<th>14d (n = 78)</th>
<th>90d (n = 78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count x 10⁹/L EDTA p value</td>
<td>223 (±50)</td>
<td>207 (±43)</td>
<td>214 (±41)</td>
</tr>
<tr>
<td>Platelet Count x 10⁹/L Citrate p value</td>
<td>166 (±43)</td>
<td>159 (±34)</td>
<td>169 (±39)</td>
</tr>
<tr>
<td>Platelet Distribution Width % EDTA p value</td>
<td>13.4 (±1.7)</td>
<td>13.1 (±1.7)</td>
<td>13.0 (±1.9)</td>
</tr>
<tr>
<td>Platelet Distribution Width % Citrate p value</td>
<td>11.6 (±2.0)</td>
<td>11.5 (±1.6)</td>
<td>11.5 (±1.49)</td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) EDTA p value</td>
<td>10.9 (±0.8)</td>
<td>10.8 (±0.8)</td>
<td>10.7 (±0.9)</td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) Citrate p value</td>
<td>9.9 (±0.8)</td>
<td>10.0 (±0.8)</td>
<td>10.0 (±0.8)</td>
</tr>
<tr>
<td>WCC x 10⁹/L EDTA p value</td>
<td>6.4 (6.0-8.0)</td>
<td>6.4 (5.7-7.8)</td>
<td>6.4 (5.6-7.5)</td>
</tr>
<tr>
<td>Neutrophils EDTA p value</td>
<td>3.8 (3.0-5.1)</td>
<td>3.9 (2.9-4.7)</td>
<td>3.6 (3.1-4.4)</td>
</tr>
<tr>
<td>Monocytes x 10⁹/L EDTA p value</td>
<td>0.6 (0.5-0.7)</td>
<td>0.6 (0.4-0.6)</td>
<td>0.5 (0.5-0.7)</td>
</tr>
<tr>
<td>Lymphocytes x 10⁹/L EDTA p value</td>
<td>2.1 (±0.8)</td>
<td>1.9 (±0.7)</td>
<td>2.0 (±0.8)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl) EDTA p value</td>
<td>14.2 (±1.6)</td>
<td>13.8 (±1.5)</td>
<td>13.8 (±1.5)</td>
</tr>
<tr>
<td>HCT (L/L) EDTA p value</td>
<td>0.42 (±0.04)</td>
<td>0.41 (±0.04)</td>
<td>0.40 (±0.04)</td>
</tr>
</tbody>
</table>

P values refer to comparison between follow-up data at 14d and 90d versus baseline values
Values are Means (±SD) or medians (25-75% Inter-quartile range)
Impact of any antiplatelet change on % RP over time in ischaemic CVD

When we combined data from all subgroups, and compared data from all CVD patients who were undergoing a change in antiplatelet therapy (N = 78), the median % RP did not increase at 14d (15.80 %, p = 0.8), but did significantly increase at 90d (19.55%, p=0.007) compared with baseline values within 4 weeks of TIA or ischaemic stroke onset (13.7%). Based on our subgroup data above, it is likely that this result was predominantly driven by the withdrawal of aspirin in the subgroup that changed from aspirin to clopidogrel.

Overall, 37/75 patients (49%) were non-responsive to their antiplatelet regimen using our longitudinal definition of non-responsiveness on the PFA-100®. There was no difference in percentage of circulating reticulated platelets between antiplatelet non-responders and responders overall at either 14d or 90 d (p≥0.2). There was no correlation between collagen-ADP or collagen-epinephrine closure times on the PFA-100 and the percentage of circulating reticulated platelets at 14 days or 90 days (p ≥ 0.06).

Mean platelet volume decreased at 90d compared with baseline in EDTA only (p = 0.003). PDW was lower at both 14d and 90d than at baseline in EDTA (p ≥ 0.03, Table 13-8), but was only lower at 90d in citrate (p = 0.01). White cell count was lower at 14d and 90d than at baseline (p ≤ 0.04). This was driven mainly by the reduction in lymphocyte counts (p ≤ 0.01). Haemoglobin and haematocrit were lower at 14d and 90d than at baseline (p ≤0.02).
There was a significant positive correlation between the MPV and % RP at 90d in both citrate- and EDTA-anticoagulated blood (Spearman’s rho ≥ 0.31, p ≤ 0.006, Figure 13-1), but not at baseline or 14d (p ≥ 0.2). There also was a significant correlation between the PDW and % RP at 90d in both citrate- and EDTA-anticoagulated blood (Spearman’s rho ≥ 0.24, p ≤ 0.03, Figure 13-2), but not at baseline or 14d (p ≥ 0.1).
Figure 13-1 Scatterplot of mean platelet volume (MPV) in citrate at 90d vs. percentage reticulated platelets at 90d in the overall CVD population. Spearman's rho = 0.33, p = 0.003.
Figure 13-2 Scatterplot of platelet distribution width (PDW) in citrate at 90d vs. percentage reticulated platelets at 90d in the overall CVD population.

Spearman's rho = 0.26, p = 0.02.
Table 13-7: Summary of flow cytometry findings – Percentage circulating reticulated platelets in overall CVD population during follow up.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall % RP</strong></td>
<td>13.50 (8.20-22.40)</td>
<td>15.80 (8.16-21.68)</td>
<td>19.55 (11.15-24.13)</td>
</tr>
<tr>
<td><strong>p value</strong>*</td>
<td>0.8</td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>12.15 (9.07-20.33)</td>
<td>11.4 (8.45-20.28)</td>
<td>18.05 (11.15-23.23)</td>
</tr>
<tr>
<td><strong>Non-responders</strong></td>
<td>17.90 (7.41-25.30)</td>
<td>18.1 (7.54-23.2)</td>
<td>21.20 (15.30-25.20)</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are medians (25-75% inter quartile range)

*P value refers to comparisons between values at 14d and 90d versus baseline

+P value refers to comparison of % RP between responders and non responders at each time point
Table 13-8 FBC parameters in overall CVD population during follow up

<table>
<thead>
<tr>
<th>FBC Parameter</th>
<th>Baseline (n = 78)</th>
<th>14d (n = 78)</th>
<th>90d (n = 78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count x 10^9/L EDTA</td>
<td>233 (±57)</td>
<td>233 (±56)</td>
<td>235 (±54)</td>
</tr>
<tr>
<td>p value</td>
<td>0.9</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Platelet Count x 10^9/L Citrate</td>
<td>172 (±49)</td>
<td>176 (±42)</td>
<td>177 (±42)</td>
</tr>
<tr>
<td>p value</td>
<td>0.4</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution Width % EDTA</td>
<td>13.4 (±1.8)</td>
<td>13.1 (±1.9)</td>
<td>12.8 (±1.87)</td>
</tr>
<tr>
<td>p value</td>
<td>0.03</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>Platelet Distribution Width % Citrate</td>
<td>11.4 (±1.6)</td>
<td>11.3 (±1.8)</td>
<td>11.0 (±1.62)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) EDTA</td>
<td>10.9 (±0.8)</td>
<td>10.8 (±1.0)</td>
<td>10.7 (±0.9)</td>
</tr>
<tr>
<td>p value</td>
<td>0.4</td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>Mean Platelet Volume (fl) Citrate</td>
<td>9.8 (±0.7)</td>
<td>9.8 (±0.9)</td>
<td>9.74 (±0.80)</td>
</tr>
<tr>
<td>p value</td>
<td>0.6</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>WCC (x 10^9/L) EDTA</td>
<td>7.4 (±1.9)</td>
<td>7.04 (±2.0)</td>
<td>7.02 (±1.79)</td>
</tr>
<tr>
<td>p value</td>
<td>0.04</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Neutrophils EDTA</td>
<td>4.2 (3.1-5.5)</td>
<td>3.9 (2.9-4.8)</td>
<td>3.9 (3.1-4.8)</td>
</tr>
<tr>
<td>p value</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Monocytes x 10^9/L EDTA</td>
<td>0.6 (0.5-0.8)</td>
<td>0.6 (0.5-0.7)</td>
<td>0.6 (0.5-0.8)</td>
</tr>
<tr>
<td>p value</td>
<td>0.08</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes x 10^9/L EDTA</td>
<td>2.1 (±0.7)</td>
<td>2.0 (±0.7)</td>
<td>2.0 (±0.7)</td>
</tr>
<tr>
<td>p value</td>
<td>0.01</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl) EDTA</td>
<td>14.3 (±1.4)</td>
<td>13.8 (±1.2)</td>
<td>13.9 (±1.3)</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.0001</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>HCT (L/L) EDTA</td>
<td>0.42 (±0.04)</td>
<td>0.41 (±0.03)</td>
<td>0.41 (±0.04)</td>
</tr>
<tr>
<td>p value</td>
<td>0.0002</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

P values refer to comparison between follow-up data at 14d and 90d versus baseline values
Values are Means (±SD) or medians (25-75% Inter-quartile range)
Figure 13-3: Scatterplot of circulating reticulated platelets at each time point in entire CVP population who underwent a change in antiplatelet therapy.

P values relate to comparison between values at 14d and 90d versus Baseline (see Table 13-7).
13.5. Discussion

To our knowledge, this is the first longitudinal study to investigate the impact of commencing commonly prescribed antiplatelet regimens on circulating reticulated platelets in a well-characterised cohort of patients following TIA or ischaemic stroke.

The addition of aspirin monotherapy was not associated with a significant reduction in the percentage of circulating reticulated platelets in this study. However, withdrawal of aspirin in patients who changed to clopidogrel monotherapy was ultimately associated with an increase in the percentage of reticulated platelets at 90d. The latter data either suggest that treatment with aspirin monotherapy may help stabilise reticulated platelet formation or their release into the circulating blood, or that treatment with clopidogrel may enhance reticulated platelet formation and/or release compared with treatment with aspirin. Prior pilot studies provide some support to the hypothesis that aspirin therapy influences the % RP in patients without ischaemic cerebrovascular disease. Patients with essential thrombocytosis (with and without thrombosis) had lower percentages of circulating reticulated platelets on aspirin therapy compared with patients not treated with aspirin (Rinder et al, 1998). In addition, patients with renal failure treated with aspirin had lower % RP than similar patients who were not on aspirin in one study (Zanazzi et al, 2010). However, the potential impact of aspirin on reticulated platelet levels has not been proven in all patients at risk of vascular events: circulating reticulated platelet levels did not differ between antiphospholipid syndrome patients who were and those who were not treated with aspirin (Joseph et al, 1998). Therefore, further work is required to explore the impact of aspirin on the percentage of circulating reticulated platelets in ischaemic CVD to confirm the findings of this study and to determine whether patients treated with aspirin who have a high percentage of circulating reticulated platelet are at an increased risk of recurrent vascular events.
Aspirin non-responders had a higher percentage of circulating reticulated platelets than aspirin responders at 14d, but the differences between non-responders and responders was not significant at 90d. These data suggest that the % RP may have an impact on ex vivo aspirin non-responsiveness early after TIA or stroke onset; the very small number of patients with 90d follow-up on aspirin monotherapy may have contributed to a type I error at that stage. Control subjects with laboratory aspirin non-responsiveness have been shown to have higher percentages of circulating reticulated platelets than controls who are responsive to aspirin (Guthikonda et al, 2007). Furthermore, control subjects on aspirin therapy with high levels of circulating reticulated platelets have also been shown to have higher platelet COX-2 expression and serum thromboxane β2 than controls with low circulating reticulated platelets (Guthikonda et al, 2007), indicating that COX-2-mediated platelet production of thromboxane A2 may be one of the mechanisms contributing to aspirin non-responsiveness in these subjects. If patients with elevated circulating reticulated platelets were to be shown to be at a higher risk of recurrent vascular events than patients with lower circulating reticulated platelets, it might be warranted to treat these patients with higher doses of aspirin, or alternate antiplatelet agents which are not dependant on cyclooxygenase inhibition.

The effect of adding dipyridamole to aspirin, or changing from aspirin to clopidogrel on circulating reticulated platelets has never been studied previously. Given that aspirin may exert some effects at the level of the megakaryocyte (van Pampus et al, 1993), whereas dipyridamole and clopidogrel may not, perhaps it should have been anticipated that these antiplatelet changes would not reduce the % RP in our patient population. The % RP did not influence the likelihood of being non-responsive to dipyridamole or
clopidogrel on the PFA-100®, although we have shown in Chapter 10 that the PFA-100® is poorly sensitive at detecting inhibition of platelet function \textit{ex vivo} with clopidogrel therapy. Further studies investigating the relationship between circulating reticulated platelets and clopidogrel non-responsiveness on more sensitive tests of platelet function, such as the VerifyNow P2Y12 analyser®, the Multiplate® ADP cuvette, or the recently-licensed PFA P2Y Innovance cartridge® are warranted. These assays were not available to us at the time of this study.

In the overall CVD patient population, mean platelet volume decreased at 90d in EDTA only, and PDW was lower at both 14d and 90d in EDTA and at 90d in citrate compared with baseline. Based on our subgroup analyses, these results appear to be predominantly due to the reduction in MPV and PDW in response to adding dipyridamole to aspirin, because these values remained stable after changing from no medication to aspirin, and from aspirin to clopidogrel. Although it is also possible that the reduction in MPV and PDW could have arisen secondary to resolution of the acute phase response, if that were the case, we would have expected to see a similar pattern in the other antiplatelet treatment groups. This will be explored further when we analyse the remaining samples from our patient subgroups in time.

There was a significant positive correlation between both the MPV and PDW and % RP at 90d as shown in our case control study, and other prior studies (McCabe \textit{et al}, 2004b), indicating that circulating reticulated platelets are larger than non-reticulated platelets in ischaemic CVD.
The neutrophil count has been shown to be transiently elevated in patients following ischaemic stroke (Marquardt et al, 2009). We did not confirm these findings in this study, but we included patients at any stage up to 4 weeks after TIA or stroke onset. The lymphocyte count was persistently elevated during follow up compared with controls, indicating ongoing chronic inflammation following symptom onset.

13.6. Conclusion
Treatment with aspirin therapy may potentially stabilise the reticulated platelet fraction in whole blood, and the percentage of reticulated platelets may also influence ex vivo non-responsiveness to aspirin on the PFA-100®. Further studies using other laboratory tests that are more sensitive and specific to platelet inhibition with clopidogrel are warranted to investigate whether circulating reticulated platelets impact on ex vivo responsiveness to antiplatelet therapy, including clopidogrel, in ischaemic CVD.
14. Assessment of von Willebrand factor and von Willebrand factor propeptide in patients following TIA and ischaemic stroke

14.1. Introduction

Activated platelets play a key role in arterial thrombus formation, and because platelets interact with both the endothelium and coagulation system, endothelial or coagulation system activation has the potential to cause or exacerbate an ischaemic insult in patients with cerebrovascular disease (CVD). Coagulation system activation will be discussed and investigated in Chapter 15 [Coagulation system potential in TIA and ischaemic stroke]. In this chapter, I will focus on a pilot study investigating some aspects of endothelial activation in patients with TIA or ischaemic stroke.

Von Willebrand factor (VWF:Ag) is a multimeric plasma glycoprotein that is synthesised in vascular endothelial cells and megakaryocytes (Nishio et al, 2004; Bongers et al, 2006). Endothelial cells secrete VWF:Ag constitutively into the circulating blood or into the subendothelial matrix, and also release VWF:Ag stored in Weibel-Palade bodies in response to endothelial cell activation (Ruggeri, 1997; Nishio et al, 2004; Bongers et al, 2006). VWF:Ag may bind to the platelet surface glycoprotein (Gp) Ib-IX-V receptor complex, thus mediating platelet adhesion to exposed subendothelial collagen and subsequent platelet-rich thrombus formation (Nishio et al, 2004; Bongers et al, 2006). VWF:Ag is an important glycoprotein in patients with vascular disease, and elevated VWF:Ag levels have been identified in both the early (Catto et al, 1997; Bath et al, 1998; Kozuka et al, 2002; Lynch et al, 2004; McCabe et al, 2004a; Nadar et al, 2005; Bongers et al, 2006; Kotzailias et al, 2007) and late phases
following an ischaemic cerebrovascular event (CVE) (Kozuka et al, 2002; Kain et al, 2002; McCabe et al, 2004a), and following an ischaemic or haemorrhagic stroke compared with healthy controls (Catto et al, 1997).

Von Willebrand Factor propeptide (VWF:Ag II) is produced by cleavage of pro-VWF into VWF:Ag and VWF:Ag II, and is also released from the endothelium (van Mourik et al, 1999; Frijns et al, 2006). Recent reports have indicated that the ratio of von Willebrand factor antigen (VWF:Ag) to von Willebrand factor propeptide (VWF:Ag II) in plasma provides an accurate measure of the degree of acute endothelial cell activation (van Mourik et al, 1999; Hollestelle et al, 2006). However, to date, VWF:Ag II levels have not been studied in both the early and late phases after an ischaemic cerebrovascular event, or in response to intensifying antiplatelet therapy after TIA or ischaemic stroke. Because VWF:Ag levels have been shown to have significant impact on the results of platelet function testing with the PFA-100®, a better understanding of the role of VWF:Ag and VWF:Ag II may improve our understanding of the mechanisms influencing ex vivo non-responsiveness on this device and potentially in the clinical setting. Although it is estimated that up to 25% of plasma VWF:Ag may be derived from platelets (McGrath et al, 2010), the majority of circulating VWF:Ag is derived from endothelium and we did not differentiate between endothelial- or platelet-derived VWF:Ag in this thesis (see Methods section on preparation of platelet poor plasma below).
14.2. Aims and Hypotheses

The aims of this pilot study were:

- To determine whether plasma VWF:Ag or VWF:Ag II levels, or VWF:Ag/VWF:Ag II ratio were elevated as a marker of endothelial activation in patients with recent TIA or ischaemic stroke compared with controls;

- To assess the impact of adding dipyridamole to aspirin on plasma levels of VWF:Ag or VWF:Ag II and VWF:Ag/VWF:Ag II ratio in ischaemic CVD patients who were followed up from the early to late phase after symptom onset;

- To determine whether VWF:Ag, VWF:Ag II or VWF:Ag/VWF:Ag II ratio influenced the prevalence of ex-vivo non-responsiveness to dipyridamole on the PFA-100® after the addition of dipyridamole to aspirin monotherapy.

We hypothesised that:

- TIA or ischaemic stroke patients would have higher VWF:Ag or VWF:Ag II levels, or VWF:Ag/VWF:Ag II ratio compared with control subjects;

- VWF:Ag or VWF:Ag II levels, or VWF:Ag/VWF:Ag II ratio would decrease after the addition of dipyridamole to aspirin in the early phase after TIA or ischaemic stroke, and any effects would be maintained during longer-term follow up

- Patients who were ‘non-responsive’ to dipyridamole in the laboratory, as assessed by our novel definition of antiplatelet responsiveness on the PFA-100® (Chapters 10 and 11), would have higher levels of endothelial activation than ‘antiplatelet responders’ i.e. higher VWF:Ag or VWF:Ag II levels or VWF:Ag/VWF:Ag II ratio
14.3. Methods

Recruitment of all patients was performed as outlined in Chapter 13 [Impact of antiplatelet agents on circulating reticulated platelets in the early and late phases after TIA or ischaemic stroke]. In brief, patients were recruited from the Rapid Access Stroke Prevention Clinic population, and from the inpatient population of the Neurology, Age-Related Health Care and Stroke Services at AMNCH.

14.3.1. Study Design and Inclusion Criteria:

We performed 2 parallel pilot studies for this aspect of my thesis:

(a) Case-control study: Consecutive eligible patients older than 18 years of age, whose treating physician decided to change their antiplatelet therapy, were recruited within four weeks of onset of a TIA or ischaemic stroke, regardless of their antiplatelet treatment regimen.

Control subjects of similar age and gender to the TIA / stroke patient population, who had no prior history of cerebrovascular disease were recruited and assessed once. Control subjects were recruited from amongst the staff and local population at AMNCH. Spouses of patients and control subjects were also recruited.

(b) Nested longitudinal study in CVD patients who were changing from aspirin monotherapy to aspirin and dipyridamole combination therapy: A subgroup of patients who had experienced a TIA or ischaemic stroke within the preceding 4 weeks, and who were taking aspirin for at least 7 days at the time of study recruitment were assessed in a longitudinal manner at three time points: at baseline on aspirin therapy (baseline); 14 days following the addition of dipyridamole MR to their aspirin regimen (14d), and again after at least 90 days after symptom onset on treatment with aspirin and...
dipyridamole combination therapy (90d). For this nested longitudinal study, the
patient’s attending physician made the decision to change antiplatelet therapy following
TIA or ischaemic stroke, and the study investigators did not influence this treatment
decision unless they were the patient’s primary attending physicians.

**Exclusion Criteria:**

We excluded CVD patients with a history of primary intracerebral haemorrhage,
myocardial infarction within the preceding 3 months, ongoing unstable angina, unstable
symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within
the preceding 3 months (haemoglobin decrease of >1g/dl in one day, or requiring
transfusion), if they had systemic vasculitis, underlying neoplasia, or a known bleeding
or clotting diathesis including known platelet disorders, platelet count < 120 x 10^9/L or
> 450 x 10^9/L, urea >10mmol/l or GFR < 30ml/min, current infection (clinical signs of
infection, white cell count > 12 x 10^9/L), or non-steroidal anti-inflammatory drug
(NSAID) use within 14 days of recruitment.

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 study Doppler ultrasound screening, or if they were on antiplatelet therapy
or NSAIDs.

Written informed consent, or assent where appropriate, was obtained from all subjects,
and this study was approved by the St. James Hospital/Adelaide and Meath Hospital
Research Ethics Committee (REC Ref: 2007/07/MA).
14.3.2. Clinical assessment

Clinical assessment of the CVD patients who were recruited within 4 weeks of symptom onset to our case control study was performed as outlined in Chapter 11 [Assessment of the impact of dipyridamole on platelet activation and function in the early and late phases after TIA or ischaemic stroke]. Clinical assessment of control subjects was performed as outlined in Chapter 12. In brief, all subjects underwent a detailed clinical assessment by at least one of three examiners, and CVD patients had TIA and stroke work-up according to ESO guidelines (European Stroke Organisation (ESO) Executive Committee & ESO Writing Committee, 2008). The underlying mechanism responsible for the ischaemic stroke or TIA in CVD patients was categorised according to TOAST classification (Adams, Jr. et al, 1993).

In our nested longitudinal study, the 90d follow-up in the large artery atherosclerotic subgroup was performed at least 3 months following carotid surgery or endovascular treatment, unless this treatment had been delayed for at least 3 months after the initial event for medical reasons.

As the majority of patients at baseline and 14d were assessed during their inpatient stay, adherence to antithrombotic therapy was confirmed by checking the drug chart. All outpatients were phoned before their appointment to stress the importance of medication adherence in the week prior to assessment. Adherence in this group was assessed by history taking alone. Patients who were not adherent to their antithrombotic regimen were invited back for reassessment after 14 days.
14.3.3. Blood sampling and laboratory tests:

Venepuncture was performed using a standardised method, as described previously (Chapter 9, General Methods). Full blood counts were performed between 2 and 4 hours in EDTA and citrate anticoagulated tubes. Platelet function was assessed with the PFA-100® to measure C-ADP closure times in citrate-anticoagulated whole blood between 2 and 2.5 hours after venepuncture, as described in Chapters 10 and 11. The maximum closure time recorded by the device is 300s, and we arbitrarily defined closure times above 300s as 301s for statistical analysis, using non-parametric tests. For our longitudinal study in patients who had dipyridamole MR added to aspirin monotherapy, we defined patients as being ‘dipyridamole non-responsive’ ex vivo on the PFA-100® if they failed to prolong their C-ADP closure time with dipyridamole compared with their baseline value on aspirin by more than twice the coefficient of variation of the C-ADP assay. As outlined in Chapter 11 [Assessment of the impact of dipyridamole on platelet activation and function in the early and late phases after TIA or ischaemic stroke], dipyridamole non-responders on the PFA-100® failed to prolong their C-ADP closure time by more than 14% of their baseline value on aspirin monotherapy.

Platelet Poor Plasma (PPP)

PPP was obtained from fresh citrate-anticoagulated whole blood by centrifugation as described earlier and stored at -70°C (Chapter 9, General Methods). Samples were thawed once at 37°C for 20 minutes before analysis in a VWF:Ag ELISA assay. Samples were then refrozen and stored at -70°C, until they were thawed once more for the VWF:Ag II ELISA assays. Because VWF:Ag II may be endothelium- or platelet-derived, as outlined above, measurement of soluble E-selectin levels, as an additional specific measure of endothelial activation, is planned in our laboratory, but had not been performed at the time these data were analysed.
**Enzyme Linked Immunosorbant Assay (ELISA)**

The concentration of VWF:Ag and VWF:Ag II in each PPP sample was quantified, as previously described (O'Donnell *et al.*, 2005; Preston *et al.*, 2009). Polyclonal Rabbit Anti-Human VWF antibody (DAKO) was used as coating antibody, and polyclonal rabbit Anti-Human VWF/HRP antibody (DAKO) as detection antibody for the VWF:Ag ELISA. M193902 CLB-Pro 35 coating antibody (Plesmanlaan 125) and M103904HRP CLB-Pro 14.3 detection antibody (Plesmanlaan 125) were used for VWF:Ag II quantification. The ELISA assay result was measured by spectrophotometry at 490nm, using a VERSA Max Tuneable Microplate Reader. VWF:Ag and VWF:Ag II levels were recorded as µg/mL.

**Statistical Methods**

Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively. The Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. Chi squared or Fisher Exact tests were used to compare proportions between groups, where appropriate. Multiple linear regression analysis was performed to examine the independent influence of certain demographic variables on the results of the comparison of coagulation system potential between patients and controls, in turn. Logarithmic transformation of the data was undertaken before regression analyses, where appropriate, so that assumptions of normality were reasonably well met. P < 0.05 was considered to be statistically significant. All statistical calculations were performed using R, version 2.10.1 (R Development Core Team, 2009).
14.4. Results

(a) Case control study:
Data from 51 ischaemic CVD patients (≤ 4 weeks of onset of a TIA or ischaemic stroke) and 18 ‘cerebrovascular disease-free’ controls were available for analysis at the time of writing up our interim case-control results. The clinical details of the study subjects, and their TIA or stroke subtypes, in addition to the clinical and demographic features of the 18 control subject are outlined in Table 14-1 and Table 14-2.

The mean (± standard deviation) interval from TIA or stroke onset to inclusion in this study was 9 ± 5 days. 7 patients (14%) were on no antiplatelet medication, 43 patients (86%) were on aspirin at a median daily dose of 300mg, and one patient (2%) was on combination therapy with aspirin and clopidogrel (75 mg of each daily).

The median VWF:Ag level was higher in patients with recent TIA or ischaemic stroke than in controls, indicative of increased endothelial activation [median 14.48 μg/mL vs. 10.37 μg/mL, p = 0.02] (Table 14-3, Figure 14-1). Certain vascular risk factors were more common in patients than in controls (Table 14-1), and could have partly accounted for the significant difference in VWF:Ag levels between patients and controls. Therefore multiple linear regression analysis was used to examine the independent influence of hypertension and smoking status on the results obtained. Having controlled for each of these variables individually, there remained a significant difference in VWF:Ag between patients and controls (p = 0.01).
Table 14-1: Demographic Data of Study Subjects at Enrolment to Case-Control Study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n = 51)</th>
<th>Controls (n = 18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>61 [±10]</td>
<td>60 [±11]</td>
<td>0.8</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>29 / 22</td>
<td>12 / 6</td>
<td>0.5</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>8 (16%)</td>
<td>0 (0%)</td>
<td>0.07</td>
</tr>
<tr>
<td>IHD</td>
<td>6 (12%)</td>
<td>0 (0%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>32 (63%)</td>
<td>3 (17%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>7 (14%)</td>
<td>0 (0%)</td>
<td>0.1</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Family History Stroke</td>
<td>25 (49%)</td>
<td>6 (12%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>3 (6%)</td>
<td>0 (0%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>3 (6%)</td>
<td>0 (0%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>1 (2%)</td>
<td>1 (6%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Migraine without aura</td>
<td>4 (8%)</td>
<td>1 (6%)</td>
<td>0.7</td>
</tr>
<tr>
<td>Current smokers (at enrolment)</td>
<td>16 (31%)</td>
<td>0 (0%)</td>
<td>0.007</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>14 (27%)</td>
<td>7 (39%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Never smoker</td>
<td>21 (41%)</td>
<td>11 (22%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspirin therapy</td>
<td>44 (86%)</td>
<td>0 (0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>29 (57%)</td>
<td>0 (0%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

P values relate to Chi Squared or Fisher exact testing between CVD patients and controls, where appropriate.
Values are Means [±SD] or absolute counts with percentages in parentheses.

Table 14-2 Aetiological subtyping by TOAST classification of early phase CVD patients

<table>
<thead>
<tr>
<th>TIA / Stroke Subtype (n=51)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Artery Atherosclerotic</td>
<td>21 (41%)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>11 (22%)</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Undetermined Aetiology</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>Other Determined</td>
<td>3 (6%)</td>
</tr>
</tbody>
</table>
Figure 14-1 Comparison of VWF:Ag levels in patients in the early phase following acute TIA or ischaemic stroke with controls. Red lines and values above indicate median values. $p$ value for difference $= 0.02$. 
There was no significant difference in VWF:Ag II levels between early phase CVD patients and controls (p = 0.4). This was still the case following correction for smoking status and hypertension (p = 0.6).

There was a trend toward elevated VWF:Ag/VWF:Ag II ratio in patients compared with controls, but this did not reach statistical significance [median 1.65 vs. 1.44, p = 0.06] (Table 14-3), and was not present following correction for smoking status and hypertension (p = 0.1).

We performed preliminary analysis to assess endothelial activation in different CVD TOAST subgroups compared with controls. Patients with TIA or stroke of large vessel aetiology (n=21) had higher VWF:Ag (p = 0.02) and VWF:Ag/VWF:Ag II ratio (p = 0.02) than controls, but similar VWF:Ag II levels (p = 0.8), suggesting enhanced endothelial activation in this subgroup (Table 14-4). There were no significant differences in endothelial activation markers between any of the other CVD subgroups and controls, although these analyses must be interpreted with extreme cautions because the numbers in each subgroup were very small (p ≥ 0.1).

Table 14-3: VWF:Ag, VWF:Ag II Levels And VWF:Ag/VWF:Ag II Ratio In Patients With Recent TIA or Ischaemic Stroke Compared With Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:Ag</td>
<td>14.48 (10.11-18.07)</td>
<td>10.37 (7.45-13.52)</td>
<td>0.02</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>8.63 (7.11-10.52)</td>
<td>7.68 (6.90-9.14)</td>
<td>0.4</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag II ratio</td>
<td>1.65 (1.37-2.08)</td>
<td>1.44 (0.86-1.92)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

P value refers to comparison between CVD patients and controls
Values are medians (25-75th percentile)
Table 14-4: Endothelial Activation in Aetiological Subgroups of Patients With Recent Ischaemic Stroke or TIA Compared With Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 21)</th>
<th>Controls (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:Ag</td>
<td>16.12 (11.39-21.66)</td>
<td>10.37 (7.45-13.52)</td>
<td>0.02</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>8.43 (6.21-11.27)</td>
<td>7.68 (6.90-9.14)</td>
<td>0.8</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag ratio II</td>
<td>1.85 (1.50-2.96)</td>
<td>1.44 (0.86-1.92)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA or stroke of large vessel aetiology vs. controls
Values are medians (25-75th percentile)

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 3)</th>
<th>Controls (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:Ag</td>
<td>17.33 (13.65-18.41)</td>
<td>10.37 (7.45-13.52)</td>
<td>0.3</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>10.06 (9.55-16.20)</td>
<td>7.68 (6.90-9.14)</td>
<td>0.1</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag ratio II</td>
<td>1.10 (0.94-1.5)</td>
<td>1.44 (0.86-1.92)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA or stroke of cardioembolic aetiology vs. controls
Values are medians (25-75th percentile)

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 11)</th>
<th>Controls (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:Ag</td>
<td>14.05 (10.56-18.04)</td>
<td>10.37 (7.45-13.52)</td>
<td>0.1</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>9.87 (8.11-12.99)</td>
<td>7.68 (6.90-9.14)</td>
<td>0.1</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag ratio II</td>
<td>1.43 (1.26-1.57)</td>
<td>1.44 (0.86-1.92)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA or stroke of small vessel aetiology vs. controls
Values are medians (25-75th percentile)
<table>
<thead>
<tr>
<th>VWF:Ag</th>
<th>Patients (n = 3)</th>
<th>Controls (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.35 (11.57-15.78)</td>
<td>10.37 (7.45-13.52)</td>
<td>0.5</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>8.05 (6.32-8.34)</td>
<td>7.68 (6.90-9.14)</td>
<td>0.8</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag II ratio</td>
<td>2.13 (1.84-2.19)</td>
<td>1.44 (0.86-1.92)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA or stroke of other determined aetiology vs. controls
Values are medians (25-75\textsuperscript{th} percentile) or means (±SD)

<table>
<thead>
<tr>
<th>VWF:Ag</th>
<th>Patients (n = 13)</th>
<th>Controls (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.68 (9.86-15.20)</td>
<td>10.37 (7.45-13.52)</td>
<td>0.3</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>8.05 (7.09-9.07)</td>
<td>7.68 (6.90-9.14)</td>
<td>0.9</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag II ratio</td>
<td>1.70 (1.58-1.81)</td>
<td>1.44 (0.86-1.92)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA or stroke of undetermined aetiology vs. controls
Values are medians (25-75\textsuperscript{th} percentile) or means (±SD)
(b) Longitudinal study in CVD patients who were changing from aspirin monotherapy to aspirin and dipyridamole combination therapy

Interim data from 23 CVD patients who underwent assessment at baseline on aspirin, and subsequently at 14d and 90d after adding dipyridamole MR 200mg BD to their aspirin treatment, were available at the time of completion of this thesis. The clinical details of the CVD patients in this ongoing longitudinal study, and their stroke subtypes are outlined in Table 14-5 and Table 14-6.

The impact of adding dipyridamole to aspirin monotherapy on endothelial activation markers are shown in Table 14-7. VWF:Ag levels decreased 14d after the addition of dipyridamole to aspirin therapy, and this reduction was maintained at 90d (p < 0.02). Of note, there was no significant change in the proportion of patients smoking at either 14d or 90d compared with baseline, or at 90d vs. 14d (p > = 0.5).

VWF:Ag II increased 14d after the addition of dipyridamole to aspirin monotherapy compared with baseline (p = 0.03), but this difference was not maintained at 90d (p = 0.6). Consequently, the VWF:Ag/VWF:Ag II ratio significantly decreased 2 weeks after the addition of dipyridamole to aspirin monotherapy (p = 0.01), but this effect was not maintained at 90d either (p = 0.1).
Impact of endothelial activation on ex vivo dipyridamole responsiveness in the early and late phases after TIA or ischaemic stroke

We compared VWF:Ag and VWF:Ag II levels, and the VWF:Ag/VWF:Ag II ratio in ‘dipyridamole non-responders’ with ‘dipyridamole responders’ at 14d (n = 14 vs. 9) and 90d (n = 11 vs. 12). There were no significant differences in any of these markers between the dipyridamole non-responders and responders on the PFA-100® at any stage during follow-up (p ≥ 0.07).

Table 14-5: Demographic Data of Patients at Enrolment to the Longitudinal Study Assessing the Impact of Adding Dipyridamole to Aspirin Therapy on Endothelial Activation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years</td>
<td>61 [±10]</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>16 / 7</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>8 (25%)</td>
</tr>
<tr>
<td>IHD</td>
<td>2(9%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>15 (65%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>3 (13%)</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Family History Stroke</td>
<td>11 (48%)</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Migraine without aura</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Current smokers (at enrolment)</td>
<td>5 (22%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>8 (35%)</td>
</tr>
<tr>
<td>Never smoker</td>
<td>10 (43%)</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>9 (39%)</td>
</tr>
</tbody>
</table>
Table 14-6: Aetiological Subtyping By TOAST Classification of CVD Patients in the Longitudinal Study Assessing the Impact of Adding Dipyridamole to Aspirin Therapy on Endothelial Activation

<table>
<thead>
<tr>
<th>Stroke/TIA subtype (n = 23)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherothrombotic</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>7 (30%)</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>Undetermined aetiology</td>
<td>11 (48%)</td>
</tr>
<tr>
<td>Other determined</td>
<td>2 (9%)</td>
</tr>
</tbody>
</table>

Table 14-7: VWF:Ag, VWF:Ag II Levels And VWF:Ag/VWF:Ag II Ratio In Ischaemic CVD Patients After Changing From Aspirin Monotherapy (Baseline, Within 4 Weeks Of Symptom Onset) To Aspirin And Dipyridamole MR Combination Therapy 14 Days (14d) And At Least 90 Days (90d) Later

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:Ag</td>
<td>14.05 (10.37-15.60)</td>
<td>11.55 (8.74-13.77)</td>
<td>11.75 (8.84-14.63)</td>
</tr>
<tr>
<td>VWF:Ag II</td>
<td>8.45 (±2.46)</td>
<td>8.83 (±3.01)</td>
<td>8.29 (±2.80)</td>
</tr>
<tr>
<td>VWF:Ag/VWF:Ag II ratio</td>
<td>1.64 (±0.40)</td>
<td>1.43 (±0.40)</td>
<td>1.47 (±0.46)</td>
</tr>
</tbody>
</table>

P value refers to comparison between baseline and follow-up data at 14d and 90d
Values are medians (25-75th percentile) or means (±SD)
Figure 14-2 Comparison of VWF:Ag levels in patients before (at baseline), 14 days after (Day 14), and 90 days after (Day 90) addition of dipyridamole MR to aspirin monotherapy. Red lines and values above indicate median values. P value indicates pairwise comparison with baseline values.
14.5. Discussion
This pilot study has yielded a number of important preliminary findings. The case-control data have again demonstrated that VWF:Ag levels are increased in patients within 4 weeks of onset of a TIA or an ischaemic stroke compared with controls, and in particular, in patients with TIA or stroke of large-artery aetiology (i.e. exposed to high shear stress in vivo), indicating excessive endothelial +/- platelet activation in our CVD patient cohort. These data are in keeping with similarly-sized (Nadar et al, 2005) and larger published case-control studies in ischaemic CVD (Bath et al, 1998; McCabe et al, 2004a; Bongers et al, 2006). This study does not allow us to comment on the relative contribution of endothelial- and platelet-derived VWF:Ag to the observed circulating VWF levels in our CVD population. However, further work is planned in collaboration with Dr James O’Donnell’s Haemostasis Research Group at St James’s Hospital to assess the potential importance of platelet-derived VWF in ischaemic CVD.

Analysis of the available data has also shown that VWF:Ag II levels transiently increased 14d after the addition of dipyridamole to aspirin monotherapy, but did not remain elevated at 90d; the reduction in the VWF:Ag/VWF:Ag II ratio at 14d mirrored this finding. This increase in VWF:Ag II was somewhat unexpected in view of the fact that VWF:Ag levels decreased during follow up, and we anticipated that VWF:Ag and VWF:Ag II which are produced in equimolar amounts (van Mourik et al, 1999) would both change in concert with one another. Although these results could potentially represent reduced clearance of VWF:Ag II relative to VWF:Ag, because VWF:Ag II is believed to have a shorter half life than VWF:Ag (van Mourik et al, 1999), the reliability of this effect is undetermined at present and may simply represent a type II error due to the small number of patients included in our longitudinal study at the time of writing. Analysis of the outstanding data from all recruited CVD patients who
changed from aspirin to dipyridamole is planned (N = 52) and will initially address this question.

This is the first, pilot longitudinal study to demonstrate that plasma VWF:Ag levels may be reduced significantly by the addition of dipyridamole to aspirin in the early phase after TIA or ischaemic stroke, and that this reduction persists for at least 3 months after commencing dipyridamole. This finding may reflect one of the putative indirect inhibitory effects of dipyridamole on endothelial activation (Eisert, 2001), but we cannot exclude the possibility that VWF:Ag levels would have fallen anyhow in association with resolution of the acute phase response. Longitudinal studies in CVD patients who have not undergone a change in antiplatelet therapy during follow up would be required to address this question; and analysis of plasma samples from patients who remained on aspirin monotherapy throughout the follow up period in the this study is planned (Chapter 10, Assessment of the impact of aspirin and clopidogrel on platelet activation and function in the early and late phases after TIA or ischaemic stroke).

We did not identify any differences in endothelial activation between dipyridamole non-responsive and responsive patients on the PFA-100®. One cannot conclude from these preliminary data that the degree of endothelial activation does not play a major role in mediating ex vivo dipyridamole non-responsiveness on the PFA-100®, nor that dipyridamole non-responders on the PFA-100® fail to exhibit a reduction in endothelial activation in response to administration of the drug. As stated above, because of the very small number of subjects in these subgroups, these data must be interpreted with extreme caution as this substudy is grossly underpowered to allow one to draw any definitive conclusions. Analysis of the remaining stored samples in our entire cohort of
patients who changed from aspirin monotherapy to aspirin and dipyridamole is ongoing to address this question, but further larger studies will be needed to investigate this in more detail.

14.6. Conclusion

Elevated ‘unadjusted’ and ‘adjusted’ plasma VWF:Ag levels, indicating excessive endothelial +/- platelet activation, are seen in TIA or ischaemic stroke patients within 4 weeks of symptom onset compared with healthy controls. Dipyridamole may reduce endothelial activation in both the early and late phases after TIA or ischaemic stroke, in addition to its proposed antiplatelet effects. Additional studies are planned to further investigate these potentially important preliminary findings.
15. Coagulation system potential in TIA and ischaemic stroke

15.1. Introduction
As discussed in Chapter 1 [Overview of Transient Ischaemic Attack (TIA) and ischaemic stroke], most ischaemic strokes and TIA's are secondary to atherothromboembolism to or thrombotic occlusion of an intracranial artery. Activated platelets play a key role in arterial thrombus formation, and because platelets interact with the endothelium and coagulation system, coagulation system activation has the potential to cause or exacerbate an ischaemic insult in patients with cerebrovascular disease (CVD).

The inter-relationship between platelet activation and coagulation system activation in acute thrombus formation, propagation and stabilisation has been discussed in Chapter 1.3 [Thrombus Formation in TIA and Ischaemic Stroke]. In brief, events downstream of initial platelet activation promote assembly of tenase and prothrombin complexes on the activated platelet surface, thus accelerating thrombin generation (van der Meijden et al, 2005). However activation of the platelet P2Y12 ADP receptor increases the time taken to achieve peak thrombin formation (van der Meijden et al, 2005). Furthermore, thrombin, a late product of coagulation system activation, is a potent platelet agonist. During thrombus formation, thrombin activates platelets by binding to the GpIb-IX-V complex (Berndt et al, 2001). Increased thrombin production has been demonstrated in platelet rich plasma from patients who were studied at least 3 months following TIA or ischaemic stroke (van der Meijden et al, 2005).
A subpopulation of activated platelets can also separate from the propagating thrombus, and bind to circulating platelet microparticles via interactions between CD62P and P-selectin glycoprotein 1 (PSGL-1). This increases the concentration of circulating microparticles at the site of thrombus formation, and these microparticles express monocyte-derived tissue factor on their surface. Activated endothelial cells and platelets at the site of vascular injury release protein disulfide isomerase, which catalyzes the ‘decryption’ of platelet microparticle-bound tissue factor, allowing it to further activate the coagulation system via the tissue factor pathway (Furie & Furie, 2008).

Prior to the commencement of this work, no studies had comprehensively assessed coagulation system potential in patients with recent TIA or ischaemic stroke, or serially assessed the potential impact of dipyridamole on coagulation system potential in ischaemic CVD. Furthermore, the influence of coagulation system potential on ex vivo antiplatelet responsiveness had not been investigated in ischaemic CVD.

This component of my thesis was performed in conjunction with the novel studies investigating von Willebrand factor antigen and von Willebrand factor propeptide levels in ischaemic CVD, as described in Chapter 14 [Assessment of von Willebrand factor and von Willebrand factor propeptide in patients following TIA and ischaemic stroke]. Although the study design was identical in each of these studies, it will be outlined again for ease of interpretation of this chapter.
15.2. Aims and Hypotheses

The **aims** of this pilot study were to:

- Determine whether patients in the early phase after TIA or ischaemic stroke had enhanced coagulation system potential compared with cerebrovascular disease-free controls;
- To assess the impact of adding dipyridamole to aspirin on coagulation system potential in ischaemic CVD patients who were followed up from the early to the late phase after symptom onset;
- To determine whether coagulation system potential results influenced the closure times on the high shear stress PFA-100® platelet function analyser, and hence the prevalence of *ex-vivo* dipyridamole non-responsiveness when dipyridamole was added to aspirin monotherapy (see Chapter 11, Assessment of the impact of dipyridamole on platelet activation and function in the early and late phases after TIA or ischaemic stroke).

The **hypotheses** were that:

- TIA or ischaemic stroke patients would have greater coagulation system potential than control subjects;
- Coagulation system potential might decrease after the addition of dipyridamole to aspirin in the early phase after TIA or ischaemic stroke, and any effects would be maintained during longer-term follow up
- Closure times on the high shear stress PFA-100®, and hence the dichotomisation of patients into ‘dipyridamole non-responders’ and ‘responders’, would not be influenced by coagulation system potential results.
Methods

15.2.1. Recruitment

Patients were recruited from the Rapid Access Stroke Prevention Clinic population, and from the inpatient population of the Neurology, Age-Related Health Care and Stroke Services at AMNCH.

Study Design and Inclusion Criteria:

We performed 2 parallel pilot studies for this aspect of the thesis:

(a) Case-control study: Consecutive eligible patients older than 18 years of age, whose treating physician decided to change their antiplatelet therapy, were recruited within four weeks of onset of a TIA or ischaemic stroke, regardless of their antiplatelet treatment regimen.

Control subjects of similar age and gender to the TIA / stroke patient population, who had no prior history of cerebrovascular disease were recruited and assessed once. Control subjects were recruited from amongst the staff and local population at AMNCH. Spouses of patients and control subjects were also recruited.

(b) Nested longitudinal study in CVD patients who were changing from aspirin monotherapy to aspirin and dipyridamole combination therapy: A subgroup of patients who had experienced a TIA or ischaemic stroke within the preceding 4 weeks, and who were taking aspirin for at least 7 days at the time of study recruitment were assessed in a longitudinal manner at three time points: at baseline on aspirin therapy (baseline); 14 days following the addition of dipyridamole MR to their aspirin regimen (14d), and again after at least 90 days after symptom onset on treatment with aspirin and dipyridamole combination therapy (90d). For this nested longitudinal study, the
patient’s attending physician made the decision to change antiplatelet therapy following TIA or ischaemic stroke, and the study investigators did not influence this treatment decision unless they were the patient’s primary attending physicians.

**Exclusion Criteria:**

We excluded CVD patients with a history of primary intracerebral haemorrhage, myocardial infarction within the preceding 3 months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding 3 months (haemoglobin decrease of >1g/dl in one day, or requiring transfusion), if they had systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis including known platelet disorders, platelet count < 120 x 10^9/L or > 450 x 10^9/L, urea >10mmol/l or GFR < 30ml/min, current infection (clinical signs of infection, white cell count > 12 x 10^9/L), or non-steroidal anti-inflammatory drug (NSAID) use within 14 days of recruitment.

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 study Doppler ultrasound screening, or if they were on antiplatelet therapy or NSAIDs.

Written informed consent, or assent where appropriate, was obtained from all subjects, and this study was approved by the St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee (REC Ref: 2007/07/MA).
15.2.2. Clinical assessment

Clinical assessment of the CVD patients who were recruited within 4 weeks of symptom onset to our case control study was performed as outlined in Chapter 11 [Assessment of the impact of dipyridamole on platelet activation and function in the early and late phases after TIA or ischaemic stroke]. Clinical assessment of control subjects was performed as outlined in Chapter 12 outlining the results of our case-control study on reticulated platelets on antiplatelet therapy in patients in the early and late phases after TIA or ischemic stroke (Reticulated platelets in patients on antiplatelet therapy in the early and late phases after TIA or ischaemic stroke). In brief, all subjects underwent a detailed clinical assessment by at least one of three examiners, and CVD patients had TIA and stroke work-up according to ESO guidelines (European Stroke Organisation (ESO) Executive Committee & ESO Writing Committee, 2008). The underlying mechanism responsible for the ischaemic stroke or TIA in CVD patients was categorised according to the TOAST classification (Adams, Jr. et al, 1993).

In our nested longitudinal study, the 90d follow-up in the large artery atherosclerotic subgroup was performed at least 3 months following carotid surgery or endovascular treatment, unless this treatment had been delayed for at least 3 months after the initial event for medical reasons.

As the majority of patients at baseline and 14d were assessed during their inpatient stay, adherence to antithrombotic therapy was confirmed by checking the drug chart. All outpatients were phoned before their appointment to stress the importance of medication adherence in the week prior to assessment. Adherence in this group was assessed by
history taking alone. Patients who were not fully adherent to their antithrombotic regimen were invited back for reassessment after 14 days.

15.2.3. Blood sampling and laboratory tests

Venepuncture was performed using a standardised method, as described previously (McCabe et al., 2004b) (Chapter 9). Full blood counts were performed between 2 and 4 hours in EDTA and citrate anticoagulated tubes. Platelet function was assessed with the PFA-100® to measure C-ADP closure times in citrate-anticoagulated whole blood between 2 and 2.5 hours after venepuncture, as described in Chapter 11. As outlined in Chapter 11, for our longitudinal study in patients who had dipyridamole MR added to aspirin monotherapy, we defined patients as having ‘dipyridamole non-responsiveness’ *ex vivo* on the PFA-100® if they failed to prolong their C-ADP closure time by more than 14% of their baseline value on aspirin monotherapy.

*Assessment of coagulation system potential*

Coagulation system potential was measured, as previously described [Faber et al. 2003]. 20 µl of 5pM PPP-Reagent, containing a mixture of phospholipids and Tissue Factor, (Thrombinscope BV, Netherlands) was added to 80 µl of platelet poor plasma in a polystyrene 96-well plate, in triplicate. In a fourth well per patient, 20 µml of Thrombin Calibrator (Thrombinscope BV, Netherlands) was added to 80 µl of platelet poor plasma from the same patient. No thrombin production took place in this well, and it served as an internal control from which thrombin production in the other 3 wells were calculated.

The 96-well plate was pre-heated to 37°C for 5 min in a Fluroskan Ascent® microplate fluorometer with Thrombinscope™ software (Thrombinscope BV, Netherlands).
Fluo-Buffer (Thrombinscope BV, Netherlands), containing Hepes (pH 7.35) and Calcium Chloride, was warmed in a water bath at 37°C prior to the experiment. Fluo-Substrate (Thrombinscope BV, Netherlands) containing the fluorogenic substrate solubilised in DMSO, was added to the warmed Fluo-Buffer shortly before the experiment, and loaded onto the Fluroskan Ascent® microplate fluorometer.

The device automatically dispensed premixed and warmed Fluo-Buffer and Fluo-Substrate into each of the wells. This mixture recalcified the citrated plasma, hence commencing thrombin generation. After addition of 20 µL FluCa, the final reaction mixture contained 5 pM Tissue Factor and 4 µM phospholipids. The fluorescent signal that corresponds to thrombin generation was automatically measured every 20 seconds. Thrombin generation curves were calculated for each of the three wells per patient, using the internal control to correct for patient-to-patient differences in colour of plasma, inner filter effect and substrate consumption. Thrombin generation curve values were averaged over the three tested wells for each patient.

'LAG time' to thrombin spark (min), 'peak thrombin' generated (nM), 'time to peak thrombin' (ttPeak; min), 'start-tail' time to end of thrombin generation (min), and endogenous thrombin generation potential (ETP, nM*min), were quantified for each patient plasma sample (Figure 15-1). Lag time represents the time to initiation of thrombin generation and happens to correspond to the clotting time because clot appears when roughly 1% of thrombin is formed; shorter lag times correspond with more procoagulant samples. Peak thrombin represents the maximum concentration of thrombin that a given sample is capable of generating. The time to peak thrombin is a measure of how quickly maximal thrombin generation can be reached; shorter time to peak
thrombin represents a more pro-coagulant sample. The start-tail represents the total time taken to generate thrombin; longer start-tail times indicate that a more persistent stimulus to thrombin generation is present, and therefore, samples with longer start-tail times are believed to be more pro-coagulant. Endogenous thrombin generation potential corresponds to the total amount of thrombin that is produced.

Figure 15-1 Example of Thrombin Generation Curve
A: Lag-time: Time to initiation of thrombin generation; B: Peak thrombin generated; C: tPeak: Time to Peak thrombin generated; D: Start-tail: Time to end point of thrombin generation; E: ETP: Area under thrombin generation curve. All times measured from t=0 min.
**Statistical Methods**

Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively. The Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, and one-way ANOVA and the Kruskal-Wallis rank sum test for comparison of multiple parametric and non-parametric variables, respectively. Chi squared or Fisher exact tests were used to compare proportions between unpaired groups, where appropriate. Multiple linear regression analysis was performed to examine the independent influence of certain demographic variables on the results of the comparison of coagulation system potential between patients and controls, in turn. \( P < 0.05 \) was considered to be statistically significant. Statistical calculations were performed using R, version 2.10.1 (R Development Core Team, 2009).
15.3. Results

15.3.1. Case-control study comparing coagulation system activation in CVD patients vs. controls

Data from 44 ischaemic CVD patients (≤ 4 weeks of onset of a TIA or ischaemic stroke) and 18 ‘cerebrovascular disease-free’ controls were available for analysis at the time of writing up our interim case-control results. The clinical details of the patients and their TIA or stroke subtypes, in addition to the clinical and demographic features of the 18 control subject are outlined in Table 15-1 and Table 15-2. The mean interval from TIA or stroke onset to inclusion in this study was 9 ± 5 days. 6/44 (14%) patients were taking no antiplatelet medication at enrolment, 37/44 (84%) patients were on aspirin monotherapy (median dose 300mg daily), and 1 (2%) patient was on combination therapy with aspirin and clopidogrel (75 mg of each daily).

Peak thrombin generation and ETP were significantly higher in patients than in controls, indicative of increased coagulation system potential in the patient cohort (p ≤ 0.02). Certain vascular risk factors were more common in patients than in controls and could have partly accounted for the significant differences in results between patients and controls. Therefore, multiple linear regression analysis was performed to examine the independent influence of hypertension and the use of aspirin and statin therapy on the results obtained. Having controlled for these variables individually the results remained significant for both ETP and peak thrombin generation following correction for statin use (p ≤ 0.04) and hypertension (p ≤ 0.003), but not following correction for aspirin use (p ≥ 0.08). Aspirin use (p ≥ 0.7) was in itself, not significant within either model, and the majority of patients with stroke were on aspirin. Stepwise model selection (backward/forward) yielded a model with group and statin use for ETP, in which ETP
was still significantly elevated in patients compared to controls. A similar stepwise model selection procedure for peak thrombin production yielded a model with group and peak thrombin production only. Peak thrombin production was significantly elevated in patients compared with controls in this model \( p = 0.005 \) However, lag time and time-to-peak were also significantly higher in patients than in controls \( p \leq 0.02 \), findings which are usually assumed to indicate reduced coagulation system potential. Start-tail time was not significantly different between the groups.

We performed preliminary analysis to assess coagulation system potential in different CVD TOAST subgroups compared with controls. Lag time was prolonged in patients with TIA or stroke of large vessel and undetermined aetiology \( p \leq 0.005 \), but not in patients with cardioembolic, small vessel, other determined or undetermined aetiologies \( p \geq 0.2 \), Table 15-4). Peak thrombin generation was elevated in patients with TIA or stroke of undetermined aetiology \( p = 0.004 \), but not in the other subtypes, when compared with controls \( p \geq 0.07 \). Time to peak thrombin was prolonged in patients with TIA or stroke of large vessel and undetermined aetiologies compared with controls \( p \leq 0.02 \), but not in other subtypes \( p \geq 0.3 \). Start tail was longer in large vessel CVD patients than in controls \( p = 0.03 \), but there was no difference between the other subgroups and controls \( p \geq 0.1 \). ETP was higher in CVD patients of undetermined aetiology than in controls \( p = 0.004 \), but this difference was not significant for other stroke subtypes \( p \geq 0.1 \).
Table 15-1: Demographic Data of Study Subjects at Enrolment to Case-Control Study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n=44)</th>
<th>Controls (n=18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>25 / 19</td>
<td>12 / 6</td>
<td>0.6</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>8 (18%)</td>
<td>0 (0%)</td>
<td>0.09</td>
</tr>
<tr>
<td>IHD</td>
<td>6 (14%)</td>
<td>0 (0%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>29 (66%)</td>
<td>3 (17%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>7 (16%)</td>
<td>0 (0%)</td>
<td>0.09</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Family History Stroke</td>
<td>22 (50%)</td>
<td>6 (33%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>3 (7%)</td>
<td>0 (0%)</td>
<td>0.5</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>3 (7%)</td>
<td>0 (0%)</td>
<td>0.5</td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>1 (2%)</td>
<td>1 (6%)</td>
<td>0.5</td>
</tr>
<tr>
<td>Migraine without aura</td>
<td>3 (7%)</td>
<td>1 (6%)</td>
<td>1</td>
</tr>
<tr>
<td>Current smokers (at enrolment)</td>
<td>16 (36%)</td>
<td>0 (0%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>11 (25%)</td>
<td>7 (39%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Never smoker</td>
<td>17 (39%)</td>
<td>11 (61%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Aspirin therapy</td>
<td>38 (86%)</td>
<td>0 (0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>27 (61%)</td>
<td>0 (0%)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

P values relate to Chi Squared or Fisher exact testing between CVD patients and controls, where appropriate.

Values are Means [±SD] or absolute counts with percentages in parentheses.

Table 15-2: Aetiological Subtyping by TOAST Classification of Early Phase CVD Patients

<table>
<thead>
<tr>
<th>TIA / Stroke Subtype (n = 44)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Artery Atherosclerotic</td>
<td>20 (45%)</td>
</tr>
<tr>
<td>Lacunar</td>
<td>9 (20%)</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Undetermined aetiology</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Other determined</td>
<td>3 (7%)</td>
</tr>
</tbody>
</table>
Table 15-3: Coagulation System Potential in Patients with Recent TIA or Ischaemic Stroke Compared With Controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 44)</th>
<th>Controls (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>3.22 (2.80-3.36)</td>
<td>2.56 (2.25-3.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>353.53 (±63.80)</td>
<td>300.40 (±67.15)</td>
<td>0.007</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>5.78 (5.30-6.36)</td>
<td>5.22 (4.75-5.97)</td>
<td>0.02</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>20.13 (±2.74)</td>
<td>18.94 (±2.39)</td>
<td>0.1</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>1853.36 (±384.90)</td>
<td>1559.06 (±444.17)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients and controls
Values are medians (25-75th percentile) or means (±SD)
Table 15-4: Coagulation System Potential in Aetiological Subgroups of Patients with Recent Ischaemic Stroke or TIA Compared With Controls

<table>
<thead>
<tr>
<th></th>
<th>Large vessel (n=20)</th>
<th>Controls (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>3.33 (2.97-3.55)</td>
<td>2.56 (2.25-3.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>340.14 (±60.90)</td>
<td>300.40 (±67.15)</td>
<td>0.07</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>6.17 (5.41- 6.45)</td>
<td>5.22 (4.75-5.97)</td>
<td>0.004</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>20.68 (±2.30)</td>
<td>18.94 (±2.39)</td>
<td>0.03</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>1769.92 (±283.00)</td>
<td>1559.06 (±444.17)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA and stroke of large vessel aetiology vs. controls. Values are medians (25-75th percentile) or means (±SD).

<table>
<thead>
<tr>
<th></th>
<th>Cardioembolic (n=2)</th>
<th>Controls (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>3.05 (2.91-3.19)</td>
<td>2.56 (2.25-3.00)</td>
<td>0.2</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>447.79 (±72.89)</td>
<td>300.40 (±67.15)</td>
<td>0.2</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>5.44 (5.22-5.67)</td>
<td>5.22 (4.75-5.97)</td>
<td>1</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>21.84 (±4.01)</td>
<td>18.94 (±2.39)</td>
<td>0.5</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>2354.50 (±817.66)</td>
<td>1559.06 (±444.17)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA and stroke of cardioembolic aetiology vs. controls. Values are medians (25-75th percentile) or means (±SD).
<table>
<thead>
<tr>
<th></th>
<th>Small vessel (n=9)</th>
<th>Controls (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>2.91 (2.11-2.99)</td>
<td>2.56 (2.25-3.00)</td>
<td>0.7</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>357.75 (±73.25)</td>
<td>300.40 (±67.15)</td>
<td>0.07</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>5.44 (4.56-5.66)</td>
<td>5.22 (4.75-5.97)</td>
<td>0.6</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>19.00 (±3.34)</td>
<td>18.94 (±2.39)</td>
<td>0.9</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>1768.30 (±452.23)</td>
<td>1559.06 (±444.17)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with stroke and TIA of small vessel aetiology vs. controls
Values are medians (25-75th percentile) or means (±SD)

<table>
<thead>
<tr>
<th></th>
<th>Other (n=3)</th>
<th>Controls (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>3.32 (2.50-4.38)</td>
<td>2.56 (2.25-3.00)</td>
<td>0.4</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>308.61 (±47.29)</td>
<td>300.40 (±67.15)</td>
<td>0.8</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>6.66 (5.44-8.22)</td>
<td>5.22 (4.75-5.97)</td>
<td>0.3</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>18.33 (±5.37)</td>
<td>18.94 (±2.39)</td>
<td>0.9</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>1762.56 (±527.36)</td>
<td>1559.06 (±444.17)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with stroke and TIA of other determined aetiology vs. controls
Values are medians (25-75th percentile) or means (±SD)
<table>
<thead>
<tr>
<th></th>
<th>Undetermined (n=10)</th>
<th>Controls (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>3.16 (3.00-3.67)</td>
<td>2.56 (2.25-3.00)</td>
<td>0.005</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>371.11 (±48.55)</td>
<td>300.40 (±67.15)</td>
<td>0.004</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>6.22 (5.59-6.58)</td>
<td>5.22 (4.75-5.97)</td>
<td>0.02</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>20.23 (±1.74)</td>
<td>18.94 (±2.39)</td>
<td>0.1</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>2023.80 (±327.58)</td>
<td>1559.06 (±444.17)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

P value refers to comparison between patients with TIA and stroke of undetermined aetiology vs. controls
Values are medians (25-75th percentile) or means (±SD)
15.3.2. Longitudinal study in CVD patients who were changing from aspirin monotherapy to aspirin and dipyridamole combination therapy

Interim data from 21 CVD patients who underwent assessment at baseline on aspirin, and subsequently at 14d and 90d after adding dipyridamole MR 200mg BD to their aspirin treatment, were available at the time of completion of this thesis. The clinical details of the CVD patients in this ongoing longitudinal study, and their stroke subtypes are outlined in Table 15-5 and Table 15-6.

The potential impact of adding dipyridamole to aspirin monotherapy on *ex vivo* coagulation system potential in platelet poor plasma are shown in Table 15-7. Lag time, peak thrombin generation and endogenous thrombin potential were all significantly reduced 14d after the addition of dipyridamole to aspirin therapy, and this reduction was maintained at 90d (p ≤ 0.01). There was no significant change in time to peak thrombin generation, or significant differences in start-tail time at 14d or 90d compared with baseline values on aspirin monotherapy.
Table 15-5: Demographic Data of Patients at Enrolment to the Longitudinal Study Assessing the Impact of Adding Dipyridamole to Aspirin Therapy on Coagulation System Potential

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years</td>
<td>62 [±10]</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>14 / 7</td>
</tr>
<tr>
<td>Prior Stroke/TIA</td>
<td>4 (19%)</td>
</tr>
<tr>
<td>IHD</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>14 (67%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>3 (14%)</td>
</tr>
<tr>
<td>A Fib/Flutter at enrolment</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Family History Stroke</td>
<td>11 (52%)</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Migraine without aura</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Current smokers (at enrolment)</td>
<td>4 (19%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>7 (33%)</td>
</tr>
<tr>
<td>Never smoker</td>
<td>10 (48%)</td>
</tr>
<tr>
<td>Statin</td>
<td>8 (38%)</td>
</tr>
</tbody>
</table>

Table 15-6: Aetiological Subtyping by TOAST Classification of CVD Patients in the Longitudinal Study Assessing the Impact of Adding Dipyridamole to Aspirin on Coagulation System Potential

<table>
<thead>
<tr>
<th>Stroke/TIA subtype (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherothrombotic</td>
</tr>
<tr>
<td>Lacunar</td>
</tr>
<tr>
<td>Cardioembolic</td>
</tr>
<tr>
<td>Undetermined aetiology</td>
</tr>
<tr>
<td>Other determined</td>
</tr>
</tbody>
</table>
Table 15-7: Coagulation System Potential in Ischaemic CVD Patients After Changing From Aspirin Monotherapy (Baseline, Within 4 Weeks of Symptom Onset) to Aspirin And Dipyridamole MR Combination Therapy 14 Days (14d) and at Least 90 Days (90d) Later.

<table>
<thead>
<tr>
<th>Coagulation System Potential</th>
<th>Baseline</th>
<th>14d</th>
<th>90d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Time (min)</td>
<td>2.91 (2.33-3.00)</td>
<td>2.88 (2.33-3.00)</td>
<td>2.67 (2.29-3.00)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.009</td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>Peak Thrombin (nM)</td>
<td>332.57 (±70.30)</td>
<td>315.98 (±71.40)</td>
<td>312.55 (±66.98)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.01</td>
<td></td>
<td>0.0009</td>
</tr>
<tr>
<td>Time to Peak Thrombin (min)</td>
<td>5.45 (±0.76)</td>
<td>5.45 (±0.90)</td>
<td>5.39 (±0.77)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.9</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Start Tail (min)</td>
<td>19.10 (±2.62)</td>
<td>19.10 (±2.40)</td>
<td>18.97 (±2.37)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.99</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>ETP (nM x min)</td>
<td>1704.72 (±248.57)</td>
<td>1627.29 (±418.79)</td>
<td>1621.23 (±427.58)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.02</td>
<td></td>
<td>0.004</td>
</tr>
</tbody>
</table>

P value refers to comparison between baseline and follow-up at 14d and 90d. Values are medians (25-75th percentile) or means (±SD).

Impact of coagulation system potential results on ex-vivo dipyridamole responsiveness in the early and late phases after TIA or ischaemic stroke

We compared thrombin generation parameters in ‘dipyridamole non-responders’ with ‘dipyridamole responders’ at 14d (n = 13 vs. 8) and 90d (n = 10 vs. 11). There were no significant differences in lag time, peak thrombin production, time to peak, start-tail time, or ETP between dipyridamole non-responders and responders on the PFA-100® at any stage during follow-up (p ≥0.4).
15.4. Discussion

This pilot study has yielded a number of novel findings that warrant further investigation. We have found evidence of (i) enhanced coagulation system potential (higher peak thrombin generation and ETP levels) and (ii) potentially reduced coagulation system potential (elevation of the lag time and time-to-peak) in the same patients in the early phase after TIA or ischaemic stroke compared with controls. The reasons for these apparently conflicting coagulation system potential results are unclear, but a number of possible mechanisms need to be considered. It is possible that the antiplatelet treatment regimens prescribed in all patients in this study (predominantly aspirin monotherapy), may have had different indirect inhibitory effects on separate components of the thrombin generation potential curve. For example, if longitudinal studies in patients studied before and after commencing aspirin were to indicate that aspirin therapy prolongs lag time and time to peak thrombin generation, but does not affect other aspects of the thrombin generation curve, this could explain why some parameters were reduced in parallel with other markers of enhanced coagulation system potential following TIA or stroke. It is possible that the relatively small sample size has lead to a type II error with respect to the lag time or the time to peak data, or a type I error with respect to the peak thrombin generation and ETP results. These questions will be explored in ongoing studies in our laboratory in larger groups of patients in the early and late phases after TIA or stroke, who underwent a treatment change from no medication to aspirin, and from aspirin to clopidogrel.

Because the study design led to the inclusion of patients predominantly with non-cardioembolic TIA or ischaemic stroke, only 2 (5%) patients in our case-control study had TIA or stroke of cardioembolic origin. Enhanced coagulation system potential is
likely to be of even greater importance in patients with cardioembolic CVD, and the majority of these patients are treated with long-term warfarin anticoagulation. Up to 50% of patients with non-valvular atrial fibrillation are not treated with warfarin (Go et al, 1999). Most of these patients are empirically treated with antiplatelet therapy instead in the clinical setting. If it were confirmed that certain patients with enhanced coagulation system potential despite treatment with a particular antithrombotic regimen are at a higher risk of recurrent vascular events than those without enhanced potential, this might facilitate altering antithrombotic therapy in individuals to optimise secondary prevention. Large studies with long-term follow up would be required to address these hypotheses. We cannot draw any definitive conclusions about coagulation system potential in individual TIA or ischaemic stroke TOAST subtypes compared with controls because the numbers of subjects involved in these subgroup analyses were far too small, although it appears that the results we have demonstrated are mainly influenced by data in large artery and undetermined CVD subgroups. Larger studies with careful patient subtyping are also required to explore this further because this study was not adequately powered to compare coagulation system potential in CVD subtypes compared with controls.

In our longitudinal substudy, we have found some evidence that dipyridamole may reduce coagulation system potential (reduced peak thrombin generation and ETP) over and above any effect that might be seen with aspirin monotherapy at both 14d and 90d after commencing treatment. If these findings are confirmed in larger studies, this would enhance of understanding of the potential indirect anti-thrombotic effects of dipyridamole in ischaemic CVD. We cannot exclude the possibility that peak thrombin generation and ETP would have fallen anyhow in association with resolution of the
acute phase response. Longitudinal studies in CVD patients who have not undergone a change in antiplatelet therapy during follow up would be required to address this question; and analysis of plasma samples from patients who remained on aspirin monotherapy throughout the follow up period in the study is planned (Chapter 10, Assessment of the impact of aspirin and clopidogrel on platelet activation and function in the early and late phases after TIA or ischaemic stroke). However, contrary to the above findings that suggested a potential ‘anticoagulant effect’, lag time also reduced following treatment with dipyridamole at 14d and 90d; this could be interpreted as indicating an unanticipated procoagulant effect following the addition of dipyridamole to aspirin. The reasons for the disparity in these findings could also relate to type II or type I errors, respectively, as alluded to in the discussion of our case control findings above, or the differential effects of dipyridamole on aspects of the coagulation system potential curve. Other thrombin generation parameters (time to peak, and start-tail) were not altered in a consistent fashion during follow-up compared with initial treatment with aspirin alone. These findings need to be confirmed in larger longitudinal studies exploring potential novel effects of dipyridamole on coagulation system activation/potential in ischaemic CVD.

As anticipated, we did not identify any differences in coagulation system potential between dipyridamole non-responsive and responsive patients on the PFA-100®. One cannot conclude from these very preliminary small subgroup comparative data that enhanced coagulation system potential does not play a role in mediating ex vivo dipyridamole non-responsiveness on the PFA-100®, although the high shear stress system of the PFA-100® is unlikely to be significantly influenced by coagulation system activation or potential. Analysis of the remaining stored samples in our entire cohort of
patients who changed from aspirin monotherapy to aspirin and dipyridamole is ongoing to further address this question.

15.5. Conclusion
To our knowledge, this is the first study to demonstrate enhanced coagulation system potential in the early phase after TIA or ischaemic stroke compared with healthy controls. Dipyridamole may reduce coagulation system potential in both the early and late phases after TIA or ischaemic stroke, in addition to its proposed antiplatelet effects, but these results could also reflect resolution of the acute phase response. Because some of the interim coagulation system potential results were conflicting, further prospective studies in a larger patient population at AMNCH is planned to reassess the interesting findings from this pilot study.
16. Future Work

The studies described in this thesis investigated the *ex vivo* response to commonly prescribed antiplatelet agents for secondary prevention following TIA and ischaemic stroke, and the impact of antiplatelet therapy on several important pathways involved in thrombosis and thrombo-embolism and vice versa. Our novel findings have inevitably raised new clinically important translational research questions. In this chapter, I will discuss proposals to investigate these outstanding research issues.

16.1. Response to Antiplatelet Agents in the Laboratory

I have described in Chapters 10 and 11 that the PFA-100® can reliably detect the effect of aspirin and dipyridamole using the C-EPI and C-ADP cartridges, respectively, although there is a substantial rate of laboratory non-responsiveness to both agents. The C-ADP cartridge is not reliable at detecting the *ex vivo* antiplatelet effects of clopidogrel. To determine whether laboratory non-responsiveness is predictive of ‘clinical non-responsiveness’, it will be necessary to undertake a longitudinal prospective study, sufficiently powered to detect differences in outcome events between antiplatelet non-responders and responders. If it can be shown that patients who are non-responsive to antiplatelet agents in the laboratory have higher rates of recurrent vascular events than patients who are responsive, this would provide a sound rationale for the design of an interventional trial to alter antiplatelet therapy to optimise secondary prevention. Based on our data, a multicentre study to investigate this hypothesis has been designed by our group.
The poor discriminatory ability of the PFA-100® C-ADP cartridge to detect the inhibitory effects of clopidogrel means that alternate devices or cartridges are required to assess ex vivo clopidogrel non-responsiveness. Potentially suitable devices exist, including the VerifyNow® P2Y12 cartridge which is discussed in Chapter 3, the PFA-100® Innovance P2Y cartridge which has recently been licensed, and the relatively new Multiplate® analyser (ADP cuvette). While the PFA-100® assesses platelet function under moderate to high shear stress conditions, responsiveness to antiplatelet agents on this device may only be relevant to stroke aetiologies in which moderate to high shear stress plays a role, such as stroke of large-artery atherosclerotic aetiology. Patients with stroke of small vessel or cardioembolic aetiology may be better risk-stratified by assessing their response to antiplatelet agents on a low shear device. The VerifyNow® assesses inhibition of platelet function at low shear stress in a stirred solution in response to agonist-induced activation of platelets to assess the response to aspirin or clopidogrel, but has not, to date, been shown to detect the ex vivo effects of dipyridamole. It is also possible that patients with TIA or stroke of large artery atherosclerotic aetiology, whose platelets are exposed to high shear stress in vivo, may be optimally monitored by a low shear stress device ex vivo to avoid inducing ‘platelet exhaustion’ upon re-exposure to further high shear stress ex vivo. Therefore, future studies assessing ex vivo responsiveness to antiplatelet therapy in ischaemic CVD should include simultaneous testing with high and low shear stress devices. These studies are also now planned in our laboratory.

Finally, because the PFA-100®, VerifyNow and Multiplate use fixed doses of agonists or agonist combinations, our platelet science collaborators at the Department of Clinical Pharmacology, Royal College of Surgeons in Ireland (RCSI) have developed a novel
Platelet ADP Secretion (PAS) assay that examines ADP release from granules in activated platelets in response to increasing doses of platelet agonists. The PAS assay provides rapid, efficient and reproducible testing of platelet function, with a classical dose-response relationship between the concentration of a platelet agonist and the \textit{ex vivo} response observed in preliminary experiments in healthy controls. We intend to assess inhibition of platelet function with this novel PAS assay in patients treated with aspirin, or aspirin and dipyridamole combination therapy, in tandem with more established platelet function tests in future planned longitudinal observational studies. Our platelet science collaborators will also assess whether this assay can be modified to assess \textit{ex vivo} non-responsiveness to clopidogrel and novel ADP-receptor antagonists. We also aim to modify our flow cytometry protocols in future studies to assess ‘inducible platelet activation/reactivity’ in patients on different antiplatelet regimens rather than assess platelet activation in unstimulated samples.

16.2. Coagulation System Activation

For the first time, we have demonstrated enhanced coagulation system potential in the early phase after TIA or ischaemic stroke, which appears to decrease after treatment with dipyridamole. To determine whether the reduction in coagulation system potential is a result of adding dipyridamole MR therapy to aspirin, we will assess coagulation system potential over time in the remaining cohort of patients who changed from aspirin to aspirin and dipyridamole combination therapy, and in patients on other stable antiplatelet regimens, including aspirin and clopidogrel monotherapy. However, studies in larger groups of patients will need to be done due to the small number of patients who remain on long term aspirin monotherapy. If coagulation system potential falls in
all subgroups over time, this may reflect a ‘class effect’ with antiplatelet agents, or it may simply reflect resolution of the acute phase response.

16.3. Endothelial +/- Platelet Activation

We have confirmed previous findings that VWF:Ag is elevated in the early phases after TIA or ischaemic stroke as a marker of endothelial +/- platelet activation. For the first time, we have described the potential impact of the addition of dipyridamole to aspirin on VWF:Ag and VWF:Ag II levels in ischaemic CVD. There were no differences in VWF:Ag levels between dipyridamole non-responders and responders, but the number of subjects in these subgroup analyses was far too small to make any definitive conclusions. Therefore, analysis of remaining stored samples that were collected during this thesis and of samples that will be prospectively collected in planned studies at our institution will be informative in this regard to assess whether these changes are also secondary to alteration of antiplatelet treatment or resolution of the acute phase response. The impact of VWF:Ag and VWF:Ag II levels on the PFA-100® results in patients on aspirin and clopidogrel monotherapy will be assessed by analysing stored PPP from the remaining cohort of patients. We anticipate that VWF:Ag levels will be shown again to influence ex vivo non-responsiveness to aspirin in keeping with data from Dr McCabe’s group in the past, and it is likely that VWF:Ag levels will influence the results of the PFA-100® in patients on clopidogrel.
16.4. Circulating reticulated platelets

This thesis includes data on the largest longitudinal study of circulating reticulated platelets in patients following TIA or ischaemic stroke, and the largest study to systematically investigate the effects of changing antiplatelet regimens on circulating reticulated platelets. Contrary to some previous reports, we have not found any difference in the percentage of circulating reticulated platelets between ischaemic CVD patients in the early phase after symptom onset and controls, but we did identify elevated reticulated platelets in the late phase. Interestingly, patients who are non-responsive to aspirin have elevated circulating reticulated platelets in the early phase following ischaemic and TIA. Treatment with aspirin therapy may potentially stabilise the reticulated platelet fraction in whole blood, and the percentage of reticulated platelets may also influence ex vivo non-responsiveness to aspirin on the PFA-100®. To investigate whether circulating reticulated platelets impact on ex vivo non-responsiveness to antiplatelet therapy, including dipyridamole and clopidogrel in ischaemic CVD, we will assess circulating reticulated platelets in conjunction with the platelet function and activation studies described above, in the context of a larger, longitudinal study in patients following TIA and ischaemic stroke. This combination of assays will improve our understanding of the underlying cellular mechanisms responsible for ex vivo non-responsiveness to antiplatelet therapy in ischaemic CVD.

We will also continue to collect serum and plasma samples for measurement of thromboxane B₂ and salicylate levels, as additional potential indirect markers of adherence, absorption and responsiveness to aspirin therapy.
These future studies will address some of the outstanding questions raised by the data that have emerged from this thesis. The ultimate aim of this work is improve our understanding of the mechanisms responsible for \textit{ex vivo} non-responsiveness to antiplatelet therapy, and to translate these findings into the clinical setting by identifying patients at high risk of recurrent vascular events on their current antiplatelet regimen with sensitive and specific tests of platelet function. This should facilitate targeting these patients with individualised antiplatelet treatment regimens to optimise secondary prevention following TIA or ischaemic stroke, and thus reduce morbidity and mortality from ischaemic cerebrovascular disease.
References


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