Assessment of Platelet Activation and Function in Ischaemic Stroke, Transient Ischaemic Attack and Asymptomatic Severe Carotid Stenosis

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by

Dominick John Henry McCabe MB, BCh, BAO, MRCPI

Stroke Research Unit,
Department of Headache, Brain Injury and Rehabilitation,
Institute of Neurology,
The National Hospital for Neurology and Neurosurgery,
Queen Square, London WC1N 3BG.
Abstract

Platelets play a pivotal role in haemostasis and thromboembolism, and a better understanding of the cellular mechanisms involved in platelet activation should improve our understanding of the pathogenesis of ischaemic cerebrovascular disease (CVD).

The purpose of this thesis was to perform a comprehensive assessment of platelet activation and function in patients with ischaemic stroke, TIA, and asymptomatic severe carotid stenosis.

We hypothesised that patients with ischaemic CVD would have excessive platelet activation compared with controls without a history of stroke or TIA. We also hypothesised that we would find excessive platelet activation in patients with recently symptomatic compared with asymptomatic severe carotid stenosis. We examined the prevalence of ‘aspirin resistance’ in ischaemic CVD using a relatively novel platelet function analyser, called the PFA-100®, and investigated the ex vivo response to increasing doses of oral aspirin in the convalescent phase after ischaemic stroke or TIA.

We found elevated levels of CD62P and monocyte-platelet complexes using whole blood flow cytometry, and elevated von Willebrand factor antigen levels using a latex agglutination assay, in the acute and convalescent phases after ischaemic stroke or TIA compared with controls. We did not find a significant increase in the percentage of reticulated platelets, or in the levels of soluble P-selectin or E-selectin in ischaemic CVD patients compared with controls. The mean platelet count and the percentage of leucocyte-platelet complexes were significantly higher in patients with recently symptomatic compared with asymptomatic severe carotid stenosis. Using the PFA-100®, 60% of patients in the acute phase and 43% of patients in the convalescent phase after ischaemic stroke or TIA were ‘resistant’ to the antiplatelet effects of aspirin ex vivo. Preliminary studies did not provide any convincing evidence that the ex vivo response to aspirin therapy was more pronounced as the dose was increased from 75 mg to 150 mg to 300 mg daily in the convalescent phase after ischaemic CVD. Preliminary platelet aggregometry studies in platelet rich plasma (PRP) revealed that two patients who were aspirin resistant on 75 mg daily using epinephrine-induced platelet aggregometry, were aspirin responsive on higher doses of the drug. The fact that sodium arachidonate-induced platelet aggregation was inhibited to a significant degree in all of our patients suggests that cyclooxygenase-independent mechanisms are important in mediating aspirin resistance using the PFA-100®. Our data suggest that elevated VWF levels in ischaemic CVD are likely to play an important role in mediating aspirin resistance using this device. There was poor concordance between the results obtained with the PFA-100® and platelet aggregometry. However, because the PFA-100 measures platelet function in whole blood at moderately high shear rates, it should more closely mimic in vivo platelet aggregation in patients with atherosclerosis in comparison with platelet aggregometry in PRP that is performed at very low shear rates.

We have confirmed that excessive platelet activation occurs in patients with ischaemic stroke or TIA, and have shown that a large proportion of ischaemic CVD patients are ‘resistant’ to the antiplatelet effects of aspirin therapy ex vivo. Further studies are required to determine whether one can use assays of platelet activation and function to monitor and / or predict the response to antiplatelet therapy in this patient population.
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<td>Anterior Cerebral Artery</td>
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<td>ECST</td>
<td>European Carotid Surgery Trial</td>
</tr>
<tr>
<td>EDV</td>
<td>End Diastolic Velocity</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Endothelial-leucocyte adhesion molecule</td>
</tr>
<tr>
<td>ESPS-2</td>
<td>Second European Stroke Prevention Study</td>
</tr>
<tr>
<td>FBC</td>
<td>Full Blood Count</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>FISS</td>
<td>Fraxiparin in Stroke Study</td>
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<tr>
<td>FISS bis</td>
<td>Fraxiparine in Ischaemic Stroke Study</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<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>
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<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>
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<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>
</tr>
<tr>
<td>H2O</td>
<td>Water</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal Carotid Artery</td>
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<tr>
<td>ICH</td>
<td>Intracerebral Haemorrhage</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</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>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>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>MPV</td>
<td>Mean Platelet Volume</td>
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<tr>
<td>MRA</td>
<td>Magnetic resonance angiography</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NASCET</td>
<td>North American Symptomatic Carotid Endarterectomy Trial</td>
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<tr>
<td>NVAF</td>
<td>Non-valvular Atrial Fibrillation</td>
</tr>
<tr>
<td>OCS</td>
<td>Open Canalicular System</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Arterial Disease</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
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<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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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PFA-100&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Platelet Function Analyser-100, Dade-Behring, Germany</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
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</table>
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
SPIRIT  Stroke Prevention in Reversible Ischaemia Trial
SS  Side Scatter
TIA  Transient Ischaemic Attack
TO  Thiazole Orange
TOAST  Trial of ORG 10172 in Acute Stroke Treatment
TxA₂  Thromboxane A₂
VWF  von Willebrand factor
WARSS  Warfarin-Aspirin Recurrent Stroke Study
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Finally, I thank all of my friends and other professional colleagues whom I look forward to spending more time with in future.
1. General Introduction

1.1 Ischaemic Stroke and Transient Ischaemic Attack (TIA)

1.1.1 Definition of stroke and TIA

A stroke is a clinical syndrome that is characterised by the sudden onset of symptoms or signs of focal neurological dysfunction, with symptoms lasting for more than 24 hours or resulting in death within this time period, that after adequate investigation are presumed to be of non-traumatic vascular origin (Bamford, 1992). However, 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. In addition, patients presenting with a stroke caused by subarachnoid haemorrhage may present with a severe headache and meningism without focal or global neurological dysfunction. The definition does not include neurological dysfunction caused by subdural or extradural haemorrhage (Whisnant et al, 1990), and some authors also exclude cerebral infarction or haemorrhage caused by an infection or a tumour (Warlow C.P et al, 1996b).

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 C.P et al, 1996b). Most TIAs last for minutes rather than hours, with the majority lasting between six and 30 minutes in one hospital-based series (Warlow C.P et al, 1996b). 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 C.P et al, 1996b). It is also important to remember that the findings on neuroimaging do not alter the definition of whether a patient has had a TIA or stroke, because up to 25% of patients with a TIA will have an infarct in an appropriate area on brain CT or MRI that does not lead to any obvious functional impairment.

1.1.2 Pathogenesis of stroke and TIA

Cerebral infarction is the underlying pathogenic mechanism responsible for approximately 80% of first strokes (Warlow C.P et al, 1996e), with 10% secondary to primary intracerebral haemorrhage and 5% secondary to subarachnoid haemorrhage. In up to 5% of patients, the mechanism responsible for the stroke may remain undetermined after investigation, although the proportion of patients in this category is likely to decrease in the future with more advanced neuroimaging techniques. Although some patients with intracranial haemorrhage may present with symptoms consistent with a TIA (Lees et al, 2000), TIAs are, by definition, secondary to inadequate cerebral or ocular blood supply as a result of arterial thrombosis or embolism (Warlow C.P et al, 1996b).

1.1.3 Aetiology of cerebral infarction and TIAs

The majority of ischaemic strokes and TIAs result from thromboembolism from an atherosclerotic plaque in a large extracranial or intracranial artery (atherothrombotic stroke), intracranial small vessel disease (lacunar stroke), or embolism from the heart (cardioembolic stroke). In the remaining patients, less common causes for the ischaemia are identified (e.g. sickle cell disease or vasculitis) or the origin of the infarction is not
established by investigation (ischaemic stroke or TIA of indeterminate aetiology). In patients with ischaemic stroke of indeterminate aetiology, it is likely that atherothrombosis is involved in the majority. In a hospital-based study of 1273 patients with ischaemic stroke, cerebral infarction was attributed to atherosclerosis in 9%, intracranial small vessel disease in 27%, embolism from the heart in 19%, and 5% of patients had more than one possible cause of their infarction (Sacco et al, 1989a). 40% of patients were classified as having infarction of indeterminate aetiology. However, the relative frequencies of the different ischaemic stroke subtypes in any clinical series depends on the criteria used for classification. For example, in another hospital based study of first stroke in Lausanne that employed different diagnostic criteria, cerebral infarction was attributed to atherosclerosis in up to 48% of patients, and only 8.3% of patients had infarction of indeterminate aetiology (Bogousslavsky et al, 1988).

In the following section, I will briefly outline the underlying pathogenic mechanisms responsible for, and the clinical features associated with, the different aetiological categories of ischaemic stroke or TIA. I will specifically deal with atherothrombotic ischaemic stroke or TIA in association with extracranial carotid artery stenosis, and will not deal with the important category of patients with symptomatic intracranial carotid or extracranial vertebral artery stenosis who were not systematically studied in this thesis. Most of the principles outlined relate to both completed ischaemic stroke and TIA, with the exception that TIAs are less likely to be associated with cerebral infarction. To avoid repetition, I will refer only to ischaemic stroke unless otherwise specified.
Atherothrombotic ischaemic stroke or TIA associated with extracranial carotid artery stenosis

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). However, in some patients, 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). In other patients with internal carotid artery occlusion, emboli may arise (i) from the occluded internal carotid stump, (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).

Patients with ischaemic stroke secondary to extracranial carotid artery stenosis usually present with clinical features of middle cerebral artery (MCA) rather than anterior cerebral artery (ACA) territory infarction. In MCA territory infarction, patients can present with unilateral weakness, hemisensory impairment, dysphasia, visual field defects or other features of higher cortical dysfunction. The most common presentation in patients with ACA territory infarction is weakness of the contralateral lower limb, often most marked distally, and sometimes associated with proximal more than distal weakness of the upper limb (Whisnant et al, 1990). Sensory impairment and other signs of higher cortical dysfunction, especially apraxia of the affected limb, can occur. In addition,
patients with severe extracranial carotid stenosis can present with ipsilateral isolated amaurosis fugax, visual blurring or retinal infarction because of distal embolisation to the ophthalmic artery (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991). Computerised tomography (CT) or magnetic resonance imaging (MRI) of the brain may show an infarct in the appropriate vascular territory. Magnetic resonance angiography (MRA), colour Doppler ultrasound, or intra-arterial digital subtraction angiography (DSA) can be used to quantify the severity of carotid stenosis.

The degree of carotid stenosis on DSA has traditionally been measured using 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 (1-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 (1-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 (1-A/N), where A = the diameter of the residual lumen at the point of maximal stenosis, and N = the normal diameter of the distal ICA at the point where the walls are 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 clinical practice, it has been suggested that the NASCET method be adopted for grading ICA stenosis (Rothwell et al, 2003).

**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 leave behind a small cystic fluid-filled cavity called a lacune (small lake or pond), and some collapse of the cavity is the rule (Fisher, 1982); (Whisnant et al, 1990). 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 (15 to 20 mm in diameter) that are more commonly symptomatic are usually caused by intracranial atherosclerosis involving arteries 200 to 900 μm in diameter (Fisher, 1982); (Lammie, 2000). 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 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 C.P et al, 1996d), 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); (Inzitari et al, 2000). Brain CT can be normal or show an anatomically compatible infarct of <1.5 cm in maximum diameter (Whisnant et al, 1990), but MRI is more likely to identify the responsible lesion, especially if it is located in the posterior fossa. With the exception of patients with a
potential proximal source of embolism, e.g. severe ipsilateral carotid stenosis, no other cause for the infarction should be identified on diagnostic imaging.

**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, 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. However, it may be difficult to be certain that a stroke is cardioembolic in origin, because approximately one third of patients with stroke in association nonvalvular atrial fibrillation (NVAF) have another potential underlying cause for their stroke (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 infarcts may also be secondary to cardiac embolism. Investigation with electrocardiography, transthoracic or transoesophageal echocardiography, and with 24-hour cardiac rhythm (holter) monitoring where appropriate, should reveal a cardiac source of embolism. Brain CT or MRI should demonstrate an infarct in a clinically appropriate location.
Ischaemic stroke of indeterminate aetiology

Patients who cannot be categorised as having any of the above ischaemic stroke subtypes on the basis of the clinical findings or results of investigations can be classified as having ischaemic stroke of indeterminate aetiology. This includes patients with two or more potential causes for their stroke e.g. atrial fibrillation and severe carotid stenosis (see chapter 3 also).

1.1.4 Epidemiology of stroke (emphasis on ischaemic stroke)

The health and economic impact of stroke on patients, their families, health services and society in general is considerable. Stroke is the commonest life-threatening neurological disorder in adults and the third most common cause of death in Western populations (Warlow C.P et al, 1996c). The annual age-and sex-standardised incidence of stroke has been calculated as 312 per 100,000 for the age group 45 to 84 years (Sudlow & Warlow, 1997), with this incidence increasing steeply with advancing years (Bonita, 1992). For this reason, the impact of stroke is likely to increase in the future because of the predicted longer life expectancy of our ageing population.

Moreover, because the majority of patients survive their first ischaemic stroke with 95% alive at seven days, 90% alive at 30 days, and 77% alive at one year (Warlow C.P et al, 1996a), 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’ (Moroney et al, 1998); (Sacco et al, 1989b); (Goldstein & Perry, 1992) 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 trial design. Early recurrence occurs in 8 to 13% of patients in the first one to three months after atherothrombotic stroke (Moroney et al, 1998); (Sacco et al, 1989b). 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%) (Moroney et al, 1998); (Sacco et al, 1989b), and although the recurrent stroke risk was as high as 9% in the first year in the Oxfordshire Community Stroke Project (Bamford et al, 1991), the overall incidence of stroke recurrence in this subgroup has also been reported to be as low as 6.5% over two years in a smaller hospital-based study (Clavier et al, 1994). 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 two weeks (International Stroke Trial Collaborative Group, 1997). Patients with 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 C.P et al, 1996a).

Therefore, the importance of improving our understanding of the pathogenesis of stroke and TIA, and of improving the therapeutic options for patients in the acute and
convalescent phases after stroke, with an emphasis on better rehabilitation and preventative strategies to decrease recurrence, cannot be overemphasised.

1.2 Asymptomatic Carotid Stenosis

1.2.1 Introduction

Five to 10 per cent of people over 65 years of age have asymptomatic stenosis (> 50%) of one extracranial carotid artery (The European Carotid Surgery Trialists Collaborative Group, 1995), and 1.6% of subjects over 50 years of age had asymptomatic > 80% carotid stenosis in one study (Ricci et al, 1991). In the European Carotid Surgery Trial (ECST), asymptomatic moderate ($ECST_{30 \text{ to } 69\%}$) or severe ($ECST_{70 \text{ to } 99\%}$) carotid stenosis of the contralateral carotid artery was noted on digital subtraction angiography in 36.7% and 5.5% of patients, respectively (The European Carotid Surgery Trialists Collaborative Group, 1995). In this trial, the risk of stroke in the distribution of the asymptomatic stenosed carotid artery varied according to the severity of the stenosis. The three year risk of stroke was higher in patients with 70 to 99% stenosis compared with those with 30 to 69% stenosis (5.7% versus 2.1%) (The European Carotid Surgery Trialists Collaborative Group, 1995). Because carotid endarterectomy has been shown to effective in reducing the risk of subsequent stroke in patients with symptomatic severe (> 70%) extracranial carotid stenosis (see section 1.3), several studies have compared the outcome after surgical treatment to best medical care of asymptomatic severe carotid stenosis (The CASANOVA Study Group, 1991); (Hobson et al, 1993); (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995); (Mayo Asymptomatic Carotid Endarterectomy Study Group, 1992). However, aspirin therapy was prescribed in both the surgical and medical groups in only three of the trials
published to date, and all of these studies included some patients with < 70% carotid stenosis (The CASANOVA Study Group, 1991); (Hobson et al, 1993); (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995). Because the design of one of these studies did not allow for a direct meaningful comparison of the effects of carotid endarterectomy with medical treatment alone (The CASANOVA Study Group, 1991), the results of two of the trials will now be discussed. This will be followed by a summary of a metaanalysis of completed trials of surgical treatment for asymptomatic carotid stenosis.

1.2.2 Review of the literature regarding the treatment of asymptomatic moderate or severe carotid stenosis

The Veterans Affairs Cooperative Study Group carried out a randomised clinical trial to investigate the outcome after treatment with carotid endarterectomy compared with best medical care in male patients with angiographically confirmed asymptomatic NASCET 50 to 99% carotid artery stenosis (Hobson et al, 1993). Two hundred and eleven patients were randomly assigned to undergo surgery and 233 to receive best medical care; both treatment groups received aspirin therapy (325 to 650 mg daily). The mean age of the patients was 64.5 years, and the mean duration of follow up was two years. Carotid endarterectomy reduced the absolute risk of ipsilateral TIA, transient monocular blindness or stroke by 12.6% compared with medical treatment alone (p < 0.001). However, there was no significant difference in the combined incidence of stroke or death between the two treatment groups during follow-up.
In the Asymptomatic Carotid Atherosclerosis Study (ACAS), patients with asymptomatic NASCET 60 to 99% carotid stenosis were randomised to undergo carotid endarterectomy (n = 825) or receive best medical care (n = 834) in 39 clinical centres in the USA and Canada (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995). The mean age of the study subjects was 67 years, and 58% of patients had 70 to 99% stenosis of the randomised carotid artery. All patients received 325 mg of aspirin daily, and follow-up was continued for a median of 2.7 years. The estimated five-year risk of ipsilateral stroke or any perioperative stroke or death was 5.1% in the surgical group and 11% in the medical group. Therefore, carotid endarterectomy reduced the absolute risk of stroke by about 1.2% per year over the 5 year follow-up period (p = 0.004). However, it is important to remember that the risk of stroke or death associated with the combination of pre-operative angiography and surgery itself was very low at 2.7% in this study.

The results of ACAS are consistent with the findings of a recent metaanalysis of five completed trials of carotid endarterectomy compared with medical treatment alone for asymptomatic 50 to 99% carotid stenosis (Benavente et al, 1998). In comparison with medical treatment, endarterectomy significantly reduced the absolute risk of ipsilateral stroke or perioperative stroke or death by 2% over 3.1 years (0.65% per year).

Therefore, the benefits of carotid endarterectomy in patients with asymptomatic NASCET 50 to 99% carotid stenosis are marginal, and up to 50 carotid endarterectomies would need to be performed to prevent one stroke over a three year period (Warlow, 1998). Further studies are required to identify patients with asymptomatic carotid stenosis who are most likely to benefit from surgery i.e. those who are at high risk of stroke with
medical treatment alone. The results of the ongoing Asymptomatic Carotid Surgery Trial should be available in the near future, and will provide more evidence to guide clinical practice in this patient population.

1.3 Review of the literature regarding the treatment of symptomatic severe carotid stenosis

1.3.1 Introduction

Five randomised controlled trials comparing the outcome after treatment with carotid endarterectomy with non-surgical treatment of symptomatic carotid stenosis have been published (Rothwell, 2000). The first two of these studies (Fields et al, 1970); (Shaw et al, 1984) included a small number of subjects, did not produce statistically significant results, and are probably not representative of current surgical practice (Rothwell, 2000). The larger Veterans Affairs study (Mayberg et al, 1991) was prematurely discontinued after publication of the interim results of two larger trials that convincingly demonstrated that carotid endarterectomy is beneficial in preventing recurrent stroke in patients with recently symptomatic severe carotid stenosis (European Carotid Surgery Trialists' Collaborative Group, 1991); (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991). Three published randomised clinical trials have also compared the outcome of treatment with carotid endarterectomy with carotid endovascular treatment in patients with symptomatic carotid stenosis (Naylor et al, 1998); (CAVATAS Investigators, 2001); (Brooks et al, 2001). However, one of these studies, that was carried out in a single-centre in the U.K., was discontinued prematurely after only 17 patients had been treated, because five out of seven patients who underwent endovascular treatment had a peri-procedural stroke (Naylor et al, 1998).
The final results of ECST and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) for patients with symptomatic severe (ECST or NASCET 70 to 100%) carotid stenosis will now be discussed. This will be followed by an overview of the results of a recent reanalysis of data comparing the outcome after surgery with best medical treatment in patients with symptomatic severe ICA stenosis (Rothwell et al, 2003). I will then discuss the main results of the Carotid and Vertebral Artery Transluminal Angioplasty Study (CAVATAS), followed by an overview of the data from the second largest published study comparing carotid endarterectomy with carotid endovascular treatment (Brooks et al, 2001). The outcome after carotid endarterectomy for recently symptomatic 0 to 69% carotid stenosis will not be described in detail, because patients with this degree of stenosis were not specifically studied in this thesis.

1.3.2 Surgery versus control for the treatment of symptomatic severe carotid stenosis

ECST was a multicentre randomised controlled trial in which patients with recently symptomatic carotid stenosis (a history of TIA, amaurosis fugax or non-disabling stroke within the preceding six months) were randomly allocated to undergo carotid surgery (surgery, n = 1811) or to avoid surgery (control, n = 1213) (European Carotid Surgery Trialists' Collaborative Group, 1998). All patients received best medical care irrespective of trial treatment allocation. The mean duration of follow-up was 6.1 years, and approximately 80% of patients were on treatment with aspirin during this time period. Overall, follow-up data were available on 356 surgery and 220 control patients with greater than ECST 80% carotid stenosis. Some patients with carotid occlusion were
randomised in error, but these patients were included in the intention-to-treat analysis. Carotid endarterectomy reduced the absolute risk of major ipsilateral stroke by 13.8% (p < 0.0001), and the absolute risk of major stroke or death at three years by 11.6% (p = 0.001) in patients with ECST 80 to 100% carotid stenosis (European Carotid Surgery Trialists' Collaborative Group, 1998). These figures included a 7% risk of major stroke or death within 30 days of carotid surgery. The authors concluded that the beneficial effects of carotid endarterectomy were seen in patients with recently symptomatic carotid stenosis with a stenosis severity greater than ECST 70 to 79%.

These results are consistent with the interim and final results of NASCET (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991) (Barnett et al, 1998). NASCET was also a multicentre trial in which patients with a history of TIA, amaurosis fugax or non-disabling stroke within the preceding four months in association with NASCET 30 to 99% ipsilateral carotid stenosis were randomly allocated to receive either carotid endarterectomy or best medical care alone. As in ECST, patients randomised to undergo surgery also received best medical care. 98% of surgical patients and 94% of medically treated patients were on aspirin; 1300 mg of aspirin daily was prescribed unless adverse effects necessitated reduction of the dose (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991). In the subgroup of patients with NASCET 70 to 99% carotid stenosis, 328 were randomised to receive surgery and 331 to receive best medical care alone (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991). The rate of perioperative stroke or death was 5.8% in this subgroup of patients. Over a two year period of follow-up, carotid endarterectomy reduced the absolute risk of ipsilateral stroke (fatal or non-fatal) by 17%
(p < 0.001), and the risk of any major stroke or death by 10.1% (p< 0.01) compared with medical treatment alone. The beneficial effect of surgery was durable, with a lower risk of any ipsilateral stroke (p < 0.001) or disabling ipsilateral stroke (p = 0.004) for up to 8 years after treatment in patients treated with surgery (Barnett et al, 1998).

If one looks at the results of ECST and NASCET together, carotid endarterectomy is beneficial in patients with recent symptoms associated with $\text{ECST}^{70}$ to 99% or $\text{NASCET}^{50}$ to 99% carotid stenosis (Rothwell, 2000), although the benefit is greatest in patients with more than $\text{ECST}^{80}$% or $\text{NASCET}^{70}$% stenosis. Further collaborative risk-modelling studies are in progress to identify patients with symptomatic severe carotid stenosis who are likely to derive most benefit from carotid endarterectomy (Rothwell, 2000).

Rothwell et al. recently published the results of an analysis of the pooled data from ECST, NASCET, and the Veterans Affairs Trial (VA309), that compared the outcomes after carotid endarterectomy (CEA) with best medical treatment in patients with recently symptomatic carotid stenosis (Rothwell et al, 2003). These results confirm the benefit of surgery in patients with recent symptoms in association with $\geq 70$ to 99% ICA stenosis. One observer remeasured the severity of carotid stenosis in all of the ECST and NASCET angiograms using the NASCET method of grading ICA stenosis. The outcome events were reclassified to allow pooling of outcome events from the three trials, and the analysis was performed on an intention-to-treat basis. Patients with near-occlusion of the ICA in ECST and NASCET were also reclassified, where possible, to perform a separate analysis of the outcome after treatment in this subgroup of patients. ICA near-occlusion was defined as severe ICA stenosis with reduced flow in the distal ICA beyond the stenosis, and post-stenotic narrowing of the ICA (Rothwell et al, 2003).
In patients with stenosis without near-occlusion, 589 patients were randomised to undergo CEA and 506 to receive best medical treatment. Carotid endarterectomy reduced the absolute risk of ipsilateral ischaemic stroke and any operative stroke or operative death by 16% compared with best medical treatment alone (relative risk reduction 61%, p < 0.00001) (Rothwell et al, 2003). The benefit from surgery was apparent in the first year, maximal at three years, but still evident at 8 years follow-up.

In NASCET and ECST, 148 patients with ICA near occlusion were randomised to undergo CEA, and 114 to receive best medical treatment (Rothwell et al, 2003). Although there was a trend towards benefit with CEA in the first 2 years after surgery (absolute reduction in risk of ipsilateral ischaemic stroke of 5.6%, p = 0.19), this trend did not persist during long-term follow up. Overall, the absolute risk of ipsilateral ischaemic stroke and any operative stroke or operative death was increased by 1.7% at 5 years with CEA. However, these data must be interpreted with caution because of the limited number of patients studied and the small number of outcome events. In addition, because 29% of patients randomised to receive best medical treatment actually underwent CEA, this may have led to an underestimation of the benefit of CEA in this subgroup of patients. The authors concluded that the benefit from surgery in patients with near-occlusion is likely to be small in the short term, and unknown in the long term.

Of interest, the pooled analysis showed that CEA increased the risk of all main outcomes for the first 2 years of follow-up in patients with NASCET 50 to 69% ICA stenosis, but was of some benefit in the long-term secondary prevention of ipsilateral ischaemic stroke. CEA reduced the absolute risk of ipsilateral ischaemic stroke and any operative
stroke or operative death at 5 years by 4.6% (relative risk reduction 25%, \( p = 0.04 \)). CEA was not beneficial in patients with < 50% ICA stenosis (Rothwell et al, 2003).

1.3.3 Endovascular compared with surgical treatment of symptomatic severe carotid stenosis

**The Carotid and Vertebral Artery Transluminal Angioplasty Study**

The Carotid and Vertebral Artery Transluminal Angioplasty Study (CAVATAS) was the first large multi-centre randomised controlled trial to compare the risks and benefits of percutaneous endovascular treatment with carotid surgery for the treatment of atherosclerotic carotid artery stenosis in patients suitable for surgery (CAVATAS Investigators, 2001). 504 patients were randomly allocated at 22 centres to undergo percutaneous endovascular treatment (\( n = 251 \)) or carotid surgery (\( n = 253 \)). The mean angiographic ICA stenosis before treatment was \(^{\text{ECST}}86.4\%\) (\(^{\text{NASCET}}77.3\%\)) and \(^{\text{ECST}}85.1\%\) (\(^{\text{NASCET}}75.2\%\)) in the endovascular and surgery groups, respectively (CAVATAS Investigators, 2001). Ninety-seven per cent of patients had symptoms that were attributable to the randomised stenosed carotid artery in the year prior to treatment, and the two treatment groups were well matched for vascular risk factors. Patients randomised to undergo endovascular treatment before 1994 were treated by percutaneous transluminal angioplasty using balloon catheters. Stents suitable for use in the carotid artery were developed during the course of the study, and stenting was allowed from 1994 onwards at the discretion of the treating radiologist. Stenting could be performed either as a primary procedure in which the lesion was stented without attempting complete balloon dilation first, or as a secondary procedure after unsatisfactory initial
balloon dilation. The type and manufacturer of the stents, guide wires, and catheters were not specified by the trial protocol and were left to the preference of the individual radiologists. Stenting was performed in 22% of those allocated endovascular treatment (26% of procedures); Wallstents were used in 53%, Palmaz stents in 34% and Strecker stents in 13% of those who were stented. The protocol specified that endovascular patients should be treated with aspirin (minimum dose 150 mg daily) or an alternative antiplatelet agent for at least 24 hours before the procedure, and anticoagulated with systemic heparin at the time of the procedure and for at least 24 hours afterwards, unless contraindicated. Carotid endarterectomy was performed by the technique routinely used by the collaborating surgeon. No requirements were specified about the use of anaesthesia, shunts or patches, or the use of perioperative heparin; peri-operative shunts or patches were used in 64% and 63% of surgical procedures, respectively. Antiplatelet therapy was continued throughout the period of follow up in both groups, and all patients received best medical care and treatment of vascular risk factors. The mean duration of clinical follow-up was approximately two years in both treatment groups. Participants were encouraged to perform Doppler ultrasound examination of the carotid arteries prior to randomisation, and at one month and one year after treatment at centres where reliable ultrasound facilities were available.

The initial results of the trial showed a comparable risk of disabling stroke or death at 30 days after the procedure in patients allocated endovascular treatment (6.4%) or surgery (5.9%) (p = 0.8). The 30-day rate of death or any stroke (lasting more than 7 days) was 10% in patients allocated endovascular treatment and 9.9% in patients allocated surgery (p=0.98). All but one of the strokes in the endovascular group, and all
of the strokes in the surgery group were ipsilateral to the treated carotid artery. In addition, there was no significant difference in the rate of death or disabling stroke, or in the rate of any ipsilateral stroke between the two treatment groups for up to three years after randomisation. Analysis of the follow-up Doppler ultrasound data showed that the overall degree of carotid stenosis was significantly higher in endovascular compared with surgery patients at both one month (p = 0.0007) and one year (p < 0.0001) after treatment (McCabe, D.J.H. et al., in preparation). At one year, 32/173 (18.5%) endovascular patients compared with 9/174 (5.2%) surgery patients had severe carotid stenosis or occlusion (p = 0.0001).

The results of a small single-centre study comparing the outcome after carotid endarterectomy with carotid angioplasty and stenting have been published recently (Brooks et al, 2001). Patients with a recent TIA or stroke in the arterial territory supplied by a > 70% ICA stenosis within the preceding 3 months were randomly allocated to undergo CEA (n = 51) or carotid stenting (n = 53). It is important to note that the majority of patients in each treatment arm had TIAs or amaurosis fugax, and only 16% of CEA patients and 17% of stenting patients had a non-disabling stroke prior to study entry. The average pre-treatment ICA stenosis was 88.2% in the CEA group and 82.4% in the endovascular group. All patients received 325 mg of aspirin and 75 mg of clopidogrel before treatment, and treatment was carried out within six weeks of the presenting symptom. Patients were followed up for two years after treatment. There were no stroke outcome events in either treatment arm during follow-up. One CEA patient had a fatal MI in the immediate post-operative period, and another patient had a wound haematoma requiring re-exploration. One stenting patient had a peri-procedural TIA, one
had a popliteal artery thrombosis requiring below-knee amputation, and three patients had
a retroperitoneal haematoma after treatment with heparin and the GpIIb/IIIa antagonist,
abciximab. The 24 month patency of the treated vessel on follow-up Doppler ultrasound
was reported to be “satisfactory” in each group, and the mean ICA/CCA ratio of < 2 in
each group suggested a mean ICA stenosis of < 70% at this stage after CEA or stenting
(Sidhu & Allan, 1997). However, further Doppler ultrasound velocity criteria, e.g. the
peak systolic velocities in the ICA, were not available to confirm or refute this
conclusion. The authors suggested that stenting is equivalent to CEA for the treatment of
symptomatic severe carotid stenosis. However, the number of patients screened for
inclusion in this study, and the reasons why some were excluded were not specified.
Furthermore, the sample size was small, the methods used for patient randomisation was
not clearly described in the paper, and the prespecified primary or secondary endpoints
were not stated.

To address the limitations of these studies, a number of larger ongoing studies,
including the International Carotid Stenting Study, are comparing the outcome after
carotid endarterectomy with primary carotid stenting in patients with recently
symptomatic severe carotid stenosis.

1.4 Platelets

1.4.1 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, Jr., 2001).
He called them the ‘globulins of chyle’. However, others have doubted 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 eighth the size of erythrocytes, that were occasionally clumped together in masses of up to 100 individual components (Owen, Jr., 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, Jr., 2001). In 1881, Professor Bizzozero of Turin 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’.

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. In the following section, I will summarise our understanding of the mechanisms involved in platelet production, and outline the basic platelet structure and the events involved in platelet activation.

1.4.2 Platelet production and lifespan

Platelets are anucleate cells that are formed by fragmentation of megakaryocyte cytoplasm (Lunetta & Penttilä, 1997); (George, 2000). There are a number of theories regarding the primary mechanism and site of platelet formation (Lunetta & Penttilä, 1997). It has been proposed that megakaryocytes are completely partitioned into ‘platelet...
territories' within the bone marrow, and that platelets are released directly into the bone marrow sinusoidal spaces, and hence into the venous circulation (Lunetta & Penttilä, 1997). It has also been suggested that megakaryocytes project pseudopodia into the marrow sinusoidal spaces, and that platelets are released into the venous blood by budding from these pseudopodia (Lunetta & Penttilä, 1997). In addition, there is some evidence that platelets are formed by further fragmentation of these megakaryocytic cytoplasmic fragments in the pulmonary microcirculation (Lunetta & Penttilä, 1997); (George, 2000).

The normal platelet count in adult blood is between 150 to 400 x 10^9/L, and this is far in excess of what is essential for haemostasis. The platelet count is regulated by humoral mechanisms in which platelet production varies depending on the number or volume of circulating platelets to maintain a constant platelet mass (the product of the platelet count and MPV) (O'Malley et al, 1995). Thrombopoietin is the predominant hormone controlling megakaryocyte development and platelet formation, although other hormones and growth factors, including interleukins 3, 6 and 11, also have some thrombopoietic activity (George, 2000). The ability of the bone marrow to respond with increased thrombopoiesis is limited to a maximal level of 3 to 5 times basal production (Ault et al, 1992), and in situations where this capacity is exceeded, the platelet count will fall.

The normal lifespan of the platelet varies between 8 and 11 days (Ault & Knowles, 1995); (Butterworth & Bath, 1998). A small fraction of platelets is constantly removed from the circulation during the process of haemostasis and maintenance of vascular integrity (George, 2000). It stands to reason that some platelets will also be
removed from the circulation in disease states involving thrombosis, e.g. ischaemic stroke or TIA. In addition, the spleen continually sequesters approximately a third of circulating platelets, and in patients with splenomegaly, the fraction of platelets retained in splenic sinusoids increases without affecting overall platelet survival time (George, 2000). Senescent platelets are removed from the circulating blood by the spleen, platelets expressing phosphatidylserine undergo apoptosis, and some platelets are engulfed and destroyed by monocytes and macrophages of the reticular endothelial system (Snyder & Rinder, 2003).

1.4.3 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 1.4.1) (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). The outer layer of the membrane bilayer is rich in sphingomyelin 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 (see below). 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 GpIa/IIa, stabilises the phospholipid bilayer, regulates the shape of the plasma membrane (Blockmans et al, 1995), and lines the channels of the surface connected open canalicular system (OCS) (Shapiro, 1999). 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) (see below).

(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). 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). They are discussed in greater detail in section 2.3.6 and chapter 6.
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 and contain platelet specific proteins (platelet factor 4, β-thromboglobulin [βTG]), adhesive glycoproteins (fibrinogen, von Willebrand factor [VWF], thrombospondin, fibronectin, and vitronectin), coagulation factors (factor V, factor XI, high molecular weight kininogen, and plasminogen activator inhibitor-1), and mitogenic factors (platelet derived growth factor, epidermal growth factor, transforming growth factor-β) (Ware & Coller, 1995). They also contain the granule membrane protein CD62P (P-selectin, PADGEM), that is expressed on the surface of activated platelets (Blockmans et al, 1995); (Shapiro, 1999); (Frijns et al, 1997). Furthermore, small amounts of plasma proteins are taken up into α-granules by either endocytosis or pinocytosis.

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 is sequestered, and where the enzymes involved in PG synthesis are localised (Blockmans *et al.*, 1995); (Shapiro, 1999). Platelets may secrete thromboxane A₂ and platelet activating factor (in response to stronger stimuli) from the dense tubular system during the activation process.
Figure 1.4.1 Diagrammatic representation of the ultrastructure of a platelet in cross section (adapted from Figure 1 in Monograph by (Shapiro, 1999))
1.4.4 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 (Blockmans et al, 1995). Binding of agonists to these receptors may generate a molecular signal within the platelet in a process referred to as ‘outside-in’ signalling (Fitzgerald, 2001). 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 (Du & Ginsberg, 1997). Before dealing with these platelet receptors, the signalling mechanisms involved in receptor dependent platelet activation and inhibition will be described.

**Guanine nucleotide binding regulatory (G) Proteins**

Many platelet receptors are coupled to the second messenger generating enzymes, phospholipase C, phospholipase A₂, and adenylate cyclase via G proteins (Blockmans et al, 1995). G proteins are composed of 3 subunits: α, β, and γ. The α 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 A₂ (see below) (Blockmans et al, 1995).

**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 (IP₃), diacylglycerol (DAG), TxA₂, 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). As outlined below (section 1.4.6), 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 (see below).

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$_2$ (section 1.4.6 and Figure 1.6.1). 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 exit 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 Gs regulatory proteins. In contrast, thromboxane, ADP, epinephrine, and thrombin stimulate Gi 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).

As outlined in more detail in section 1.6.8 (see below), soluble guanylate cyclase catalyses the conversion of guanosine triphosphate (GTP) to guanosine 3', 5'-cyclic monophosphate (cGMP) (Figure 1.6.3). 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 (Blockmans et al, 1995). Both cAMP and cGMP are broken down by phosphodiesterases (see section 1.6.8 and Figure 1.6.3).
1.4.5 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 GpIa/IIa 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 (Berndt et al, 2001). Each receptor contains two GpIbα, two GpIbβ and two GpIX subunits, but only one GpV subunit (Andrews et al, 1997). 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 (Escolar & White, 2000) per platelet, and approximately half as many copies of GpV (Andrews et al, 1997). 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 at high shear rates (Ruggeri, 1997); (Clemetson, 1997); (Escolar & White, 2000) (see below). GpIbα also possesses binding sites for thrombin, Mac-1, CD62P, high molecular weight kininogen and Factor XII (Berndt et al, 2001). The cytoplasmic tail of GpIbα contains a binding site for actin-binding proteins, 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); (Berndt et al, 2001). In addition to these functions of the receptor subunits, GpIbα, GpIbβ and GpIX are all required for stable surface expression of the receptor complex (Andrews et al, 1997). Until 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 Gplbα 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 Gplb-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). These signalling events ultimately result in 'inside-out' activation of the GpIIb/IIIa receptor and platelet aggregation (see below) (Blockmans et al, 1995); (Berndt et al, 2001).

GpIIb/IIIa (αIIbβ3 integrin) is the predominant platelet receptor, with up to 80,000 copies on each platelet surface (Wagner et al, 1996). Some GpIIb/IIIa receptors are also located in the OCS and in platelet α-granules (Calvete, 1995); (Litjens et al, 2000), but can be translocated to the platelet membrane surface on platelet activation (Blockmans et al, 1995). 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 (Du & Ginsberg, 1997). The association between the GpIIb and GpIIIa subunits depends on a submicromolar concentration of extracellular calcium (Calvete, 1995). 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 (Abrams & Shattil,
1991); (Moran & FitzGerald, 1994); (Du & Ginsberg, 1997). Thus, the receptor complex can bind several different ligands including fibrinogen, VWF, fibronectin, vitronectin and thrombospondin (Du & Ginsberg, 1997). 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 (Moran & FitzGerald, 1994). 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 (Litjens et al, 2000). 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 (Moran & FitzGerald, 1994). 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 & Leavitt, 2001). However, the second messenger systems mediating the effects of ligand binding to GpIIb/IIIa are not fully understood (Blockmans et al, 1995). 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 (Shattil & Leavitt, 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 (Clemetson & Clemetson, 2001). There are between 1000 and 7000 copies of
GpIa/IIa (Blockmans et al, 1995), 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 (Chen et al, 2002), but may predominantly be involved in mediating intraplatelet signalling responses to collagen, with subsequent activation and facilitation of collagen binding to GpIa/IIa (Clemetson & Clemetson, 2001). Under conditions of high shear stress, neither GpIa/IIa nor GpVI can mediate platelet adhesion to collagen. However, VWF +/- platelet surface GpIb undergo a conformational change in response to high shear stress, facilitating platelet GpIb-VWF binding. Subsequently, the GpIb-VWF complex may bind to subendothelial collagen via a receptor for collagen on VWF (see later). 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 (Clemetson & Clemetson, 2001) (Table 1.4.1). 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 (α5β1), and laminin to GpIc'/IIa (α6β1) (Clemetson & Clemetson, 2001).
<table>
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<tr>
<th>Receptor</th>
<th>Agonist (s)</th>
<th>Major Function (s) in Platelets</th>
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<tr>
<td>GpIb-IX-V</td>
<td>VWF, Thrombin, CD62P, MAC-1</td>
<td>Adhesion, Aggregation</td>
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<tr>
<td>GpIIb/IIIa $(\alpha_\text{IIb}\beta_3)$</td>
<td>Fibrinogen (VWF, Fibronectin, Vitronectin)</td>
<td>Aggregation, Adhesion</td>
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<td>$(\alpha_\text{V}\beta_3)$</td>
<td>Vitronectin, Thrombospondin</td>
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<td>GpIa/IIa $(\alpha_2\beta_1)$</td>
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<td>GpIV (GpIIIb, CD36)</td>
<td>Thrombospondin, Collagen</td>
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<td>GpVI</td>
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<td>Adhesion</td>
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<td>GpIc'/IIa $(\alpha_4\beta_1)$</td>
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<td>CD31 (PECAM-1)</td>
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1.4.6 Excitatory ‘non-glycoprotein’ platelet receptors

The main ‘non-glycoprotein’ platelet receptor agonists that were studied 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.

**Thromboxane A₂ receptor(s)**

Thromboxane A₂ (TXA₂) 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. As outlined in Figure 1.6.1 (see below), arachidonic acid is metabolised to form thromboxane A₂ (TXA₂), which has a short half-life of less than 1 minute before being rapidly metabolised to form inactive thromboxane B₂. TxA₂ 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 PIP₂, and elevation of intraplatelet free calcium (Blockmans *et al*, 1995); (Shapiro, 1999).

**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 (Gachet, 2001). These P2 receptors are divided into two main groups: the G protein-coupled P2Y receptors, and the ligand-gated ion channel P2X receptors. Three separate ADP P2 platelet receptors have been cloned (Daniel et al, 1998), (Jin et al, 1998), (Hollopeter et al, 2001). These receptors are termed P2Y1 (Gq- coupled), P2Y12 (Gi-coupled) and P2X1, and they mediate different aspects of the response to this agonist (Gachet, 2001).

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 (Gachet, 2001). When ADP binds to P2Y1, calcium is mobilised from intraplatelet stores, thus triggering platelet shape change and pseudopod formation (Jin et al, 1998) (see below), 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 (Gachet, 2001). The thienopyridines exert their antiplatelet effects by selective and irreversible inhibition of the P2Y12 ADP receptor (see section 1.6.9). The P2X1 receptor has been reported to mediate ADP-induced rapid calcium influx into the platelet. It may synergise the calcium influx mediated by the P2Y1 receptor, especially under conditions where cAMP levels are elevated. However, the exact role of this receptor in the platelet activation process is not well understood (Gachet, 2001), and some authors have suggested that ATP, and not ADP, is the agonist at P2X1 (Mahaut-Smith et al, 2000).
**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 (chapter 5). Platelets have stimulatory $\alpha$-2 adrenoceptors and inhibitory $\beta$-adrenoceptors, and in most patients, the $\alpha$-2 adrenoceptors predominate (Blockmans et al, 1995). There are approximately 200 to 300 copies of the $\alpha$-2 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 (Blockmans et al, 1995). It is also likely that epinephrine induces platelet aggregation using TxA$_2$ 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 (Helgason et al, 1993). Epinephrine may also sensitize 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 (Blockmans et al, 1995).

**Thrombin receptors**

Thrombin is the main protease involved in the coagulation cascade, and is arguably the most effective platelet agonist *ex vivo* (Coughlin, 2001). However, the relative importance of thrombin-induced platelet activation *in vivo* is unknown. Laboratory studies have shown that thrombin induces shape change, secretion of the contents of platelet granules, synthesis and release of TxA$_2$, mobilisation of CD62P to the platelet surface, and activation of GpIIb/IIIa (Coughlin, 2001). It also induces the expression of
procoagulant factors on platelets, which stimulates further thrombin generation (Coughlin, 2001). Moreover, it has effects on endothelial cells, vascular smooth muscle cells and other blood components.

Thrombin signalling is mediated, at least in part, by binding to protease activated receptors (PARs), and human platelets express PAR1 and PAR4 (Coughlin, 2001). When thrombin binds to PAR1 on platelets, it cleaves part of the receptor and unmasks a new amino terminal domain, which in turn binds to the body of the receptor itself and activates it (Coughlin, 2001). Therefore, PAR1 is a peptide receptor that utilises an intriguing mechanism to activate itself after agonist binding. 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 (Coughlin, 2001). As mentioned above, thrombin also binds to the GpIb-IX-V complex, and it has been postulated that thrombin binding to GpIbα may facilitate cleavage of PARs on the platelet surface (Coughlin, 2001). 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).

**Platelet activating factor (PAF) receptor**

PAF is formed in certain cells, including platelets, by the consecutive actions of phospholipase A2 and acyltransferase on a platelet membrane phospholipid (Blockmans et al, 1995). PAF causes platelet shape change and aggregation, but because it is dependent on TxA2 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).

**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).

**Vasopressin receptor**

Platelets possess vasopressin (V$_1$) receptors that mediate platelet aggregation via phosphatidylinositol metabolism and an increase in the intraplatelet calcium concentration (Blockmans et al, 1995).

### 1.4.7 Inhibitory ‘non-glycoprotein’ platelet receptors

As described in detail in section 1.6.8, dipyridamole mediates some of its actions via the adenosine receptor, and because several patients who were studied in this thesis were on treatment with this agent, the adenosine receptor will be described in the following section. In addition, because most patients were on treatment with aspirin, receptors for PGs that inhibit platelet function are also referred to briefly.

**Adenosine receptor**

Adenosine inhibits platelet shape change, aggregation and secretion by its action on the G protein-linked adenosine A$_2$ 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).

**PGI₂ and PGE₁ receptor**

Binding of PGI₂ and perhaps PGE₁ 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).

**PGD₂ receptor**

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

**PGE₂ receptor**

It has been proposed that PGE₂ may inhibit platelet activation either by binding to its own receptor or the PGI₂ receptor (Blockmans et al, 1995). However, depending on the experimental conditions, PGE₂ may also potentiate platelet activation by activating protein kinase C or inhibiting cAMP formation (Blockmans et al, 1995).

**1.4.8 Platelet function**

Platelets ‘survey’ the endothelial lining of normal blood vessels, and may ‘roll’ on the endothelium, without adhering to it under resting conditions (Ruggeri, 1997). Factors released from the endothelium, e.g. PGI₂, nitric oxide, and adenosine may inhibit platelet adhesiveness as outlined above in section 1.4.4 and 1.4.7. However, when the continuity of the vessel lining is interrupted by an injury, subendothelial components are exposed,
blood begins to leak outside the vessel, and the process of arresting this bleeding (haemostasis) ensues (Ruggeri, 2000). Platelets are responsible for this initial repair mechanism, referred to as ‘primary haemostasis’, with subsequent activation of the coagulation system, and the formation of insoluble fibrin in the phase of ‘secondary haemostasis’ (Shapiro, 1999). However, neither platelets nor other components of the haemostatic process, can distinguish between traumatic wounds and other lesions within the vessel wall (Ruggeri, 2000). Therefore, if an atherosclerotic plaque ruptures, subendothelial components may also be exposed, and the usual beneficial function that limits blood loss after an injury, may promote the development of a potentially life-threatening thrombus (Ruggeri, 2000). Therefore, platelets play a pivotal role in both haemostasis and thrombosis. To engage in this process, platelets need to be activated, predominantly via receptor-mediated mechanisms as outlined above. The process of platelet activation includes adhesion, shape change, aggregation, and secretion of granule contents, with expression of procoagulant factors on the platelet surface (Moran & FitzGerald, 1994). These phases of platelet activation will now be described, but it is important to remember that some or all of these phases may be involved, and that the sequence of events described below may vary depending on the stimulus to platelet activation in vivo. In addition, the receptors and ligands involved in the initial phases of platelet activation vary depending on the shearing forces at play within the vessel.

**Platelet adhesion**

Endothelial cells may be activated by various cytokines, with the expression of selectin molecules, including P-selectin (McEver et al, 1989); (Bonfanti et al, 1989); (Fijnheer et al, 1997) (see section 2.6.3). In addition, endothelial cells may be denuded with the
exposure of subendothelial components; the two most important subendothelial components are collagen and VWF (Fitzgerald, 2001). Platelets are normally pushed towards the periphery of the flowing blood stream by red blood cells, thus facilitating interaction of platelets with vascular endothelium. Under conditions of low shear stress (< 1000 s⁻¹) that may be seen in veins, large arterioles, and normal arteries (Kroll et al, 1996), platelet Gplα/IIa and probably GPVI receptors bind to subendothelial collagen in a damaged or diseased vessel, and facilitate the adhesion of platelets to the thrombogenic surface (Berndt et al, 2001). In this situation, platelet adhesion is largely independent of the interaction between platelet Gplb-IX-V and VWF (Berndt et al, 2001). When shear stress levels rise e.g. in smaller arterioles and stenosed arteries (up to 10,000 s⁻¹), initial platelet adhesion to the subendothelium is mediated by the interaction of platelet Gplbα subunit with subendothelial VWF (Berndt et al, 2001). This initial adhesion process is also facilitated by platelet rolling on the endothelium, and mediated by the interaction of endothelial surface P-selectin with platelet Gplb-IX-V or platelet P-selectin glycoprotein ligand-1 (PSGL-1) (Berndt et al, 2001). In addition, as mentioned above, circulating platelet Gplb-VWF complexes may also bind to subendothelial collagen via collagen receptors on VWF, thus facilitating further platelet adhesion to the subendothelium (section 1.4.5). This adhesion process, in turn, triggers signalling processes that promote further platelet activation, including activation of the GpIIb/IIIa complex with an increase in its affinity for binding ligands (see below). Activation of platelet GpIIb/IIIa may then enable GpIIb/IIIa binding to the RGD sequence in subendothelial VWF, with firm platelet adhesion to the subendothelium (Ruggeri, 1997).
Platelet shape change

After platelet adhesion, platelet microtubular bundles depolymerise and actin polymerisation begins (Blockmans et al, 1995). Because the GpIb-IX-V and GpIa/IIa receptors are connected to the submembrane network of actin filaments in resting platelets, shape change begins early on in the platelet activation process (Blockmans et al, 1995). After platelets begin to aggregate (see below), the GpIIb/IIIa receptor also associates with cytoplasmic actin filaments, thus mediating further shape change (Blockmans et al, 1995). These processes involve complex biochemical processes including PIP$_2$ metabolism, DAG formation, and require the presence of certain membrane phospholipids, and may be calcium dependent or independent depending on the agonists involved (Blockmans et al, 1995). These processes are also referred to in the section on platelet secretion below. Overall, platelets change from a discoid to a more spherical shape, with centralisation of their organelles and granules by a tight fitting ring of reassembled microtubules, and they spread out with extension of spiny pseudopodia (Blockmans et al, 1995).

Platelet aggregation

Binding of two adjacent platelets to one another is referred to as platelet aggregation, and as mentioned earlier, adhesion and aggregation can occur independently of shape change (Shapiro, 1999). After initial platelet adhesion to the endothelium or subendothelial components, circulating platelets may form aggregates with adherent platelets. However, only a small percentage of adherent platelets form irreversible and stationary aggregates with circulating platelets, and this percentage falls with increasing levels of shear stress in vitro (Kulkarni et al, 2000). Under low shear stress conditions, the main receptor
involved in initial platelet-platelet aggregation is GpIIb/IIIa (Berndt et al, 2001), and the main ligand for this receptor is fibrinogen, although fibronectin may also be important (Blockmans et al, 1995). However, recent evidence suggests that VWF binding to GpIbα also plays a very important role in platelet aggregation at low shear rates (Kulkarni et al, 2000). At higher shear rates, initial platelet aggregation is largely dependent on plasma VWF crosslinking two adjacent platelets by binding to the GpIIb/IIIa receptor on each platelet (Kulkarni et al, 2000). In addition, VWF expressed on the surface of adherent platelets 'tethers' more circulating platelets by binding to GpIbα, although many of these tethered platelets subsequently detach (Kulkarni et al, 2000). Signalling mechanisms within the platelet then lead to further activation of the GpIIb/IIIa receptor, release of granule contents, and stable platelet aggregate formation by subsequent binding of VWF and fibrinogen to GpIIb/IIIa on adherent and circulating platelets (Kulkarni et al, 2000). Furthermore, thrombospondin, released from platelet α-granules, may stabilise platelet aggregates by binding to GpIV (Blockmans et al, 1995). As the thrombus forms and the vessel lumen narrows, the shear stresses to which adherent and circulating platelets are exposed changes, and this may further activate platelets (Kroll et al, 1996). Therefore, if large unstable platelet aggregates (thrombi) form over the surface of a ruptured carotid atherosclerotic plaque, one can clearly see how distal thromboembolism could lead to either transient or permanent ischaemia in the region supplied by that artery.

Platelet secretion

During the process of receptor-dependent platelet activation, secretion of granule contents (the release reaction) may occur (Shapiro, 1999). To enable secretion, platelet granule membranes fuse with the platelet membrane or with the membranes of the OCS,
with diffusion of the internal granular membrane proteins into the plasma membrane (Blockmans et al, 1995). During this process, myosin phosphorylation occurs via pathways that are dependent on DAG, IP$_3$, and calcium (Blockmans et al, 1995). Phosphorylated myosin filaments interact with actin filaments, they slide past one another, surround the granules and generate a contractile force that facilitates movement of the granules (Blockmans et al, 1995). This interaction between actin and myosin is inhibited by tropomyosin in the pseudopodia of activated platelets, and dephosphorylation of myosin may occur, during which time platelets regain their discoid shape (Blockmans et al, 1995). After granular fusion with the platelet membrane, the proteins will either be translocated to and expressed on the platelet surface membrane (Blockmans et al, 1995), or shed directly into the circulating blood. It is important to note that dense granules release their contents in response to weak stimuli, α-granule secretion requires higher agonist concentrations, and lysosomal granule secretion only occurs in response to strong stimuli (Blockmans et al, 1995). The intensity of the platelet aggregation response is also influenced by the contents secreted by the platelet. Weak aggregating agents, or lower concentrations of strong agonists, tend to cause reversible ('primary') aggregation in vitro, whereas stronger agonists cause irreversible ('secondary') aggregation in association with TxA$_2$ formation and secretion of platelet granule contents. TxA$_2$ and ADP are the main substances responsible for recruitment of other platelets into the forming platelet plug. Under conditions of high shear stress, ADP released from platelet dense granules plays an important role in enhancing irreversible aggregation induced by other agonists (Blockmans et al, 1995). The granular proteins
studied in this thesis were CD62P, CD63 and VWF, and their function is discussed in greater detail in section 2.3.3 and chapter 3.

**Platelet procoagulant activity**

The complex interactions between platelets and the coagulation cascade were not investigated in this thesis, and therefore, a very brief introduction to this interaction is summarised below. When a large vessel is injured or damaged e.g. by rupture of an atherosclerotic plaque, reinforcement and stabilisation of the platelet plug by secondary haemostasis and fibrin formation is required. As outlined in section 1.4.3, the inner layer of the platelet membrane contains negatively charged phospholipids, and following platelet activation, ‘flip-flop’ exposure of these procoagulant negatively charged membrane phospholipids (Shapiro, 1999), especially PS and PTE occurs (Sims & Wiedmer, 2001). A number of coagulation factors, including factors II and X, bind to these procoagulant factors, facilitating secondary activation of the coagulation cascade (Blockmans et al, 1995). Furthermore, platelet CD62P has been shown to induce the expression of tissue factor on monocytes (Celi et al, 1994), which could, in turn, lead to activation of the intrinsic coagulation pathway. The interaction of coagulation factors on the surface of activated platelets protects them from naturally occurring inhibitors and localises them to the site of vascular injury.

**Leucocyte-platelet interactions**

The concept and importance of the interactions between leucocytes and platelets to form leucocyte-platelet aggregates are discussed in detail in chapters 3 and 4.
1.5 Review of the literature on platelet activation in ischaemic stroke and TIA in humans

Within the past three decades, several studies have suggested that platelets are excessively activated in the acute (Dougherty, Jr. et al, 1977); (Koudstaal et al, 1993); (van Kooten et al, 1994); (Iwamoto et al, 1995); (McConnell et al, 2001) and convalescent phases (Iwamoto et al, 1995); (van Kooten et al, 1999) after cerebral ischaemia. However, these conclusions are predominantly based on the results of platelet aggregometry studies in PRP (Dougherty, Jr. et al, 1977), plasma assays of soluble platelet activation markers, e.g. β-thromboglobulin (Iwamoto et al, 1995), or by measuring the urinary metabolites of thromboxane A2 (Koudstaal et al, 1993); (van Kooten et al, 1994); (van Kooten et al, 1999); (McConnell et al, 2001). Most of these studies have not assessed platelet activation or function in the physiological milieu of whole blood. Platelet aggregometry studies are semiquantitative, and although they may show that platelet ‘reactivity’ is altered in patients with cerebrovascular disease, they cannot determine whether the condition directly activates platelets (Michelson, 1996). Plasma assays of platelet activation markers such as β-thromboglobulin and platelet factor 4 involve sample centrifugation and manipulation, and are subject to artifactual in vitro platelet activation with the potential loss of important platelet subpopulations (Michelson, 1996). In addition, urinary assays of metabolites of thromboxane A2 only provide an indirect measure of platelet activation in these subjects. A better understanding of the cellular mechanisms involved in platelet activation in ischaemic stroke and TIA might facilitate more effective secondary prevention of vascular events in this patient population.
In the first two years after an ischaemic stroke, the risk of stroke recurrence is highest in patients with symptomatic severe carotid stenosis (atherothrombotic stroke), intermediate in patients with cardioembolic stroke, and lowest in patients with lacunar stroke (see section 1.1.4). It is difficult to predict the risk of stroke recurrence in patients with ischaemic stroke of indeterminate aetiology, because this subgroup includes a heterogeneous selection of patients that cannot be classified under any of the above categories. However, these individuals probably have a risk of stroke recurrence that is lower than patients with symptomatic severe carotid stenosis and higher than patients with lacunar stroke e.g. about 10% over 2 years.

This difference in the risk of stroke recurrence between subgroups probably reflects the different underlying pathogenic mechanisms responsible for the first stroke. Shear stress is defined as ‘the force per unit area between laminae’ (Kroll et al, 1996). In flowing viscous blood, each lamina of blood exerts a certain degree of shear stress on its neighbour (liquid shear stress) and the vessel wall also exerts a certain degree of shear stress on the flowing blood (wall shear stress). The ‘shear rate’ (in cm / sec per cm, or s⁻¹) is calculated by dividing shear stress / viscosity (Kroll et al, 1996). Platelets may be activated by high shear stress caused by increased blood velocity and turbulence as blood flows past an arterial stenosis (Kroll et al, 1996). In experimental models, wall shear rates of between 300 to 800 s⁻¹ have been seen in normal arteries, increasing to between 500 and 1600 s⁻¹ in normal arterioles and 800 to 10000 s⁻¹ in stenosed arteries (Kroll et al, 1996). The wall shear rate exerted by the vessel wall on blood platelets is likely to be higher in patients with atherothrombotic stroke in association with severe extracranial carotid stenosis than in patients with cardioembolic stroke or small lacunar infarcts.
Moreover, rupture of an atherosclerotic plaque in the internal carotid artery in patients with atherothrombotic stroke may expose subendothelial components that activate blood platelets in addition to shear-induced platelet activation. However, in larger lacunar infarcts that are believed to be secondary to intracranial atherosclerosis (Fisher, 1982); (Lammie, 2000) (see section 1.1.3), high shear stress and exposure of subendothelial components may play a role in platelet activation. In contrast, activation of the coagulation system may play a more important role than platelet activation in the pathogenesis of cardioembolic ischaemic stroke, because fibrin-rich thrombi are formed in the heart chambers in these patients (Uchiyama et al, 1994); (Yamazaki et al, 2001).

Consistent with these hypotheses, a number of studies have reported platelet hyper-reactivity or excessive platelet activation in atherothrombotic but not in cardioembolic (Uchiyama et al, 1994); (Zeller et al, 1999) or lacunar ischaemic stroke (Uchiyama et al, 1994); (Konstantopoulos et al, 1995).

Uchiyama et al. studied shear-induced platelet aggregation in 75 patients within three weeks of onset of an ischaemic stroke or TIA, and in 26 younger control subjects (Uchiyama et al, 1994). The platelets were activated in a cone-plate chamber using a high shear rate of 10800 s⁻¹, but they were studied in PRP rather than in the physiological milieu of whole blood. Shear-induced platelet aggregation was increased in patients with TIA or atherothrombotic stroke compared with patients with lacunar stroke, cardioembolic stroke or normal controls, suggestive of platelet hyper-reactivity in TIA or atherothrombotic stroke. It was of interest to note that shear-induced platelet aggregation was not inhibited by aspirin (81 mg daily for seven days, n = 5), but it was inhibited by ticlopidine (200 mg daily for seven days, n = 6). The authors also reported that there was
no correlation between the degree of shear-induced platelet aggregation and the plasma levels of VWF, but that aggregation was enhanced in patients with higher plasma levels of large VWF multimers. These multimers play an important role in platelet aggregation at high shear rates and mediate their effects by binding to both Gplb and GpIIb/IIIa on the platelet surface (Uchiyama et al, 1994). The patients were not reassessed after the acute phase.

In 1999, Zeller et al. reported the results of a study using flow cytometry to measure ex-vivo platelet activation in patients with acute ischaemic stroke or TIA (Zeller et al, 1999). Blood samples were obtained within 36 hours of symptom onset from 47 patients with “atherosclerotic” stroke or TIA in association with ≥ 50% stenosis of the relevant feeding artery, and from 25 patients with cardioembolic stroke or TIA. Patients with lacunar stroke were not included in this study. The platelet surface expression of CD62P, CD63, and thrombospondin was measured in fixed washed platelets prepared from PRP, and compared with that seen in 72 younger control subjects without symptoms of vascular disease. All three platelet activation markers were significantly elevated in patients with atherosclerotic stroke or TIA compared with controls, but the differences between the cardioembolic patients and the control subjects did not reach statistical significance. In addition, the percentage expression of CD62P and CD63 was significantly higher in the atherosclerotic patient subgroup than in patients with cardioembolic stroke or TIA. Flow cytometric analysis was not repeated after the acute phase to assess whether these changes were transient or persistent.

Konstantopoulos et al. examined shear-induced platelet aggregation in whole blood in 23 patients within 3 days of onset of an ischaemic stroke who were on treatment
with 325 mg of aspirin daily (Konstantopoulos et al, 1995). A cone and plate viscometer was used to assess shear-induced platelet aggregation in this study, and the platelets were exposed to a more moderate shear rate of 3000 s⁻¹. The assays were repeated in 21 subjects at least four weeks after stroke onset, along with whole blood flow cytometric analysis of platelet CD62P expression and the percentage of circulating neutrophil-platelet aggregates. The results were compared with those obtained from a control group of 26 younger healthy volunteers. Patients were classified as having “atherosclerotic” (n = 15) or lacunar ischaemic stroke (n = 8), although 60% of subjects in the atherosclerotic subgroup had an ischaemic stroke of indeterminate aetiology. The authors reported that shear-induced platelet aggregation was enhanced in the acute and convalescent phases after atherosclerotic ischaemic stroke, but not after lacunar ischaemic stroke. In addition, the percentage expression of CD62P and the percentage of circulating neutrophils that were bound to platelets were elevated in the convalescent phase in the atherosclerotic stroke subgroup but not in the lacunar subgroup. However, the results of this study must be interpreted with caution because of the small sample size and the imprecise classification of the majority of patients in the atherosclerotic stroke subgroup.

In contrast, three further studies have not consistently shown that platelets are excessively activated in atherothrombotic compared with cardioembolic (Grau et al, 1998); (Yamazaki et al, 2001) or lacunar ischaemic stroke (Yamazaki et al, 2001); (Grau et al, 2003).

Grau et al. used whole blood flow cytometry to measure the expression of CD62P and CD63 in 24 patients within 24 hours of onset of an ischaemic stroke or TIA, and in 46 patients at least three months after symptom onset (Grau et al, 1998). In the latter
group, 23 patients were classified as having atherothrombotic stroke, 18 had stroke of cardioembolic origin, and five had stroke of indeterminate aetiology. None of the patients in the acute phase after cerebral ischaemia were on treatment with antiplatelet or anticoagulant therapy, but all patients in the convalescent phase were either on aspirin (n = 25, 100 to 250 mg daily) or anticoagulant therapy (n = 21, INR 2 to 3). The results were compared with those obtained from 35 age- and sex-matched control subjects who did not have vascular disease. The expression of CD62P and CD63 was significantly elevated in the acute and convalescent patient groups compared with control subjects. In the convalescent patient group, there was no significant difference in platelet activation status between patients with atherothrombotic or cardioembolic stroke or TIA, or between patients who were on treatment with either antiplatelet or anticoagulant therapy. Although the number of patients in this study was small, these data suggested ongoing platelet activation despite treatment with antiplatelet or anticoagulant therapy in patients with a history of ischaemic stroke or TIA.

More recently, whole blood flow cytometry was used to examine the platelet surface expression of CD62P and platelet fibrinogen binding in 254 patients at least one month after ischaemic stroke (Yamazaki et al., 2001). Stroke patients were classified as having atherothrombotic, cardioembolic or lacunar infarction and the results were compared with those obtained from 30 younger neurological control subjects without a history of cerebrovascular disease. CD62P expression was significantly increased in all subtypes of ischaemic stroke compared with controls. However, patients with atherothrombotic and cardioembolic stroke had significantly higher levels of CD62P expression than patients with lacunar stroke. In addition, platelet fibrinogen binding was
increased in patients with atherothrombotic and lacunar stroke compared with patients with cardioembolic stroke or controls. These results suggest that the cellular pathways involved in platelet activation depend on the underlying mechanism responsible for the stroke syndrome. In the atherothrombotic subgroup, patients who were on treatment with ticlopidine monotherapy (100 to 200 mg daily) had a significantly reduced expression of CD62P compared with those who were not on treatment, but platelet fibrinogen binding was not significantly reduced with ticlopidine therapy. Aspirin therapy did not significantly affect CD62P expression or platelet fibrinogen binding in either the atherothrombotic or lacunar subgroup.

Grau et al. also reported that the expression of CD62P and CD63 in fixed whole blood was similar in patients in the convalescent phase after atherothrombotic (n = 20) compared with lacunar ischaemic stroke (n = 11) (p > 0.25) (Grau et al., 2003). The expression of these activation markers was not significantly different on treatment with aspirin (100 to 300 mg daily), clopidogrel (75 mg daily), or combination therapy with both drugs. In addition, the results were similar in patients on 300 mg of aspirin daily (n = 26) compared with treatment with < 300 mg daily (n = 5) (p > 0.3). The expression of CD63, but not CD62P, was significantly higher in convalescent stroke patients compared with controls.

Meiklejohn et al. carried out a study to examine the effects of certain platelet polymorphisms on platelet activation in 150 patients with non-cardioembolic ischaemic stroke (Meiklejohn et al., 2001). CD62P expression and platelet fibrinogen binding were elevated in both the acute and convalescent phases after ischaemic stroke or TIA.
compared with controls, but the study was not designed to compare the degree of platelet activation between different stroke subtypes.

Marquardt et al. have recently reported the results of another flow cytometry study that examined the expression of CD62P and CD63 in fixed whole blood at different time points within the first 90 days after an ischaemic stroke (n = 50) (Marquardt et al., 2002). CD62P expression was significantly elevated on day 1, but not on days 14 or 90 after symptom onset compared with healthy controls (n = 30) or risk factor-matched control subjects (n = 20). CD63 expression was significantly higher in patients compared with both control groups on days 1, 14 and 90 after stroke. The cardioembolic stroke subgroup had the highest expression of CD63 on day 1 after symptom onset, and the expression of this activation marker was similar in patients with large artery atherosclerosis, small vessel disease and stroke of indeterminate origin. The authors did advise caution in interpreting these results because of the small numbers of patients in each stroke subgroup. On day 90 after stroke onset, CD62P and CD63 expression was similar in patients on treatment with aspirin, clopidogrel, or anticoagulants. However, the distribution of stroke subtypes in the three antithrombotic treatment subgroups was not specified, and one must keep in mind that the underlying mechanism responsible for the stroke may have influenced the results obtained from these subgroups. The authors concluded that CD63 is a more sensitive marker of platelet activation than CD62P in the subacute phase after ischaemic stroke.

In summary, the available data have yielded conflicting results regarding the role of platelet activation in different subtypes of ischaemic stroke. However, most studies involving whole blood flow cytometric analysis of a small number of platelet activation
markers have suggested that platelets are excessively activated in the acute (Grau et al, 1998); (Zeller et al, 1999); (Meiklejohn et al, 2001); (Marquardt et al, 2002), subacute (Yamazaki et al, 2001); (Marquardt et al, 2002), and convalescent phases (Konstantopoulos et al, 1995); (Grau et al, 1998); (Meiklejohn et al, 2001); (Grau et al, 2003) after ischaemic stroke or TIA.

It is also of interest that the risk of stroke in the distribution of a severely stenosed extracranial carotid artery is much higher in recently symptomatic compared with asymptomatic patients, despite a comparable degree of stenosis in the two groups (26% vs. 4% over two years) (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991); (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995). It has been hypothesised that the plaque becomes 'active' intermittently due to fissuring, ulceration, or rupture of its rigid cap, or perhaps due to haemorrhage within the plaque (Warlow C.P et al, 1996d). Any of these events could increase the thrombogenic potential of the plaque, and lead to a higher risk of subsequent thrombosis in symptomatic compared with asymptomatic carotid stenosis patients. However, because of plaque 'healing', this mechanism is unlikely to explain all of the increase in incidence of subsequent events in symptomatic patients for at least a two year period. Moreover, as the plaque heals, one would expect blood platelets to be exposed to similar levels of shear stress in both symptomatic and asymptomatic patients. It is possible that other mechanisms lead to platelet hyper-reactivity, thus conferring a hypercoagulable tendency and contributing to stroke risk. None of the published studies have compared platelet activation between patients with symptomatic and asymptomatic severe carotid stenosis.
1.6 Antiplatelet therapy in ischaemic stroke, TIA and asymptomatic carotid stenosis

1.6.1 Introduction

Treatment of vascular risk factors (e.g. hypertension and diabetes) and lifestyle changes (e.g. smoking cessation, taking regular exercise) are very important strategies in patients with cerebrovascular disease to reduce the risk of subsequent vascular events. However, because there is some evidence that platelets are excessively activated in patients with ischaemic stroke and TIA (see chapter 3), and because platelets have a pivotal role in haemostasis and thromboembolism, antiplatelet agents have the potential to play a vital role in secondary stroke prevention.

Aspirin is the most commonly prescribed antiplatelet agent for the secondary prevention of vascular events in patients presenting with ischaemic stroke or TIA, but more recently, new approaches to antiplatelet therapy have been explored. In the following section, I will summarise the evidence regarding the outcome of treatment with aspirin and a number of other established and novel antiplatelet agents for the secondary prevention of stroke and other vascular events in patients with ischaemic stroke, TIA, and asymptomatic carotid stenosis.

1.6.2 Aspirin – historical background and mechanism of action

Historical Background

Felix Hoffmann was a chemist with Friedrich Bayer & Co. in Elberfeld, Germany in the late 19th century. In 1897, he succeeded in acetyllating the phenol group of salicylic acid to produce a pure and stable compound called acetylsalicylic acid (Jack, 1997). The analgesic, anti-inflammatory and antipyretic properties of the drug in humans and
animals were soon recognised, and the drug was marketed under the name ‘aspirin’ by Bayer on February 1st, 1899 (Jack, 1997). 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 of aspirin as an antiplatelet agent**

Prostaglandin (PG) H synthase is the key enzyme involved in PG biosynthesis (Roth & Calverley, 1994). The enzyme possesses both cyclooxygenase and hydroperoxidase activities, and two isoforms of PGH synthase exist: PGH synthase-1 and PGH synthase-2 (also known as cyclooxygenase-1 and cyclooxygenase-2, respectively) (Roth & Calverley, 1994); (Weber et al, 1999); (FitzGerald & Patrono, 2001). Cyclooxygenase-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, cyclooxygenase-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 (Schror, 1997); (Weber et al, 1999). It has traditionally been accepted that the only isoform of the enzyme present in platelets is cyclooxygenase-1 (FitzGerald & Patrono, 2001). Although Weber et al. have recently published data using Western blotting techniques suggesting that cyclooxygenase-2 mRNA is also present in platelets (Weber et
al, 1999), this has not been confirmed by others (Patrignani et al, 1999); (Reiter et al, 2001).

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). Aspirin selectively and irreversibly inhibits the cyclooxygenase-mediated breakdown of arachidonic acid (Harker, 1998), thus inhibiting the subsequent formation of thromboxane A2 (a potent platelet aggregator and vasoconstrictor) (see Figure 1.6.1). 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 cyclooxygenase-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 cyclooxygenase-1 and may continue to synthesise PGs via cyclooxygenase-2 mediated pathways after administration of aspirin (Harker, 1998). It has been shown that aspirin is approximately 170-fold less
potent at inhibiting cyclooxygenase-2 than cyclooxygenase-1 (Weber et al, 1999); (Reiter et al, 2001).

**Figure 1.6.1 Mechanism of action of aspirin in platelets**

**Legend for Figure 1.6.1** After being released from phospholipid membranes, the conversion of arachidonic acid into prostaglandin (PG) G2 and subsequently PGH2 is catalysed by the enzyme PGH synthase*. PGH2 can be converted into thromboxane A2, and the subsequent binding of thromboxane A2 to the thromboxane A2 receptor (TxA2 R) can trigger intracellular signalling events that activate the glycoprotein (Gp) IIb/IIIa receptor. The GpIIb/IIIa receptor can then undergo a conformational change to facilitate binding of its predominant ligands, fibrinogen or VWF, thus mediating platelet to platelet aggregation. Aspirin irreversibly inhibits the cyclooxygenase-1 mediated breakdown of arachidonic acid to PGG2, but does not affect the peroxidase function of PGH synthase.
1.6.3 Early secondary prevention with aspirin in ischaemic stroke and TIA

Until recently, antiplatelet therapy was not routinely given in the acute phase of ischaemic stroke and the benefits of early secondary prevention were unknown. One small randomised trial failed to show any significant benefit of early aspirin therapy in this setting (Multicentre Acute Stroke Trial--Italy (MAST-I) Group, 1995). However, two large randomised trials of aspirin therapy given within 48 hours of acute ischaemic stroke have confirmed that aspirin does have a small, but important, role to play in early secondary prevention (International Stroke Trial Collaborative Group, 1997; CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). The results of these three trials will be discussed independently in the first instance, and a metaanalysis of the studies considered thereafter. Patients were included in these studies regardless of the underlying mechanism responsible for their ischaemic stroke.

In the Multicentre Acute Stroke Trial--Italy (MAST-I) study, patients were randomised to one of four treatment groups within six hours of acute ischaemic stroke: (i) 1.5 MU of intravenous streptokinase, (ii) aspirin 300 mg daily, (iii) streptokinase plus aspirin or (iv) neither treatment regimen (Multicentre Acute Stroke Trial--Italy (MAST-I) Group, 1995). All patients had a brain CT performed before randomisation to exclude intracerebral haemorrhage. This study was prematurely discontinued after 622 subjects (approximately 150 patients in each subgroup) had been randomised because of an excess 10-day case fatality in the streptokinase-aspirin group compared with those given neither treatment (34% vs. 13%, 2p < 0.00001). The case fatality and disability rates and the rate of haemorrhagic transformation of the infarct in patients receiving aspirin alone were not significantly different from those who received neither treatment. The number of early...
recurrent cerebral infarcts in the aspirin alone (1 patient) and control groups (0 patients) was too limited to make any meaningful conclusions about the efficacy of aspirin in early secondary prevention of ischaemic stroke. Therefore, there was no convincing evidence of a short or long-term net benefit from early aspirin over control in acute stroke in this small study.

In the Chinese Acute Stroke Trial (CAST), the outcome of treatment with 160mg of aspirin daily, administered within 48 hours of suspected acute ischaemic stroke and continued for up to four weeks, was compared with placebo (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). Outcome data were available on 10,335 patients randomised to receive aspirin and 10,320 randomised to receive placebo; patients were followed up for four weeks unless they died or were discharged earlier. The effects of early aspirin therapy on the rate of death or recurrent stroke are outlined in table 1.6.1. There was a significant reduction in the rate of death (0.54% absolute risk reduction, \(2p = 0.04\)) and in the combined endpoint of death or non-fatal stroke (0.68% absolute risk reduction, \(2p = 0.03\)) in the aspirin group compared with those allocated placebo. Although aspirin significantly reduced the absolute rate of recurrent ischaemic stroke by 0.47% compared with placebo (\(2p = 0.01\)), its beneficial effect on recurrent stroke overall was reduced by a non–significant increase in haemorrhagic stroke associated with its use (0.21%, \(2p > 0.1\)). Aspirin did not significantly reduce the percentage of patients dead or dependent at discharge (\(2p = 0.08\)), and was associated with an excess of transfused or fatal extracranial bleeding compared with placebo (0.27%, \(2p = 0.02\)). This study could be criticised for its lack of definitive inclusion or exclusion criteria and due to the considerable proportion of patients (13%) who did not have a brain CT before
randomisation. However, the diagnosis of ischaemic stroke was eventually confirmed in 98% of patients overall (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). The relatively young age of the patient population (72% under 70 years old), and the fact that intracranial small vessel disease is more frequent than extracranial carotid artery disease in Chinese patients (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997), may limit the application of these results to a non-Chinese population. Also, concomitant therapy with thrombolysis (22%) and anticoagulants (17%) was not contraindicated, both of which could have increased the risk of haemorrhage and reduced the benefit of early aspirin therapy. In addition, Chinese herbal products were administered to 51% of patients in this study, although some may affect platelets (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). Finally, the overall mortality rate in this study was low (table 1.6.1), partly because of the young age of the patient population and partly because of the exclusion of patients with severe stroke in some centres. Despite the limitations of this study, there was a modest improvement in outcome with early aspirin treatment.
Table 1.6.1 Outcome events at the end of 4 weeks, or earlier discharge or death, in CAST (values are percentages)

<table>
<thead>
<tr>
<th></th>
<th>Aspirin (n = 10,335)</th>
<th>Placebo (n = 10,320)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deaths</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Due to initial stroke</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Due to recurrent stroke</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>(any type)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Due to other or unknown</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>causes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3.3</td>
<td>3.9 *</td>
</tr>
<tr>
<td><strong>Recurrent stroke</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fatal or non-fatal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischaemic</td>
<td>1.6</td>
<td>2.1 **</td>
</tr>
<tr>
<td>Haemorrhagic †</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>All</td>
<td>3.2</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Death or recurrent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-fatal stroke</td>
<td>5.3</td>
<td>5.9 *</td>
</tr>
</tbody>
</table>

**Legend for Table 1.6.1:** † Includes cerebral haemorrhage or haemorrhagic transformation of the original infarct.

* 2p < 0.05; ** 2p ≤ 0.01; all other differences between the aspirin and placebo groups were non-significant.

The results of CAST were published alongside those from the International Stroke Trial (IST) (International Stroke Trial Collaborative Group, 1997). IST was designed to assess the safety and efficacy of aspirin and unfractionated heparin in acute ischaemic stroke. 19,345 patients were randomised to receive one of six different treatment regimens:
- aspirin 300 mg daily
- low dose subcutaneous heparin 5000 IU twice daily
- medium dose subcutaneous heparin 12,500 IU twice daily
- aspirin plus low dose heparin
- aspirin plus medium dose heparin
- no aspirin and no heparin (control group).

Treatment was administered within 48 hours of acute stroke onset and was continued for 14 days or until death or discharge. The treating physicians, who were not blind to the treatment allocated to the patients, performed the early outcome assessments. In an attempt to avoid bias, the six-month outcome assessment in many countries was performed by means of a validated mail questionnaire or by a telephone call from an independent assessor at the coordinating centre. In these cases, the follow up was blind to treatment allocation. The major outcome events in patients allocated aspirin vs. those allocated to “avoid aspirin” are outlined in table 1.6.2. If one compares the clinical outcome in patients on aspirin to those randomised to avoid aspirin (regardless of whether they received subcutaneous heparin or not), early secondary prevention with aspirin was again of some benefit. Aspirin therapy was associated with an absolute reduction of 1.1% in the rate of recurrent ischaemic stroke ($2p < 0.001$), and this beneficial effect was not offset by an increased risk of haemorrhagic stroke. However, aspirin did significantly increase the risk of transfused or fatal extracranial bleeding by 0.5% ($2p = 0.0004$). In contrast to CAST, aspirin did not reduce the rate of death during the study period in IST, and its effect on the unadjusted percentage of patients dead or dependent at six months just failed to reach statistical significance ($2p = 0.06$). Although
aspirin reduced the absolute risk of the combined endpoint of death or non-fatal recurrent stroke within 14 days by 1.1% (2p = 0.02), this endpoint was not a prespecified protocol outcome (International Stroke Trial Collaborative Group, 1997); (Bousser, 1997). Therefore, this study showed that aspirin is an effective therapy for early secondary prevention of recurrent ischaemic stroke, but did not prove that aspirin is beneficial as an acute treatment of cerebral infarction (Bousser, 1997). As in CAST, there were no definite inclusion or exclusion criteria in IST. The fundamental reason for eligibility for entering the trial was "that the physician was uncertain whether or not to administer either or both of the trial treatments to that particular patient" (International Stroke Trial Collaborative Group, 1997). Thirty three per cent of patients did not have a brain CT before randomisation, the trial was not placebo-controlled, and concomitant use of other nonsteroidal anti-inflammatory drugs (which theoretically could increase the risk of haemorrhagic complications associated with aspirin) was not prohibited by the study protocol. In addition, patients randomised to receive heparin did not have obligatory monitoring of their APTT. The authors justified this decision on the basis of the “low” bleeding rates on medium dose heparin in two previous trials, despite a significant excess of bleeding on this dose of heparin in both of these studies (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico, 1990); (ISIS-3 (Third International Study of Infarct Survival) Collaborative Group, 1992).

CAST and IST were conducted concurrently, and the results of the aspirin and non-aspirin arms of these two studies and of the smaller MAST-I study were combined in a metaanalysis published with the CAST results (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). The trend in CAST and IST towards a beneficial effect of
aspirin on the rate of death or dependency became significant in this metaanalysis (Figure 1.6.2). Early aspirin (160 mg–300 mg daily) significantly reduced the rate of recurrent ischaemic stroke by 7 per 1000 ($2p < 0.001$), the rate of death or non-fatal stroke by 9 per 1000 ($2p = 0.001$), and the rate of death or dependency by 13 per 1000 ($2p = 0.007$) compared with control. Thus, roughly one in 100 acute stroke patients benefits substantially from early aspirin treatment. Because aspirin is cheap, and stroke is common, this small benefit translates into substantial cost utility.
Table 1.6.2 Outcome events during the first 14 days in IST (values are percentages)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Aspirin (n = 9719)</th>
<th>Avoid Aspirin (n = 9714)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deaths and likely causes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial stroke</td>
<td>6.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Recurrent ischaemic stroke</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Haemorrhagic stroke</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Extracranial haemorrhage</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Other vascular</td>
<td>0.4</td>
<td>0.6*</td>
</tr>
<tr>
<td>Non-vascular</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total (any cause)</strong></td>
<td>9.0</td>
<td>9.4</td>
</tr>
<tr>
<td><strong>Fatal and non-fatal events</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrent ischaemic stroke (RS)</td>
<td>2.8</td>
<td>3.9***</td>
</tr>
<tr>
<td>Haemorrhagic stroke (HS)</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>RS or HS †</td>
<td>3.7</td>
<td>4.6**</td>
</tr>
<tr>
<td>Death or non-fatal stroke</td>
<td>11.3</td>
<td>12.4*</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Transfused or fatal extracranial haemorrhage</td>
<td>1.1</td>
<td>0.6***</td>
</tr>
</tbody>
</table>

**Legend for table 1.6.2:** † HS includes symptomatic intracranial haemorrhage or symptomatic haemorrhagic transformation of infarct confirmed by CT scan, MRI or necropsy.
* 2p < 0.05; ** 2p < 0.01; *** 2p < 0.001.
Figure 1.6.2 Overview of the effects of aspirin on death or dependency after acute ischaemic stroke at 6 months for MAST-I and IST, or at discharge for CAST

<table>
<thead>
<tr>
<th></th>
<th>Events/Patients</th>
<th>Odds ratio &amp; C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aspirin</td>
<td>Control</td>
</tr>
<tr>
<td><strong>MAST I</strong></td>
<td>94/153</td>
<td>106/156</td>
</tr>
<tr>
<td><strong>CAST</strong></td>
<td>3153/10335</td>
<td>3266/10320</td>
</tr>
<tr>
<td><strong>IST, aspirin vs nil</strong></td>
<td>2998/4858</td>
<td>3064/4859</td>
</tr>
<tr>
<td><strong>IST, aspirin &amp; heparin vs heparin</strong></td>
<td>3002/4861</td>
<td>3061/4855</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>9247/20207</td>
<td>9497/20190</td>
</tr>
<tr>
<td></td>
<td>(45.8%)</td>
<td>(47.0%)</td>
</tr>
</tbody>
</table>

O.R. = 5%
2p = 0.007

Legend for Figure 1.6.2: C.I., 99% confidence interval; ◆, 95% confidence interval for total; O.R., odds reduction. Data redrawn from CAST (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997).
1.6.4 Long-term secondary prevention with aspirin in ischaemic stroke and TIA

There is now good evidence that aspirin is effective in the long-term secondary prevention of stroke and other vascular events. The outcome of treatment with different doses of aspirin in secondary stroke prevention has been studied in four large randomised clinical trials.

The UK – TIA trial compared the efficacy of treatment with aspirin (300 mg or 1200 mg daily) or placebo in patients with a recent history of TIA or minor stroke; the mean duration of follow up was four years (Farrell et al, 1991). There was no significant difference in efficacy between the two different doses of aspirin. Combining the two aspirin groups together and comparing the outcome with aspirin versus placebo, there was a non-significant reduction of 15% in the odds of major stroke, MI or vascular death with aspirin. This study showed that there were significantly more upper gastrointestinal symptoms with the 1200 mg dose compared with 300 mg aspirin daily (41% vs. 31%, odds ratio 1.54). There was a non-significant trend towards a higher rate of gastrointestinal haemorrhage with high dose compared with low dose aspirin, and gastrointestinal haemorrhage occurred more frequently with low dose aspirin compared with placebo (odds ratio 2.57).

The Swedish Aspirin Low-dose Trial (SALT) compared the outcome of treatment with 75 mg of aspirin daily to placebo in patients with a recent TIA, minor ischaemic stroke or retinal artery occlusion (The SALT Collaborative Group, 1991). Patients were followed up for a median duration of 32 months. Aspirin reduced the relative risk of stroke, MI and vascular death by 17% compared with placebo (p = 0.03), the benefit of which outweighed the excess risk of severe bleeding (p = 0.04) and fatal haemorrhagic
stroke (p = 0.03) associated with aspirin use. However, for the endpoint of stroke, there was no significant reduction in risk associated with aspirin therapy (p = 0.11).

The Dutch TIA Trial compared the efficacy and tolerability of 30 mg with 283 mg of aspirin daily in patients with a TIA or minor ischaemic stroke within the preceding three months over a mean follow up period of 2.6 years (The Dutch TIA Trial Study Group, 1991). The two doses of aspirin were equally effective at preventing non fatal stroke, non fatal MI or vascular death, but minor bleeding, gastric discomfort and other minor adverse effects were significantly less common in the 30 mg group (17% risk reduction).

In the Second European Stroke Prevention Study (ESPS-2), 6602 patients with recent stroke or TIA were randomly assigned to one of four treatment arms: placebo, aspirin (25 mg twice daily), modified–release dipyridamole (200 mg twice daily), or aspirin plus dipyridamole (Diener et al, 1996). This was the first study to show that very low dose aspirin (50 mg daily) was more effective than placebo in secondary stroke prevention. The relative risk of recurrent stroke (fatal or non fatal) was reduced by 18.1% (p = 0.013), and the combined risk of stroke or death was reduced by 13.2% (p = 0.016) with low dose aspirin compared with placebo. However, even this dose of aspirin was associated with a significant excess of bleeding complications compared with placebo. Bleeding from any site was approximately twice as common in the two aspirin groups (8.2 % in the aspirin alone group; 8.7 % in the aspirin plus dipyridamole group) compared with placebo (4.5 %). However, this was responsible for treatment withdrawal in only 1.2 % of patients on aspirin alone, and 1.3 % of patients on aspirin plus dipyridamole.
In 1996, Algra and van Gijn performed a metaanalysis of 10 randomised trials of aspirin therapy versus control treatment in patients who had a history of TIA or non-disabling stroke (Algra & van Gijn, 1996). The relative reduction in the risk of subsequent vascular events was 13% with low dose (< 100 mg daily), 9% with medium dose (300 mg daily) and 14% with high dose aspirin treatment (> 900 mg daily). They concluded that doses of aspirin between 30 mg and 1500 mg daily were equally effective for secondary prevention after cerebral ischaemia, but cautioned that true differences in efficacy between the treatment regimens could not be excluded because of wide confidence intervals. These findings are consistent with those of a metaregression analysis of 11 randomised, placebo-controlled trials of aspirin therapy in patients with a recent TIA, stroke or retinal artery occlusion (Johnson et al., 1999). Aspirin therapy, across a broad range of doses from 50 mg to 1500 mg daily, significantly reduced the risk of stroke by about 15% compared with placebo (95% confidence interval, 6% - 23%). There was no significant difference in efficacy between lower and higher doses of aspirin.

The beneficial effects of aspirin compared with placebo reported in these studies and metaanalyses is consistent with the overall results of the most recent metaanalysis performed by The Antithrombotic Trialists' Collaboration (Antithrombotic Trialists' Collaboration, 2002). The Trialists analysed data from 197 trials of short- or long-term antiplatelet therapy for any vascular indication available by September 1997. Aspirin (≤ 75 mg to 1500 mg daily) was the most widely tested drug in this metaanalysis (Warlow, 2002). When antiplatelet therapy was continued for more than one month, events occurring during the scheduled treatment period were included in the analysis. If the
scheduled treatment period was shorter, the Trialists analysed events occurring as close as possible to one month after randomisation. It should be noted that the "odds reductions" in outcome events associated with treatment, as calculated by the Antithrombotic Trialists, translate into slightly lower relative risk reductions quoted in other studies (Antiplatelet Trialists' Collaboration, 1994); (Algra & van Gijn, 1996). Antiplatelet therapy was associated with a proportional odds reduction in important vascular events (non fatal stroke, non fatal MI, vascular death, or death of indeterminate aetiology) of 22% in patients with a history of transient ischaemic attack or stroke (p < 0.0001) (Antithrombotic Trialists' Collaboration, 2002). The Trialists did not publish data on the effects of different aspirin doses on outcome in the subgroup of patients with prior ischaemic stroke or TIA. However, low (75 – 150 mg daily) or medium dose (160 – 325 mg daily) aspirin were similarly effective at preventing recurrent vascular events during follow up as higher (500 – 1500 mg daily) and more gastrotoxic doses of the drug amongst different groups of patients at high risk of vascular events (Antithrombotic Trialists' Collaboration, 2002); (Farrell et al, 1991). The benefit of secondary prevention with doses of aspirin < 75 mg was less clear (p = 0.05) (Antithrombotic Trialists' Collaboration, 2002).

In summary, 160 to 300 mg of aspirin daily, administered within 48 hours of an acute ischaemic stroke, reduces the relative risk of recurrent ischaemic stroke in the first 14 to 28 days by only 24 to 28% (International Stroke Trial Collaborative Group, 1997;CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). However, long-term secondary prevention with 30 to 1500 mg of aspirin daily reduces the relative risk of subsequent vascular events by only 13 to 18% (Diener et al, 1996); (Algra & van Gijn,
1996). It is reasonable to assume that if there is uncertainty about the efficacy of very low
dose aspirin (< 75 mg daily) in patients at high risk of vascular events overall, that low or
medium dose aspirin should be used in patients with ischaemic stroke or TIA (see
below).

1.6.5 Secondary prevention with aspirin therapy in patients with symptomatic
severe carotid stenosis
As outlined in section 1.3, ECST and NASCET compared the outcome of treatment with
carotid endarterectomy with best medical care in patients who had a recent ischaemic
stroke, TIA or amaurosis fugax in association with > 70% carotid stenosis. 79% and 94%
of medically treated patients were taking aspirin therapy during follow-up in ECST and
NASCET, respectively (European Carotid Surgery Trialists' Collaborative Group, 1998);
Despite aspirin therapy in the majority of these patients, the risk of major ipsilateral
stroke was 20.6% over three years in ECST, and 26% over two years in NASCET. This
may, in part, be related to the fact that an ulcerated atherosclerotic plaque causing vessel
stenosis exposes platelets to increased shear stress (Kroll et al, 1996), and aspirin has
been reported to be ineffective at preventing shear-induced platelet aggregation
(Uchiyama et al, 1994) (see also section 1.6.6)

Recently, the Aspirin and Carotid Endarterectomy (ACE) Trial Collaborators
have shown that patients undergoing carotid endarterectomy benefit more from lower
than higher doses of aspirin in the perioperative period (Taylor et al, 1999). The
combined rate of stroke, MI or death at three months was significantly lower at 6.2% in
patients receiving lower dose aspirin (81 mg or 325 mg) compared with 8.4% with higher doses of 650 mg or 1300 mg daily (p = 0.03).

1.6.6 Aspirin therapy in patients with asymptomatic carotid stenosis

The Asymptomatic Cervical Bruit Study investigated the outcome of treatment with aspirin compared with placebo in patients with asymptomatic carotid stenosis (Côté et al, 1995). This was a double blind study in which 372 patients with > 50% carotid stenosis on duplex ultrasonography were randomised to receive 325 mg of enteric-coated aspirin daily (n = 188) or placebo (n = 184). The mean duration of follow up was 2.4 years. The primary end-point was the composite outcome of TIA, stroke, myocardial infarction (MI), unstable angina, or death; the rates of stroke and TIA were included among the secondary end-points in the study. Approximately one third of patients in each group had 80% to 99% carotid stenosis, but carotid occlusion was slightly less common in the aspirin than placebo treated patients (8% vs. 12% respectively). There was no significant difference in the rate of occurrence of the primary outcome measure between the aspirin and placebo groups during follow-up. Aspirin did not significantly reduce the overall risk of stroke compared with placebo (5.8% vs. 5.4%), and the reduction in the rate of TIA with aspirin (8.5% vs. 12.5%) did not reach statistical significance. However, the low rate of outcome events, the small sample size, and the small proportion of patients with >80% carotid stenosis limit the application of these results to patients with severe (> 70%) asymptomatic carotid stenosis.
1.6.7 ‘Aspirin resistance’ in ischaemic stroke and TIA

There is no universally accepted definition of what constitutes ‘aspirin resistance’, and the term is often interpreted differently by basic scientists and clinicians. At a cellular level, aspirin resistance could refer to incomplete inhibition, or lack of inhibition of platelet cyclooxygenase-1 \textit{ex vivo} in patients on aspirin therapy. Cyclooxygenase function can be assessed by measuring the levels of thromboxane A$_2$ metabolites in plasma or urine, but technical problems related to sample collection and processing have limited the clinical usefulness of these assays (Abrams & Shattil, 1991). The degree of platelet aggregation in response to \textit{in vitro} stimulation by arachidonic acid is also frequently used to assess cyclooxygenase inhibition by aspirin. However, platelet aggregometry is usually performed in platelet rich plasma (PRP), rather than in the physiological milieu of whole blood, and the assays are semiquantitative and subject to artifactual \textit{in vitro} platelet activation during sample collection and processing (Michelson, 1996). Moreover, there is no standard reference range for arachidonic acid-induced platelet aggregation in patients on aspirin that would allow one to consistently classify these patients as either ‘aspirin resistant’ or ‘aspirin responsive’ \textit{ex vivo} (Helgason \textit{et al}, 1993); (Gum \textit{et al}, 2001).

As mentioned above, the majority of patients with ischaemic stroke or TIA are not protected from further vascular events with aspirin therapy. In the clinical setting, ‘aspirin resistance’ might be defined as the recurrence of vascular events during follow-up in patients taking aspirin. I prefer to describe these patients as ‘aspirin failures’ (Chyatte & Chen, 1990); (Bornstein \textit{et al}, 1994) in the absence of laboratory assessment of platelet function, because platelets can be activated via cyclooxygenase-independent pathways.
Furthermore, recurrent events may occur in subjects who are ‘responsive’ to the antiplatelet effects of aspirin *ex vivo* (Helgason *et al.*, 1993). From a practical clinical viewpoint, one could consider patients to be aspirin resistant if they have excessive platelet activation or hyper-reactivity *ex vivo* despite treatment with aspirin. If one could identify patients who are resistant to the antiplatelet effects of aspirin with a simple and reliable laboratory test in whole blood, and advance our understanding of the mechanisms responsible for aspirin resistance or aspirin failure in the clinical setting, this could improve secondary prevention in this patient population. The literature regarding aspirin failure and aspirin resistance in ischaemic stroke and TIA will now be discussed.

Dougherty *et al.* reported that platelets become activated and form spontaneous platelet aggregates *in vivo* in patients with acute cerebral ischaemia (Dougherty, Jr. *et al.*, 1977). The percentage of circulating platelet-platelet aggregates was elevated in the acute (<10 days; n = 82) but not in the convalescent phase (10 days to 6 weeks; n = 34) after an ischaemic stroke or TIA compared with age-matched neurological control subjects (n = 30). A sub-study was performed to examine the effects of antiplatelet therapy on platelet aggregate formation. The percentage of circulating platelet aggregates was similar in seven TIA patients who were on treatment with 1200 mg of aspirin and 100 mg of dipyridamole daily for up to 72 hours compared with 22 TIA patients who were not on antiplatelet therapy. The authors also suggested that platelets were hyper-reactive in patients with acute cerebral ischaemia, but that this hyper-reactivity was short-lived. *In vitro* platelet aggregation in PRP in response to ADP or epinephrine was enhanced in the acute phase, but not in the convalescent phase, after an ischaemic stroke or TIA.
Although these data are suggestive of excessive platelet activation, true aspirin +/- dipyridamole resistance in vivo, and platelet hyper-reactivity in vitro in the acute phase after an ischaemic stroke or TIA, this study had several limitations. The methodology used to measure the percentage of circulating platelet aggregates was crude and indirect, and is subject to artifactual in vitro platelet activation. The percentage of subjects in the main study who were on antiplatelet therapy at the time of the analysis was not specified. In addition, the number of TIA patients who were on treatment with antiplatelet therapy in the sub-study was too small to draw any firm conclusions about the relevance of these findings.

A subsequent study involving 43 patients who had undergone carotid endarterectomy three months earlier for a TIA or completed stroke examined the in vitro and in vivo effects of treatment with 50 to 70 mg of aspirin daily (Boysen et al, 1984). 9.3% of patients had insufficient inhibition of in vitro platelet aggregation in PRP, and lack of prolongation of the in vivo bleeding time despite treatment with aspirin, suggestive of aspirin resistance in vitro and in vivo.

Tohgi et al. provided some evidence of in vitro aspirin resistance in a study of 19 “poststroke” patients (Tohgi et al, 1988). It was not clearly specified whether these patients had a history of ischaemic or haemorrhagic stroke, or whether the patients were studied in the acute or convalescent phase after symptom onset. Platelet aggregation in PRP in response to stimulation with 2 μg / ml of collagen was not inhibited at all in one patient, and was only partially inhibited in a significant number of other patients despite treatment with 40 mg of aspirin daily for one to four weeks. In addition, some patients exhibited only partial inhibition of platelet aggregation on aspirin therapy, despite a
marked reduction in thromboxane biosynthesis. These data suggest that thromboxane-independent pathways might be involved in mediating *in vitro* aspirin resistance in some patients.

In 1991, Grotemeyer reported the results of a study on *in vitro* ‘platelet reactivity’ in 82 patients with a history of stroke who were on treatment with 500 mg of aspirin three times per day (Grotemeyer, 1991). Although it is not clear from the report, the subjects appear to have been subsequently treated with single doses of aspirin between 50 to 1000 mg daily. The author did not specify the interval between stroke onset and blood sampling, or whether the patients had an ischaemic or haemorrhagic stroke, although one assumes that only patients with ischaemic stroke were included. It was suggested that suppression of platelet reactivity *in vitro* was more marked with 200 to 1000 mg of aspirin than with 50 to 100 mg of aspirin. In addition, it was shown that the results varied depending on the interval between aspirin administration and blood testing. However, the laboratory method used to assess platelet reactivity in this study was crude and involved sample centrifugation + / - fixation. In addition, the study methodology was flawed because the author did not allow for an adequate ‘aspirin washout period’ between dosing increments (only 24 hours) to investigate the effects of increasing doses of aspirin on platelet function.

Grotemeyer and colleagues also reported the results of a two year follow-up study in 174 patients with ischaemic stroke who were initially assessed within 49 days of stroke onset (Grotemeyer *et al*, 1993). Using the same technique referred to above, ‘aspirin responders’ were defined as patients with ‘normal’ platelet reactivity 12 hours after ingestion of 500 mg of aspirin, whereas ‘secondary aspirin non-responders’ had
‘enhanced’ platelet reactivity despite aspirin therapy. Patients were subsequently treated with 500 mg of aspirin TDS, and the outcome events in the two groups were determined by telephone calls to the patients, their family doctors, or by chart review. 4% of aspirin responders compared with 40% of secondary aspirin non-responders had a major vascular event (MI, stroke or vascular death) during follow-up (p < 0.0001). However, platelet reactivity was not reassessed at the time of the follow-up event, and compliance with aspirin therapy was not assessed by laboratory testing. For these reasons, and because of the limitations of the method used to assess platelet function (see above), the results of this study should be interpreted with caution.

Helgason et al. carried out two separate studies to investigate the responsiveness to aspirin therapy ex vivo in patients with ischaemic stroke (Helgason et al, 1993); (Helgason et al, 1994). In the first of these two studies, 113 outpatients with a prior history of ischaemic stroke, and 24 inpatients who had an acute ischaemic stroke on aspirin therapy were assessed (Helgason et al, 1993). Platelet aggregation in PRP was measured in response to stimulation by arachidonic acid (500 µM), ADP (5 µM), epinephrine (5 µM), and collagen (0.8 µg / ml). Most of the outpatients were on treatment with ≤ 325 mg of enteric coated aspirin daily, but the aspirin dose used in the inpatient group was not specified. The authors clearly defined what they regarded as ‘incomplete’ or ‘complete’ inhibition of platelet aggregation by aspirin in response to the different agonists used. If there was incomplete inhibition of platelet aggregation on the initial dose of aspirin, the dose was increased by 325 mg until complete inhibition was achieved, up to a maximum of 1300 mg daily. ‘Aspirin resistance’, which was defined as incomplete inhibition of platelet aggregation on 1300 mg of aspirin daily, was seen in
three (2.7%) outpatients and one inpatient (4.2%) with acute stroke. It was of interest that the majority of inpatients (79.2%) who had an acute stroke on aspirin therapy (aspirin failures) had complete inhibition of platelet aggregation *ex vivo*, suggesting that thromboxane-independent mechanisms were involved in the pathogenesis of stroke in these individuals. Therefore, this study did not enforce the argument that platelet aggregometry might be useful in identifying patients at high risk of stroke recurrence on aspirin therapy.

In a subsequent study by the same group, the *ex vivo* inhibition of platelet aggregation by aspirin was measured in 306 patients with ischaemic stroke who were on treatment with ≥ 325 mg of enteric coated aspirin daily for prevention of recurrent stroke (Helgason *et al*, 1994). 171 patients had repeat testing approximately six months later. The same platelet aggregometry methodology, the same guidelines for dosage escalation, and the same definition of aspirin resistance were used in this study. In addition, resistance to a particular dose of aspirin was defined as ‘incomplete inhibition of platelet aggregation on that dose’. Seventy eight patients (25.5%) had resistance to the dose of aspirin that was initially prescribed. Of the 171 patients who had repeat testing performed, 14 (8.2%) were aspirin resistant despite dosage escalation to 1300 mg daily. 30.5% of patients who were initially on a dose of aspirin sufficient to completely inhibit platelet aggregation, had incomplete inhibition of platelet aggregation on the same dose of aspirin at some stage during follow-up. The mechanisms responsible for fluctuation in the *ex vivo* response to aspirin therapy in a significant number of patients during follow-up were not determined. Based on the results of this study, one could predict finding *ex*
vivo ‘aspirin resistance’ in at least 25% of patients with ischaemic stroke on treatment with 75 to 300 mg of aspirin daily.

More recently, the ex vivo response to aspirin was measured in 14 ischaemic stroke patients with a history of stroke recurrence on aspirin (‘aspirin failures’) and 25 patients without stroke recurrence on aspirin (‘aspirin responders’) (Chamorro et al, 1999a). Platelet aggregometry was performed using 1.4 μM arachidonic acid, and 10 μM epinephrine after seven days treatment with 300 mg and 600 mg of aspirin daily, respectively. A daily dose of 300 mg of aspirin inhibited arachidonic acid- and epinephrine-induced platelet aggregation less effectively in the aspirin failures than in the aspirin responders (p < 0.01). More complete inhibition of platelet aggregation was seen in the aspirin failures when the dose was increased from 300 to 600 mg daily (p < 0.01, 600 vs. 300mg). Because of the limited number of subjects and the lack of clinical follow-up after dose escalation, the results of this study cannot be generalised to recommend the use of higher aspirin doses in patients with stroke recurrence on 300 mg of aspirin daily. However, this issue deserves further study. The potential mechanisms involved in aspirin resistance in vivo and ex vivo are discussed further in chapter 5.

1.6.8 Dipyridamole

**Mechanism of action**

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) (Figure 1.6.3). 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 (a platelet-inhibiting vasodilator) (Muller, 2001; FitzGerald, 1987), and adenosine mediates its antiplatelet effects by stimulation of adenylate cyclase (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).

Dipyridamole exerts its antiplatelet effects by inhibiting PDE5 in platelets, thus increasing intraplatelet levels of cGMP and cAMP (Diener et al, 1996), and by inhibiting the uptake and metabolism of adenosine by erythrocytes and endothelial cells (FitzGerald, 1987) (Figure 1.6.4). This increases the availability of adenosine in the vascular microenvironment. It has also been reported to inhibit cAMP phosphodiesterases in platelets, thus increasing intraplatelet cAMP levels even further (FitzGerald, 1987). In addition, 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 (Eisert, 2001). It has also been reported that dipyridamole inhibits lipid peroxidation and may protect the endothelium from damage by peroxiradicals (Eisert, 2001).
**Dipyridamole monotherapy and combination therapy with aspirin and dipyridamole**

Early trials investigating the relative effects of the combination of dipyridamole and aspirin versus aspirin alone were inconclusive, mainly due to their small sample size (Diener, 1998); (ESPS Group, 1990). However, the European Stroke Prevention Study (ESPS) found that the combination of 225 mg dipyridamole and 990 mg aspirin daily reduced the relative risk of death or recurrent stroke by an impressive 33.5% compared
Figure 1.6.3. Pathways involved in the intraplatelet formation and breakdown of cAMP and cGMP

Legend for Figure 1.6.3: ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; cAMP, Adenosine 3', 5'-cyclic monophosphate; PDE, Phosphodiesterase; GTP, Guanosine triphosphate; cGMP, Guanosine 3', 5'-cyclic monophosphate; PKG, cGMP-dependent protein kinases; +, Stimulatory effect; -, Inhibitory effect; The actions of enzymes are shown by single headed blue arrows, and reversible regulatory effects by double headed black arrows.
with placebo in patients with a recent TIA or ischaemic stroke (p < 0.001) (ESPS Group, 1990). However, this study had a number of methodological flaws and did not include a dipyridamole-only or an aspirin-only arm. A subsequent metaanalysis of the trials of combination antiplatelet therapy, including aspirin and dipyridamole, did not prove that
these regimens were more beneficial than aspirin alone, but did not exclude this possibility either (Antiplatelet Trialists' Collaboration, 1994).

The Second European Stroke Prevention Study (ESPS - 2), which was set up to address a number of issues raised by ESPS, showed that dipyridamole, aspirin, or a combination of dipyridamole and aspirin were superior to placebo in secondary stroke prevention (Diener et al, 1996). The trial design and the results from the 'aspirin-alone' arm of the trial have been discussed in section 1.6.4. In comparison with placebo, the overall reduction in stroke risk was 16% with dipyridamole alone, which was very similar to the 18% reduction associated with aspirin alone. The combination of dipyridamole and aspirin led to a 37% reduction in stroke risk compared with placebo; this was significantly more beneficial than treatment with either agent alone (Figure 1.6.5). The relative risk reductions for the combined endpoint of stroke or death were 15% with dipyridamole, and 24% with combination therapy. However, there was no significant reduction in the incidence of subsequent MI with any of the treatment regimens. In addition, treatment was very poorly tolerated initially with 57% of placebo treated patients and over 60% of patients on dipyridamole, aspirin or combination therapy experiencing some adverse event. Treatment withdrawal occurred in 22% of patients in the placebo or aspirin-only groups, and in 29% of patients on dipyridamole (alone or in combination with aspirin). The most common adverse events leading to treatment withdrawal were headache (8%) and gastrointestinal disturbance (6 - 7%) in the dipyridamole groups. There was no excess of bleeding in patients treated with dipyridamole alone compared with placebo (4.7% versus 4.5%).
A recent metaanalysis of 15 trials supports the view that the combination of dipyridamole and aspirin is superior to aspirin alone in preventing nonfatal stroke in patients with a history of vascular disease (Wilterdink & Easton, 1999). Combination therapy significantly reduced the odds of nonfatal recurrent stroke by 23% compared with aspirin alone, but did not significantly reduce the odds of having a subsequent MI or other vascular events. Because ESPS 2 was the first study to show an unequivocal benefit of combination therapy with dipyridamole and aspirin over aspirin alone, and because of the unexplained and disparate effects of dipyridamole on the cerebrovascular and coronary circulation, further studies are warranted to confirm these findings. The efficacy and safety of the combination of dipyridamole and aspirin is currently being reassessed in an ongoing study called the European and Australian Stroke Prevention in Reversible Ischaemia Trial (ESPRIT) (ESPRIT investigators, 1999).
Figure 1.6.5 Survival curves showing the probability of survival free of stroke over a two year treatment period on treatment with placebo, aspirin (ASA), dipyridamole (DP) or combination therapy (DP-ASA) in ESPS-2 (Diener et al, 1996)
1.6.9 The thienopyridine derivatives: Ticlopidine and clopidogrel

Introduction

As mentioned in section 1.4.6, three separate ADP receptors have been identified on platelets that mediate different aspects of the response to this agonist (Daniel et al, 1998); (Jin et al, 1998); (Hollopeter et al, 2001). Ticlopidine and clopidogrel are thienopyridine derivatives that selectively and irreversibly inhibit the P2Y₁₂ ADP receptor on platelets, thus interfering with ADP-induced activation of the GpIIb/IIIa receptor complex (Figure 1.6.6). Binding to this receptor prevents ADP-induced inhibition of adenylate cyclase, thus increasing intraplatelet levels of cAMP (Brass, 2001). However, the antiplatelet action of these drugs is also mediated by a number of other mechanisms (Quinn & Fitzgerald, 1999); (Gachet, 2001).

Ticlopidine

Ticlopidine is inactive in vitro, and must undergo hepatic metabolism by the cytochrome P450-1A enzyme system to exhibit its antiplatelet effects (Quinn & Fitzgerald, 1999); (Gachet, 2001). It rapidly and extensively metabolised after oral administration, with 13 metabolites identified in humans (Harker, 1998), but the maximal antiplatelet effects of the drug are only seen after 5 to 6 days of repeated oral therapy (Schrör, 1993). Ticlopidine has been shown to be more effective than placebo in reducing vascular events (Gent et al, 1989), and more effective than aspirin in secondary stroke prevention in patients with recent TIA or minor stroke (Hass et al, 1989).
Figure 1.6.6: Mechanism of action of the thienopyridines – Ticlopidine and clopidogrel

Legend for Figure 1.6.6: ADP, adenosine diphosphate released from platelets and erythrocytes; GpIIb/IIIa, glycoprotein IIb/IIIa platelet receptor; P2Y$_{12}$ ADP-R, P2Y$_{12}$ ADP platelet receptor
In the Canadian American Ticlopidine Study, patients with recent non-cardioembolic ischaemic stroke were randomised to receive either ticlopidine 250 mg twice daily (n = 525) or placebo (n = 528) (Gent et al, 1989). The average period of follow-up was 24 months, and the mean duration of treatment was 17 months and 19 months in the ticlopidine and placebo groups, respectively. Ticlopidine reduced the combined risk of stroke, myocardial infarction or vascular death by 23% compared with placebo (p = 0.02).

In the Ticlopidine Aspirin Stroke Study, the outcome of treatment with ticlopidine (250 mg twice daily) was compared with that seen with aspirin (650 mg twice daily) in patients with a history of TIA, amaurosis fugax, reversible ischaemic neurologic deficit, or minor stroke within the preceding three months (Hass et al, 1989). Patients with symptoms that were presumed to be cardioembolic in origin were also excluded from this study. Ticlopidine significantly reduced the three-year risk of recurrent stroke by 21% compared with aspirin. However, side effects were common in the ticlopidine group with diarrhoea occurring in 20% of patients, necessitating discontinuation of the drug in 6%. Gastritis and gastrointestinal haemorrhage were more common in the aspirin group, but ‘all site’ haemorrhage was equally common with ticlopidine (9%) and aspirin (10%). More importantly, neutropenia occurred in 2.3% of patients on ticlopidine and was severe in 0.9%, necessitating regular haematological monitoring of patients. In most cases, neutropenia was first noted between one and three months after commencing treatment, and resolved within three weeks of cessation of the drug. Ticlopidine treatment was also associated with the development of hypercholesterolaemia (mean increase in total cholesterol level of 9 +/- 20%), although the long-term implications of this finding are
unknown. A rare, but important adverse effect associated with ticlopidine therapy is thrombotic thrombocytopenic purpura, with an estimated incidence of between 1 in 1600 to 1 in 5000 (Bennett et al, 2000a), and a mortality rate of up to 21%. Although recently licensed in the U.K., ticlopidine is rarely used for secondary stroke prevention due to the more favourable side effect profile associated with its chemically related compound, clopidogrel.

**Clopidogrel**

Clopidogrel is a new thienopyridine derivative, chemically related to ticlopidine, but with antithrombotic activity in animal models greater than ticlopidine (CAPRIE Steering Committee, 1996). Clopidogrel is also inactive in its native form, but it is metabolised in the liver to form an active thiol derivative that covalently binds to the P2Y$_{12}$ receptor in vivo (Savi et al, 2000); (Gachet, 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).

A modest clinical benefit of clopidogrel over aspirin has been demonstrated in the CAPRIE trial (CAPRIE Steering Committee, 1996). 19,185 patients with recent ischaemic stroke, MI or symptomatic atherosclerotic peripheral arterial disease were randomised to receive clopidogrel (75 mg daily) or aspirin (325 mg daily). The relative risk reduction in the average annual incidence of ischaemic stroke, MI or vascular death was 8.7% with clopidogrel compared with aspirin (absolute risk reduction of 0.51%, p = 0.04 (Figure 1.6.7). The trend towards a reduction in the relative risk of subsequent events with clopidogrel compared with aspirin in the subgroup of patients presenting with
stroke was not statistically significant (7.3%, p = 0.26), but the study was not adequately powered to detect treatment effects in different subgroups. However, among the subgroup of patients presenting with ischaemic stroke or peripheral vascular disease who had a previous history of MI, clopidogrel significantly reduced the relative risk of subsequent vascular events by 22.7% compared with aspirin.

In comparison with aspirin, severe rash (0.3%) and diarrhoea (4.5%) occurred more commonly with clopidogrel, but there was no excess of neutropenia (0.1%) or hypercholesterolaemia. Gastrointestinal haemorrhage was significantly less common with clopidogrel than aspirin (1.99% vs. 2.66%, p < 0.05), and there was a non-significant trend towards a lower rate of intracranial haemorrhage with clopidogrel (0.35% vs. 0.49%, p = 0.23). Recently, there have been reports of thrombotic thrombocytopenic purpura associated with clopidogrel (Bennett et al, 2000a) with an estimated incidence of approximately 1 in 15000 users (Bennett et al, 2000b). The majority of cases occurred with two weeks of initiation of treatment, although some of the reported cases were also receiving other drugs that may have contributed to the development of the syndrome (Bennett et al, 2000a); (Cheung, 2000). The mortality rate among patients with clopidogrel-associated thrombotic thrombocytopenic purpura who were treated with plasma exchange was 9% in one series (Bennett et al, 2000a), and it has been suggested that weekly full blood counts be performed in the first few weeks of initiating therapy (Bennett et al, 2000b).

In summary, it is reasonable to consider clopidogrel for secondary prevention of vascular events in patients with ischaemic stroke who are intolerant of aspirin or dipyridamole, or in patients with a history of ischaemic heart disease, in whom
dipyridamole has not been shown to be effective (McCabe & Brown, 2000). One must remember that its cost is considerable when compared with aspirin. 200 patients with a risk profile similar to those included in CAPRIE would have to be treated with clopidogrel rather than aspirin to prevent one additional vascular event (CAPRIE Steering Committee, 1996). However, clopidogrel should be more cost effective if used for secondary prevention in subgroups of patients at high risk of vascular disease.

1.6.10 Glycoprotein IIb/IIIa antagonists

Platelet to platelet aggregation is ultimately mediated by ligand binding to the glycoprotein IIb/IIIa (GpIIb/IIIa) receptor (Wagner et al, 1996). Platelet activation leads to a conformational change in this receptor that facilitates ligand binding, and this change is the final common pathway in platelet aggregation (Moran & FitzGerald, 1994). However, GpIIb/IIIa antagonists bind to this receptor on both resting and activated platelets, and intravenous preparations have been shown to improve outcome in acute coronary syndromes and in patients undergoing percutaneous coronary interventional procedures (Topol et al, 1999).

The results of the first randomised, placebo-controlled, dose-escalation trial of an intravenous GpIIb/IIIa antagonist (abciximab) in acute ischaemic stroke have recently been published (The Abciximab in Ischemic Stroke Investigators, 2000). In this trial, 54 patients were randomised to receive one of four doses of abciximab and 20 patients were randomised to receive placebo within 24 hours of onset of an ischaemic stroke. All patients had a brain CT before randomisation to exclude intracranial haemorrhage or another contraindication to treatment. Subsequent CT scans were obtained 24 to 36 hours
Figure 1.6.7: Percentage relative risk reduction in the primary outcome of ischaemic stroke, myocardial infarction (MI), or vascular death in the subgroups of patients presenting with stroke, MI, or peripheral arterial disease (PAD) in the CAPRIE Trial (CAPRIE Steering Committee, 1996)

Relative-risk reduction (%)

- **7.3%**  
  $p = 0.26$

- **-3.7%**  
  $p = 0.66$

- **23.8%**  
  $p = 0.003$

- **8.7%**  
  $p = 0.043$
after administration of the study agent, and thereafter at the discretion of the treating physician. The patients were followed up for three months. Although there was a trend towards a lower rate of stroke recurrence (2% versus 5%) and a higher rate of functional recovery at three months with abciximab compared with placebo, the overall risk of asymptomatic haemorrhagic transformation of the original infarct was much higher with abciximab therapy than placebo (19% versus 5%). Seven per cent of patients treated with abciximab had moderate thrombocytopenia during the first five days of treatment. Although thrombocytopenia is a well recognised complication of treatment with GpIIb/IIIa antagonists, this did not predispose to a higher rate of extracranial bleeding with abciximab compared with placebo, and none of the patients with thrombocytopenia had intracranial haemorrhage. The investigators are planning a further study of abciximab in acute ischaemic stroke, but at the present time, there is insufficient evidence to support the routine use of GpIIb/IIIa antagonists for the purpose of early secondary prevention in this patient population.

The outcome of treatment with oral GpIIb/IIIa antagonists in patients with ischaemic heart disease has been universally disappointing to date (Coller, 2001). The Blockade of the glycoprotein IIb/IIIa Receptor to Avoid Vascular Occlusion (BRAVO) trial was the first study to examine the effects of an oral GpIIb/IIIa antagonist (lotrafiban) for the secondary prevention of vascular events in patients with cerebrovascular disease (Topol et al, 2003). The study included patients with recent ischaemic stroke or TIA, recent acute myocardial infarction or unstable angina, or peripheral vascular disease in association with either cerebrovascular or ischaemic heart disease (“double bed” vascular disease). Overall, 41% of subjects had cerebrovascular disease at study entry. Patients
were randomly assigned to receive lotrafiban (n = 4600) or matching placebo (n = 4590), and were concomitantly treated with aspirin (75 to 325 mg daily), with the prescribed dose left to the discretion of the treating physician. The trial was prematurely discontinued after a median follow-up period of 366 days because of a higher mortality rate with lotrafiban than placebo (3 vs. 2.3%), with this difference primarily accounted for by an excess of vascular deaths with lotrafiban (n = 107) compared with placebo (n = 78). The rate of stroke during follow-up was similar with lotrafiban and placebo (2.1 vs 2.4%, p = 0.35), and the lotrafiban group did not have an increased risk of hemorrhagic stroke or hemorrhagic infarct transformation. However, significant thrombocytopenia and other serious bleeding complications were more common with lotrafiban (p ≤ 0.001) (Topol et al, 2003).

Therefore, there is currently insufficient evidence to support the routine use of intravenous GpIIb/IIIa antagonists for early secondary prevention, and there is no evidence to support the use of oral GpIIb/IIIa antagonists for long term secondary prevention after ischemic stroke or TIA.

1.7 Acute Treatment and Secondary Prevention with Anticoagulant Therapy in Patients with Ischaemic Stroke and TIA

1.7.1 Introduction

Anticoagulants that could potentially play a role in the secondary prevention of recurrent ischaemic stroke or TIA include unfractionated heparin, low molecular weight heparins (LMWHs), heparinoids and warfarin. These agents could theoretically limit the extent of cerebral infarction and thus improve outcome by preventing propagation of a newly formed thrombus in patients with acute thromboembolic ischaemic stroke. In addition,
they could prevent early stroke recurrence by preventing further embolisation from a freshly formed thrombus e.g. superimposed on a ruptured atherosclerotic carotid plaque.

In 1993, Sandercock et al. published an overview of 11 completed controlled trials that compared anticoagulant therapy with control in acute presumed ischaemic stroke (Sandercock et al, 1993). Unfractionated heparin was studied in six trials, LMWHs in four and oral anticoagulants in one; the duration of treatment ranged from 6 to 21 days. The reviewers concluded that there was insufficient evidence at that time to justify routine anticoagulation in patients with acute ischaemic stroke because of an uncertain balance of risks and benefits associated with treatment. Further studies have been completed since this overview was published, and the currently available evidence regarding the use of anticoagulation in acute ischaemic stroke and its effect on stroke recurrence will be discussed in the following section. Specific attention will then be focused on the evidence regarding anticoagulation in the subgroup of patients with acute cardioembolic ischaemic stroke associated with NVAF. This data is of particular relevance to the study population in this thesis, because some patients were treated with unfractionated heparin, some with low molecular weight heparin, and others with warfarin (see Chapters 3, 4 and 6).

1.7.2 Unfractionated heparin in acute ischaemic stroke

Unfractionated heparin is not a homogeneous substance; it consists of a mixture of glycosaminoglycan chains of between 5,000 and 30,000 Da (Sherman, 1998). Heparin itself has little anticoagulant effect but acts as a cofactor for antithrombin III (Salzman et
After binding to antithrombin III, the complex mediates its anticoagulant action by inhibiting activated clotting factors IIa and Xa.

In the International Stroke Trial (IST), 4861 patients received low dose and 4856 patients received medium dose subcutaneous unfractionated heparin alone or in combination with aspirin (International Stroke Trial Collaborative Group, 1997). If one compares the combined outcome in patients on heparin (low or medium dose, n = 9717) to those randomised to avoid heparin (n = 9718), early anticoagulation was neither safe nor effective overall (Table 1.7.1). Heparin significantly increased the risk of fatal haemorrhagic stroke (2p = 0.04) or fatal extracranial bleeding (2p = 0.02), and increased the absolute rate of transfused or fatal extracranial bleeding by 0.9% (2p < 0.00001) (International Stroke Trial Collaborative Group, 1997). Although anticoagulation reduced the absolute risk of recurrent ischaemic stroke within 14 days by 0.9% (2p = 0.005), this was offset by an increased rate of haemorrhagic stroke (0.8%, 2p < 0.00001). However, compared with the subgroup randomised to “avoid heparin”, low dose heparin reduced the rate of recurrent ischaemic stroke by 1.2% (2p < 0.001) without a significant excess of transfused or fatal extracranial bleeding. Increasing the heparin dose from 5,000 IU to 12,500 IU twice daily did not reduce the rate of recurrent ischaemic stroke further (2p = NS), but did significantly increase the rate of haemorrhagic stroke and extracranial bleeding (International Stroke Trial Collaborative Group, 1997). Therefore, low dose unfractionated heparin was proven to be of modest benefit in the early prevention of recurrent ischaemic stroke without a significant increase in haemorrhagic complications. However, because this conclusion is based on a subgroup analysis of only 4861 patients on low dose heparin compared with 9718 patients who did not receive heparin, and
because there was no significant benefit in terms of long term outcome at six months with this dose of heparin, these results should be interpreted with caution and further studies are warranted to confirm these findings. Until then, one cannot routinely recommend subcutaneous unfractionated low dose heparin for early secondary prevention of ischaemic stroke.
Table 1.7.1 Outcome events during the first 14 days in patients randomised to heparin (low or medium dose) or “avoid heparin” in IST (International Stroke Trial Collaborative Group, 1997); (values are percentages)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Heparin (n = 9716)</th>
<th>Avoid Heparin (n = 9717)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deaths and likely causes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial stroke</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Recurrent ischaemic stroke</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Haemorrhagic stroke</td>
<td>0.3</td>
<td>0.2*</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Extracranial haemorrhage</td>
<td>0.1</td>
<td>0.0*</td>
</tr>
<tr>
<td>Other vascular</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Non-vascular</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Total (any cause)</td>
<td>9.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Fatal and non-fatal events</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrent ischaemic stroke (RS)</td>
<td>2.9</td>
<td>3.8**</td>
</tr>
<tr>
<td>Haemorrhagic stroke (HS)</td>
<td>1.2</td>
<td>0.4***</td>
</tr>
<tr>
<td>RS or HS</td>
<td>4.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Death or non-fatal stroke</td>
<td>11.7</td>
<td>12.0</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>0.5</td>
<td>0.8*</td>
</tr>
<tr>
<td>Transfused or fatal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracranial haemorrhage</td>
<td>1.3</td>
<td>0.4***</td>
</tr>
</tbody>
</table>

Legend for table 1.7.1: ^ HS includes symptomatic intracranial haemorrhage or symptomatic haemorrhagic transformation of an infarct confirmed by CT scan, MRI or necropsy.

* 2p < 0.05; ** 2p < 0.01; *** 2p < 0.00001.
1.7.3 Low molecular weight heparins and heparinoids in acute ischaemic stroke

LMWHs are fragments of larger heparins, between 4,000 and 8000 Da, and heparinoids are sulphated glycosaminoglycans of similar size (Sherman, 1998). Both LMWHs and heparinoids bind to antithrombin III and inhibit clotting factor Xa, but compounds with a low concentration of glycosaminoglycans > 5000 Da in size exhibit limited activity against clotting factor IIa (Sherman, 1998). Therefore, there is theoretically a lower risk of bleeding associated with the use of LMWHs and heparinoids compared with unfractionated heparin (Sherman, 1998), and these compounds should be safer in patients with recent ischaemic stroke. LMWHs and heparinoids are almost completely absorbed after subcutaneous administration and they have a longer half life (1.4 to 5.9 hours, and 17 to 28 hours respectively) than unfractionated heparin (Sherman, 1998). This facilitates once or twice daily administration and their anticoagulant effect does not need to be monitored by laboratory testing (Kay et al, 1995).

Low molecular weight heparins

The efficacy of nadroparin calcium (fraxiparine, Sanofi-Winthrop, Gentilly, France), a LMWH given within 48 hours of acute ischaemic stroke, was assessed in a randomised, double-blind placebo-controlled trial known as the Fraxiparin in Stroke Study (FISS) (Kay et al, 1995). Patients were included regardless of the severity of their stroke, and were randomised to receive high-dose nadroparin (4100 anti-factor Xa IU twice daily, n = 102), low-dose nadroparin (4100 IU once daily, n = 101), or placebo (n = 105) subcutaneously for 10 days. All patients in the study were Chinese and randomisation was performed after admission to one of four participating centres in Hong Kong. Stroke subtype was determined according to the classification of Bamford et al (Bamford et al,
The primary outcome measure was death or dependency at six months. Secondary outcome measures included death, haemorrhagic transformation of cerebral infarction, any other complication within ten days of treatment (including recurrent ischaemic stroke), and death or dependency three months after randomisation. There was a significant 31% (95% confidence interval: 10 – 46%) reduction in the relative risk of death or dependency at six months in the high dose nadroparin group compared with those receiving placebo, whereas the difference between the low dose and placebo groups did not reach statistical significance. Early death, dependency, or early complications were equally common in the three subgroups of patients, as was the incidence of haemorrhagic transformation (observed in 9% of patients overall). The number of patients with early recurrent ischaemic stroke in each of the subgroups was too small to make any meaningful conclusions about the effect of nadroparin on early secondary stroke prevention. These encouraging results at six months led to a larger trial known as the Fraxiparine in Ischaemic Stroke Study (FISS bis) (Hommel, 1998). This larger trial, which contained only 766 patients, did not confirm the findings of the smaller study, although the results have only been published in abstract form. Patients were randomised to receive fraxiparine (86 IU / kg daily (low dose) or 86 IU / kg twice daily (high dose)) or placebo within 24 hours of onset of confirmed ischaemic stroke. Treatment was continued for 10 days, and patients with mild stroke or coma were excluded. Fraxiparine (low or high dose) did not significantly affect the rate of death or dependency compared with placebo, but “all site” haemorrhage and intracranial haemorrhage were more common with high dose fraxiparine compared with placebo. Although the authors reported that low dose fraxiparine was not associated with an excess of haemorrhage, the
significance levels or confidence intervals that facilitate this conclusion were not stated in the abstract. Therefore, there is currently insufficient evidence to recommend LMWH as a treatment modality to prevent early recurrent ischaemic stroke. However, further studies may be warranted to reassess the effects of LMWHs in early secondary stroke prevention.

**Heparinoids**

Heparinoids have not been proven to be of benefit for early secondary prevention in patients with acute ischaemic stroke. The TOAST study (Trial of ORG 10172 in Acute Stroke Treatment) was a randomised, double-blind placebo-controlled trial of a low molecular weight heparinoid, danaparoid sodium, in patients with acute ischaemic stroke (The Publications Committee for the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators, 1998). 1281 patients between 18 and 85 years of age received either the active drug or placebo intravenously within 24 hours of acute or progressing ischaemic stroke. An intravenous bolus of danaparoid was given and the dose adjusted after 24 hours to maintain the anti-factor Xa activity at 0.6 – 0.8 anti-factor Xa U/mL; treatment was continued as an infusion for seven days (Sherman, 1998); (The Publications Committee for the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators, 1998). The primary outcome measure was a favourable outcome at three months. Secondary outcome measures included a favourable or very favourable outcome at 7 days and three months, mortality at seven days and three months, or recurrent stroke or worsening neurological dysfunction within seven days. There was no significant difference in the primary outcome measure between the two groups (p = 0.49). Although significantly more patients in the danaparoid group had a very favourable outcome at 7
days compared with placebo (33.9% versus 27.8%, p = 0.01), active drug treatment was associated with more adverse events overall (8.5% versus 5.4%, p < 0.05). Danaparoid therapy increased the risk of major bleeding complications overall (3.4% versus 1%, p < 0.005) and the rate of major intracranial haemorrhage within the first 10 days compared with placebo (2.4% versus 0.8%, p < 0.05). Danaparoid did not reduce the rate of recurrent ischaemic stroke overall, but the authors reported the intriguing finding that patients with stroke secondary to large artery atherosclerosis appeared to benefit from active treatment. In this subgroup, patients treated with danaparoid more commonly had a favourable (p = 0.04) or very favourable (p = 0.02) outcome at 3 months after stroke compared with placebo. It was postulated that anticoagulation in these patients might help maintain collateral flow or prevent progression of thrombus in a stenosed vessel. However, it was disappointing that danaparoid did not significantly reduce the rate of early or late recurrent stroke in this subgroup, as these patients are at a particularly high risk of early stroke recurrence from artery-to-artery embolism (Moroney et al, 1998); (Sacco et al, 1989b). Therefore, the safety and efficacy profile of danaparoid is unfavourable in patients with acute ischaemic stroke overall, and there is no evidence that treatment significantly reduces early stroke recurrence. However, further large-scale studies are warranted to re-examine the potential benefit of this treatment in patients with acute atherothrombotic ischaemic stroke.
1.7.4 Anticoagulation in acute cardioembolic ischaemic stroke associated with nonvalvular atrial fibrillation

Atrial fibrillation is the most common cause of cardioembolic ischaemic stroke (Brickner, 1996), and the incidence of atrial fibrillation in the general population increases with advancing age (6% of people over 65 years and 10% of people over 75 years of age) (Feinberg, 1998). Approximately 20% of patients with atrial fibrillation have rheumatic heart disease, 70% have NVAF associated with underlying non-rheumatic heart disease, and 10% have lone atrial fibrillation without obvious structural heart disease (Brickner, 1996). A number of studies have addressed this issue of early anticoagulation in patients with acute cardioembolic ischaemic stroke. Overall, none of the studies provide any convincing evidence of benefit from early anticoagulant therapy in this group of patients.

In 1983, the Cerebral Embolism Study Group performed a randomised prospective study to assess the risks and benefits of anticoagulation within 48 hours of cardioembolic stroke (Cerebral Embolism Study Group, 1983). Patients were randomised to receive immediate anticoagulation with heparin or no anticoagulation for 10 days, and 56% of patients had NVAF as the presumed embolic source. The anticoagulation group received a bolus injection of intravenous heparin (5,000 – 10,000 IU) followed by a continuous infusion of heparin adjusted to prolong the Activated Partial Thromboplastin Time (APTT) to 1½ to 2½ times the pretreatment level. All patients had a repeat brain CT after 4 to 10 days of therapy, unless an earlier scan was indicated. The study was terminated after only 24 and 21 patients were randomised to receive anticoagulation or no anticoagulation respectively, because the authors were convinced by their preliminary data and by the results from other retrospective studies that immediate anticoagulation
was safe and effective in this setting (Cerebral Embolism Study Group, 1983). Nevertheless, from the data that had been collected at that stage, the authors concluded that early anticoagulation reduced the rate of early recurrent embolism from 10% to 0% without any associated major bleeding complications.

In a separate analysis of five retrospective studies, the risk of recurrent cardioembolic stroke was reduced from 15% to 5% with early anticoagulation (Cerebral Embolism Study Group, 1984). These investigators also published the results of a separate analysis of 30 cases of cardioembolic ischaemic stroke in which there had been either haemorrhagic transformation of the infarct or intracerebral haematoma formation (Cerebral Embolism Study Group, 1984). Most of the cases (77%) were studied retrospectively, and only 19 patients had received anticoagulant therapy (heparin or coumadin). The data were compared with those from a group of cardioembolic stroke patients without haemorrhagic infarct transformation to elucidate potential risk factors for intracerebral haemorrhage (ICH) after ischaemic stroke. It was estimated that 5% of cardioembolic stroke patients who are immediately anticoagulated experience clinical deterioration due to ICH, with this risk increasing to 20% in patients with large cardioembolic infarcts (Cerebral Embolism Study Group, 1984). The authors suggested that bolus doses of heparin or excessive anticoagulation be avoided to reduce the risk of ICH. Immediate anticoagulation was recommended for small or moderate sized embolic infarcts if a brain CT 24 hours after stroke onset had excluded ICH, suggesting that this is a safe and acceptable treatment modality. In addition, it was suggested that anticoagulation be postponed for 5 to 7 days in patients with a large cerebral infarct due to the higher risk of haemorrhagic transformation in these patients. If one accepts the
authors’ estimate that the risk of early stroke recurrence is between 10% to 15% without treatment, and the risk of ICH with immediate anticoagulation lies somewhere between 5 and 20% depending on the infarct size, then the risk–benefit ratio does not favour early treatment with anticoagulation except in those with small infarcts.

A number of other reports have suggested that early heparin therapy for acute cardioembolic stroke may be safe (Rothrock et al, 1989); (Chamorro et al, 1995) but perhaps of no clear benefit (Rothrock et al, 1989). In IST, 3169 patients were in atrial fibrillation at the time of randomisation. This is the largest group of patients with acute ischaemic stroke associated with atrial fibrillation randomised to receive unfractionated heparin or no heparin (International Stroke Trial Collaborative Group, 1997). Although heparin therapy reduced the rate of recurrent ischaemic stroke by 2.1% (2p < 0.01), this beneficial effect was negated by a higher rate of haemorrhagic stroke associated with its use (1.7%, 2p < 0.001).

In 1999, Chamorro et al. published a case series in which patients with acute ischaemic stroke and NVAF were treated with intravenous unfractionated heparin (n = 171, target APTT Ratio of 1.5 to 2) or 12,500 IU of subcutaneous heparin every 12 hours (n = 60) (Chamorro et al, 1999b). The purpose of this study was to investigate whether the timing of therapy influenced the outcome of treatment with anticoagulation in patients with acute cardioembolic ischaemic stroke. All patients had a brain CT performed before commencing treatment to exclude intracranial haemorrhage, and the decision to use intravenous or subcutaneous unfractionated heparin was left to the discretion of the treating physician. Thirty two per cent of patients received anticoagulation within six hours, and 68% between six and 48 hours of symptom onset. Treatment was continued
for five to ten days until a stable degree of oral anticoagulation was achieved. Therapeutic APTT ratios were achieved 24 hours after commencing treatment in 49% and 43% of patients on intravenous and subcutaneous heparin, respectively, but excessively high APTT ratios were more common with intravenous heparin therapy (33% versus 14%). Good functional recovery (modified Rankin score ≤ 1) was more common in patients treated within six hours compared with those treated between six and 48 hours after stroke onset. The mean APTT ratio was lower in patients with early recurrent ischaemic stroke compared with those without recurrent events (1.2 versus 1.6, p = 0.02), and higher in patients with haemorrhagic changes on follow up brain CT imaging compared with those without haemorrhage (2.1 versus 1.6, p < 0.01). Early infarct signs on the pre-treatment CT were equally common in patients with or without subsequent intracranial haemorrhage on anticoagulant therapy (63% versus 65%). The authors concluded that the improved functional outcome observed in patients treated with early (< 6 hours) compared with delayed (6 to 48 hours) anticoagulation suggested a neuroprotective effect of heparin. This study had several limitations in that the number of patients included was relatively small, they were not randomised to early or delayed anticoagulation or to the route of administration of heparin, and no placebo group was included. Although patients receiving subcutaneous heparin did have APTT monitoring in this study, it was not clearly specified how frequently this was performed or how treatment was modified according to the results that were obtained. In addition, the low rate of early stroke recurrence in this study (2.16% overall) precluded any firm conclusions regarding the relative effectiveness of intravenous or unfractionated heparin for early secondary prevention in patients with ischaemic stroke associated with NVAF.
There were too few patients with cardioembolic stroke (n = 43) in FISS to assess the effect of this LMWH on the incidence of early stroke recurrence and intracerebral haemorrhage (Kay et al, 1995). The Heparin in Acute Embolic Stroke Trial (HAEST) was the first trial to examine the effects of treatment with a low molecular weight heparin (dalteparin) in patients with acute cardioembolic ischaemic stroke (Berge et al, 2000). However, this trial failed to show that dalteparin was superior to aspirin at preventing early ischaemic stroke recurrence in this group of patients. Patients were randomised within 30 hours of onset of an acute ischaemic stroke in association with atrial fibrillation to receive either:

- 100 IU / kg of dalteparin subcutaneously twice daily and a placebo tablet every day (n = 224) or
- 160 mg of aspirin daily and placebo ampoules subcutaneously twice daily (n = 225).

Eighty nine per cent of patients in each treatment group were in atrial fibrillation at the time of assessment and almost all the remaining patients had documented atrial fibrillation within the two-year period prior to presentation. The percentage of patients with NVAF was not specified. All patients had a brain CT performed before randomisation to exclude intracranial haemorrhage, and repeat CT scanning was performed seven days after randomisation or at the time of clinical deterioration. The median duration of therapy was 13 days, and compliance with treatment was excellent (> 98%). The rates of recurrent ischaemic stroke (8.5% versus 7.5%), intracerebral haemorrhage (11.6% versus 14.2%), and death or dependency at 14 days and six months (approximately 60%) were comparable in the dalteparin and aspirin treated patients, respectively. However, intracerebral haemorrhage tended to be more severe, and
extracranial haemorrhage was more common with dalteparin compared with aspirin (5.8% versus 1.8%). Therefore, there is no evidence that dalteparin is superior to aspirin at preventing early recurrent ischaemic stroke associated with atrial fibrillation, and the higher risk of bleeding associated with dalteparin therapy does not justify its use in these patients.

In the cardioembolic stroke subgroup in the TOAST study, there was no significant difference in outcome between patients treated with danaparoid (n = 143) or placebo (n = 123) (The Publications Committee for the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators, 1998). However, there were too few recurrent cardioembolic ischaemic strokes within the first seven days in this study to draw any firm conclusions about the efficacy of danaparoid in this setting (0% vs. 1.6% in the danaparoid and placebo groups respectively).

1.7.5 Secondary prevention with warfarin in patients with ischaemic stroke and TIA

Warfarin exerts its anticoagulant action by inhibiting the vitamin K-dependent clotting factors II, VII, IX and X (Hirsh et al, 1994). Two large randomised clinical trials have examined the outcome of treatment with warfarin compared with aspirin for long-term secondary prevention of vascular events in patients with ischaemic stroke or TIA. Neither study provided evidence to support the routine use of warfarin in this patient population.

The Stroke Prevention in Reversible Ischaemia Trial (SPIRIT) was a randomised open-label multi-centre study in which patients with a minor ischaemic stroke or TIA within the preceding six months were randomly allocated to receive treatment with oral anticoagulant therapy or aspirin (The Stroke Prevention in Reversible Ischemia Trial
Patients with cerebral ischaemia in association with severe carotid stenosis or atrial fibrillation were not included in the study. The mean age of the patients was 63 years, and the mean duration of follow up was 14 months. Most patients in the anticoagulation group received phenprocoumon, although some were treated with acenocoumarol or warfarin. The target INR was 3.5 (range 3.0 to 4.5). In the aspirin group, 95% of patients received 30 mg daily, 3% received 75 mg daily and 2% received 100 mg daily. The primary outcome measure was the composite outcome of non-fatal stroke, non-fatal myocardial infarction, non-fatal major bleeding complications, or vascular death. The authors also compared the rates of major ischaemic events during follow up in the two treatment groups. The trial was discontinued prematurely when only 651 and 665 patients had been allocated to receive anticoagulant therapy or aspirin, respectively because of an excess of primary outcome events in the anticoagulant group (12.4% versus 5.4%, hazard ratio 2.3). There was a significant excess of major bleeding complications with anticoagulation compared with aspirin (8.1% versus 0.9%, hazard ration 9.3), without a significant reduction in major ischaemic events (4.1% in each group). The majority of major bleeding complications were intracranial (51%), and the risk of haemorrhage increased in association with an increasing INR. It was also of interest that an age greater than 65 years (hazard ratio 2.5) and the presence of leukoaraisosis on the baseline brain CT (hazard ratio 7.5) were important risk factors for the development of intracerebral haemorrhage on anticoagulant therapy.

The results of the Warfarin-Aspirin Recurrent Stroke Study (WARSS) have recently been published (Mohr et al, 2001). WARSS was a double-blind multicentre clinical trial in which patients were randomised to receive treatment with aspirin (325 mg
daily, n = 1103) or warfarin (INR 1.4 to 2.8, n = 1103) within 30 days of onset of an ischaemic stroke. Patients with severe carotid stenosis or presumed cardioembolic stroke were also excluded from this study. The primary outcome measure was the combined endpoint of recurrent ischaemic stroke or death from any cause. The mean age of the study subjects was 63 years and the mean duration of follow up was 2 years. The mean daily INR for patients treated with warfarin was 2.1 (median = 1.9). There was no significant difference in the percentage of patients who reached the primary endpoint between the aspirin (16%) and anticoagulation groups (17.8%) during the study period (p = 0.25). Although the rates of major haemorrhagic complications were similar in the aspirin and warfarin treated patients (1.5 versus 2.2 per 100 patient-years, respectively, p = 0.1), minor haemorrhages were less likely to occur with aspirin compared with warfarin (12.9 versus 20.8 per 100 patient-years, p < 0.001).

In contrast to non-cardioembolic ischaemic stroke, warfarin is the drug of first choice for long-term secondary prevention in patients who have had a recent TIA or minor ischaemic stroke associated with NVAF (EAFT (European Atrial Fibrillation Trial) Study Group, 1993). In the European Atrial Fibrillation Trial, 1007 patients were randomised to receive anticoagulation with warfarin (target INR 3.0, n = 225), aspirin (300 mg daily, n = 404) or placebo (n = 378) within three months of experiencing a TIA or minor ischaemic stroke (EAFT (European Atrial Fibrillation Trial) Study Group, 1993). Forty three per cent of patients were randomised within two weeks of the onset of their neurological symptoms. The annual risk of recurrent stroke was reduced from 12% with placebo to 4% with warfarin (p < 0.001). However, although there was a trend towards a beneficial effect consistent with other trials of aspirin in stroke patients without
atrial fibrillation, aspirin did not significantly reduce the risk of recurrent stroke compared with placebo (10% vs. 12% per annum, \( p = 0.31 \)). Anticoagulation was more hazardous than antiplatelet therapy, with an annual incidence of major bleeding complications of 2.8% and 0.9% in patients on warfarin and aspirin respectively. Although some authors suggest commencing immediate anticoagulation with warfarin in patients with cardioembolic ischaemic stroke associated with NVAF, there are a number of reasons why one cannot extrapolate the results of this study to support this conclusion. The majority of patients in the study received delayed anticoagulation, and depending on the dose, the optimal anticoagulant effect of warfarin may be delayed for 1-7 days after the drug is administered (Hirsh \textit{et al}, 1994). Thus, patients may be denied treatment that is proven to be of modest benefit (i.e. aspirin) (International Stroke Trial Collaborative Group, 1997); (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997) during this therapeutic window period if warfarin alone is given. If heparin is commenced concurrently with warfarin in the acute phase after cardioembolic ischaemic stroke, the increased risk of bleeding associated with both of these treatment regimens (EAFT (European Atrial Fibrillation Trial) Study Group, 1993); (International Stroke Trial Collaborative Group, 1997) could theoretically increase the risk of intracerebral haemorrhage or haemorrhagic transformation of the infarct. It has also been reported that approximately one third of patients with stroke in association NVAF have another underlying cause for their stroke (Bogousslavsky \textit{et al}, 1990), and early anticoagulation in these patients may be inappropriate.

In summary, although there is some suggestion that low dose unfractionated subcutaneous heparin is of some benefit in preventing recurrent ischaemic stroke overall
(International Stroke Trial Collaborative Group, 1997), this cannot be recommended routinely for patients with acute ischaemic stroke at the present time. In addition, the available randomised evidence suggests that there is no net benefit from immediate anticoagulation with unfractionated heparin, LMWH or heparinoids in patients with ischaemic stroke with or without associated atrial fibrillation, except perhaps in those with small areas of infarction. These conclusions are consistent with the results of a recent overview of completed trials of anticoagulation in patients with acute ischaemic stroke (Gubitz et al, 1999). The anticoagulants studied included unfractionated heparin, LMWHs, subcutaneous and intravenous heparinoids, and specific thrombin inhibitors. The effects of treatment on the rate of recurrent ischaemic stroke or symptomatic (fatal and non-fatal) intracranial haemorrhage were calculated from an analysis of 21,605 and 22,794 patients respectively (Gubitz et al, 1999). Immediate anticoagulation was associated with about 9 fewer recurrent ischaemic strokes per 1000 patients treated, but this was offset by an identical increase in the rate of symptomatic intracranial haemorrhage. Therefore, immediate anticoagulation did not lead to any net reduction in the risk of early stroke recurrence. In addition, the available evidence does not support the routine use of warfarin in preference to aspirin for secondary prevention in patients with non-cardioembolic ischaemic stroke because of the additional cost, the risk of haemorrhagic complications and the need for close INR monitoring. Although warfarin is the treatment of first choice for long-term secondary prevention in patients with proven cardioembolic ischaemic stroke, there is still uncertainty regarding the optimal time to commence anticoagulant therapy in these patients.
1.8 Aims of thesis

The studies described in this thesis were designed to investigate several important aspects of platelet activation and function in ischaemic stroke, TIA and asymptomatic severe (≥ 70%) carotid stenosis that had not been fully addressed by previous studies in the literature. We carried out a number of observational analytical studies in our patient population to address the following issues:

1. To compare the degree of platelet and endothelial activation in patients in the acute and convalescent phases after ischaemic stroke or TIA with that seen in control subjects without a history of stroke, TIA or asymptomatic carotid stenosis.

2. To compare the degree of platelet and endothelial activation in patients with asymptomatic severe carotid stenosis with those with symptomatic severe carotid stenosis.

3. To determine whether a novel platelet function analyser, called the PFA-100®, could identify platelet hyper-reactivity ex vivo in patients with ischaemic stroke or TIA compared with a group of control subjects.

4. To assess the percentage of patients who are ‘responsive’ to the antiplatelet effects of aspirin therapy ex vivo using the PFA-100®, and to investigate the influence of von Willebrand factor levels on the results of this assay in ischaemic CVD patients.

5. To investigate whether the percentage of reticulated platelets is increased in the acute and convalescent phases after an ischaemic stroke or TIA, and to examine the influence of different antiplatelet regimens and different aspirin doses on the results of this assay.

6. To investigate whether the ex vivo response to aspirin therapy varies with incremental doses of the drug using both the PFA-100 and the established method of optical platelet
aggregometry. We also carried out a pilot study to determine whether the flow cytometric expression of a number of platelet activation markers varied with incremental doses of aspirin.
2. General Methods

2.1 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.

2.1.1 Patients with ischaemic stroke or TIA

Inclusion criteria

Patients who were older than 18 years of age, had experienced an ischaemic stroke or transient ischaemic attack (TIA) within the preceding four weeks, and were likely to be available for follow-up at least three months after symptom onset, were included in the study. The underlying mechanism responsible for the ischaemic stroke or TIA was categorised as atherothrombotic (Whisnant et al, 1990), lacunar (Whisnant et al, 1990; Inzitari et al, 2000), cardioembolic (Whisnant et al, 1990), or indeterminate using slight modifications of the National Institute of Neurological Disorders and Stroke diagnostic criteria (Whisnant et al, 1990). Patients were classified as having (i) atherothrombotic stroke or TIA in association with severe ipsilateral (≥ 70%) carotid stenosis or occlusion on colour Doppler Ultrasound imaging (see section 3.2.3); (ii) lacunar stroke or TIA i.e. one of the 5 classical lacunar syndromes in association with an anatomically compatible infarct on CT or MRI < 1.5 cm in maximum diameter or normal neuroimaging findings (see section 1.1.3), or a lacunar infarct visible on CT or MRI in association with an appropriate clinical presentation; (iii) cardioembolic stroke or TIA in association with atrial fibrillation or a definite cardiac source of embolism e.g. a mobile
atrial septal aneurysm and a patent foramen ovale with no other cause for embolism identified; or (iv) indeterminate stroke or TIA – could not be classified under any of the above categories, or more than one potential cause for stroke / TIA identified.

The patients were recruited from the inpatient population in the Acute Brain Injury Unit at the National Hospital for Neurology and Neurosurgery, and from University College Hospital, The Middlesex Hospital, and Atkinson Morley’s Hospital, London. Suitable outpatients were recruited from the One-Stop Stroke Clinic at the National Hospital for Neurology and Neurosurgery.

2.1.2 Patients with asymptomatic severe carotid stenosis

Inclusion criteria

Patients were defined as having asymptomatic severe carotid stenosis if they had evidence of severe (≥ 70%) stenosis of the internal or common carotid artery noted on colour Doppler ultrasound that was not associated with symptoms of stroke or TIA in that or another vascular territory within the preceding three year period. Some of these patients also had an extracranial MRA to quantify the degree of carotid stenosis noted on colour Doppler ultrasound. One patient in this category had asymptomatic severe carotid restenosis noted on follow-up colour Doppler ultrasound examination 5 years after undergoing balloon angioplasty without stenting for asymptomatic severe atherosclerotic carotid stenosis. Other patients had a history of contralateral symptomatic severe carotid stenosis requiring carotid endarterectomy or endovascular treatment in the past, but all of these patients were free of symptoms of stroke or TIA for at least the preceding three years. Patients with asymptomatic severe carotid stenosis were recruited from an
established population of patients known to Professor Martin M. Brown at St. George’s Hospital and Atkinson Morley’s Hospital, London, including some patients who were participating in the medical arm of the ongoing Asymptomatic Carotid Surgery Trial at University College London Hospitals. Other patients with asymptomatic severe carotid stenosis were recruited from the One-Stop Stroke Clinic at the National Hospital for Neurology and Neurosurgery, London.

All patients with ischaemic stroke, TIA or asymptomatic severe carotid stenosis were clinically assessed by the same examiner (DJHM) using a standardised protocol that was designed at the outset of the study. A detailed history of the presenting symptom(s) was taken. Information regarding vascular risk factors (previous stroke or TIA, ischaemic heart disease, hypertension, diabetes mellitus, peripheral vascular disease, atrial fibrillation or flutter, hyperlipidaemia, family history of stroke, history of migraine or prior venous thrombosis), smoking status (non-smoker, ex-smoker or current smoker) and alcohol intake was collected prospectively on all subjects. Hypertension was defined as a systolic or diastolic blood pressure reading greater than 140 mmHg or 90 mmHg, respectively, or a prior diagnosis of hypertension requiring treatment. Patients were considered to be diabetic if they had a definite prior diagnosis or a fasting blood glucose greater than 6.7 mmol/L. Peripheral vascular disease was diagnosed if patients had symptoms of intermittent claudication with reduced or absent pedal pulses, or if they had undergone prior treatment for peripheral vascular disease. Atrial fibrillation or flutter was classified as ongoing or paroxysmal. Patients were classified as hyperlipidaemic if the fasting cholesterol level was greater than 5.2 mmol/L, the fasting triglyceride level was
greater than 2 mmol/L, or if the diagnosis had been established prior to study entry. Information regarding medication intake, including statin use, was recorded.

**Exclusion Criteria**

Patients were excluded from the study if they had a history of primary intracerebral haemorrhage, a myocardial infarct within the preceding three months, major surgery or systemic haemorrhage within the preceding three months, or if they had systemic vasculitis, underlying neoplasia, or a known clotting diathesis. Patients with ongoing unstable angina, or unstable symptomatic peripheral vascular disease were also excluded.

### 2.1.3 Controls

**Inclusion criteria**

Control subjects of similar age to the stroke / TIA patient population were recruited from the staff at The Haemostasis Research Laboratory at the Department of Haematology, University College London Hospitals and from the local population. Spouses of patients and control subjects were also recruited. Control subjects were clinically assessed by a single examiner (DJHM) using a standardised protocol, and colour Doppler ultrasound of carotid and vertebral arteries was performed to rule out severe carotid or vertebral stenosis.

**Exclusion Criteria**

Subjects were excluded from the control group if they had a history of stroke or TIA in the past, had evidence of carotid or vertebral artery stenosis, or if they had a myocardial infarction or major surgery within the preceding three months. They were also excluded
if they had ongoing symptoms of unstable angina or peripheral vascular disease, or if they had a history of systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis.

2.1.4 Ethical approval
The study was approved by the Local Research Ethics Committee at the National Hospital for Neurology and Neurosurgery, University College London Hospitals NHS Trust, and St George's Hospital NHS Trust. 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 of the patient, where appropriate) was obtained in all cases.

2.2 Sample collection and separation

2.2.1 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 I performed careful venepuncture 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 into a sterile Vacutainer® tube containing 7.2 mg of K₂ EDTA or 0.054 ml of 15% K₃ EDTA. This sample was used for measurement of a full blood count (FBC), including measurement of the mean platelet volume (MPV). Four further 4.5 ml samples were collected into sterile Vacutainer® tubes containing 0.5 ml of 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 4.5 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). The next two citrated samples were used to prepare platelet poor plasma (PPP) (see section 2.2.2). The remaining sample was used for measurement of the platelet count and MPV in citrate-anticoagulated whole blood. We planned to perform FBC measurements between 2 and 4 hours after venepuncture, because EDTA-induced platelet swelling is maximal in the first two hours, and increases in the first 24 hours after blood sampling (O'Malley et al, 1995).

When aggregometry was performed, three additional 4.5 ml samples were drawn into 0.105 M sodium citrate Vacutainer® tubes to prepare platelet rich plasma (PRP) and PPP (see section 2.2.2).

2.2.2 Sample separation

Separation of plasma samples was performed in all subjects within 60 minutes of venepuncture and the samples were frozen within 90 minutes of venepuncture, unless stated otherwise. Buffy coat was prepared within 4 hours of sample collection.

Platelet poor plasma (PPP)

PPP was prepared from two 0.105 M buffered sodium citrate anticoagulated blood samples. The samples were centrifuged at 2250 g 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 5 ml Röhren® polypropylene tube (Sarstedt®, Germany). The sample was centrifuged again at 2250 g for 15 min. Double-spun PPP was then recovered.
from the upper two thirds of this sample, aliquoted into four 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.

**Platelet rich plasma (PRP)**

PRP was prepared from three 0.105 M buffered sodium citrate anticoagulated blood samples. Samples were centrifuged at 190 g for 10 minutes at room temperature. PRP was removed from the upper two thirds of the samples with a plastic Pasteur pipette and stored at room temperature in a capped polystyrene tube (L.I.P. Ltd, U.K.). The residual blood samples were spun at 2250 g for 15 min to obtain PPP. Autologous PPP was then added to the PRP sample to adjust the platelet count to 200 x 10^9/L for use in aggregometry experiments.

**Buffy Coat**

Buffy Coat was prepared from the K_2 or K_3 EDTA anticoagulated whole blood sample by centrifugation at 2250 g for 15 min. The PPP was initially discarded, and the buffy coat was carefully removed using a plastic Pasteur pipette, placed in a polypropylene tube and immediately frozen at -70°C (Sarstedt®, Germany).

**2.3. Flow Cytometry**

**2.3.1 General principle**

The flow cytometer used in this study was a Coulter® EPICS® XL-MCL (Beckman Coulter United Kingdom Ltd.), and the information presented 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, 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 (Figure 2.3.1).

A 488 nm argon ion laser is used in the Coulter® EPICS® XL-MCL. 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 (Figure 2.3.2). 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, 1994).

Figure 2.3.1 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 artifactual in vitro platelet activation and potential loss of platelet subpopulations than methods which use PRP or washed platelets (Michelson, 1996).

The fluorochromes and fluorescent dye (Thiazole Orange (TO)) used for whole blood flow cytometric analysis in this thesis are listed in table 2.3.1. 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, 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 and 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
Figure 2.3.2: Diagrammatic representation of the optical system of a Coulter® EPICS® XL flow cytometer (redrawn from reference: Beckman Coulter United Kingdom Ltd, 1999)

Legend for Figure 2.3.2: FS = forward scatter; SS = side scatter, PMT = photomultiplier tube. FL1, FL2, FL3 and FL4 = PMTs that detect light at 525 nm, 575 nm, 620 nm and 675 nm, respectively
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 section 2.3.6). For each assay, the ‘antibody positive population’ was determined by using an analysis marker placed to the right of a histogram from a matched control sample (Michelson and Shattil, 1996) (see section 2.3.3). 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, 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 and 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 period.
2.3.2 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 ≤1.6 on the relevant histograms was deemed acceptable.

Table 2.3.1 Fluorochromes / fluorescent dye used in flow cytometry

<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 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-phycoerhthrin-Cy5 (RPE-Cy5)*</td>
<td>670</td>
<td>Red</td>
<td>FL4</td>
</tr>
</tbody>
</table>

Legend for Table 2.3.1 *RPE-Cy5 is a combination of two fluorochromes (R-phycoerythrin (RPE) and Cyanine 5 (Cy5)) that are covalently coupled to one another. The argon ion Laser excites the RPE at 488 nm, and the emitted light energy excites the Cy5 closely bound to the RPE molecule. The Cy5 then fluoresces at 670 nm and the FL4 detector detects this fluorescence.
2.3.3 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. As mentioned earlier (section 1.4.5), the GpIbα (CD42b) subunit of the GpIb-IX-V complex is the predominant receptor for VWF 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, 1998). 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 (see below), the reduction in GpIb binding was not quantified in this thesis.

**Reagents**

- Anti-IgG1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France)
- Anti-CD42b-PE mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France)
- 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 49.75 ml of 0.9% NaCl. The fixative was freshly prepared each day and filtered with a 0.2 μm filter to remove dust particles and other debris

**Method**

5 μl of citrate anticoagulated whole blood was aliquoted into the control and test polypropylene tube within 45 minutes of venepuncture. 5 μl of 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 on the flow cytometer (Figure 2.3.3 I). 10,000 platelet events
were analysed in this and all other assays using a low flow rate on the flow cytometer,
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 (Figure 2.3.3 II). To confirm that the
cells within the gate in the test sample were platelets (Figure 2.3.3 III), 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% (Figure 2.3.3 IV). If the
% GpIb binding was < 95%, the process was repeated until ≥ 95% GpIb positivity was
obtained for the test sample. The median %GpIb binding for all of the samples analysed
in the study was 99% (interquartile range 98.5% - 99.4%).

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 GpIb positive cells. This facilitated single
labelling of platelets with the fluorochrome-conjugated monoclonal antibody of interest.
**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 α-or dense-granule secretion, and the CD63 antigen is expressed on the platelet surface after the release of 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, flow cytometry of whole blood 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)
- Anti-CD62P-PE mouse monoclonal antibody (IgG₁) (Immunotech, Beckman Coulter, Marseille, France)
- 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-IgG1-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 (Figure 2.3.4 I). The non-specific fluorescence of the control sample was calculated, and the % CD62P positivity was then measured in FL2 (Figure 2.3.4 II).

**CD63**

**Reagents**

- Anti-IgG1-FITC isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France)
- Anti-CD63-FITC mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France)
- 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-IgG1-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 (Figures 2.3.5 I and 2.3.5 II).
Figure 2.3.3 I: Scatterplot of log FS versus log SS from a Gplb control sample identifies platelets within region B by their characteristic light scatter profiles; gate 'A' is manually positioned around the platelet cloud.

Figure 2.3.3 II: Histogram of cell count (Count) versus log fluorescence detected in FL2 from an anti-IgG1-PE isotype control sample to calculate the degree of non-specific background fluorescence of the control sample. The positive analysis function includes 0.5% of the control sample in region E.
Figure 2.3.3 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 2.3 I.

Figure 2.3.3 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
Figure 2.3.4 I: Scatterplot of log FS versus log SS from a CD62P test sample.

Figure 2.3.4 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.
**Figure 2.3.5 I:** Scatterplot of log FS versus log SS from a CD63 test sample.

**Figure 2.3.5 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.
2.3.4 PAC1 Binding

**Principle**

Platelet activation leads to a conformational change in the GpIIb/IIIa receptor (GpIIb/IIIa) that facilitates ligand binding, and this change is the final common pathway in platelet aggregation (Moran & FitzGerald, 1994). 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 (Abrams & Shattil, 1991; Moran & FitzGerald, 1994; Du & Ginsberg, 1997). Most monoclonal antibodies directed against GpIIb/IIIa bind to resting platelets. However, PAC1 is an IgM murine monoclonal antibody that does not bind to resting platelets, but appears to bind specifically to this RGD recognition site in GpIIb/IIIa exposed by a conformational change during platelet activation (Shattil et al., 1985; Abrams & Shattil, 1991). PAC1 and fibrinogen inhibit each other’s binding to GpIIb/IIIa, and small RGD containing peptides inhibit the binding of both proteins to the receptor (Abrams & Shattil, 1991). Therefore, 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. However, it must be remembered that PAC1 binds to only 10,000 to 25,000 sites on the platelet surface (Abrams & Shattil, 1991), whereas individual platelets possess up to 80,000 copies of the GpIIb/IIIa receptor on their surface membrane (Wagner et al., 1996).

**Reagents**

- Anti-IgM-FITC isotype control mouse monoclonal antibody (Sigma-Aldrich Inc., Saint Louis, USA)
- PAC1-FITC mouse monoclonal antibody (IgM κ) (Becton Dickinson, San Jose, USA)
- Phosphate Buffered Saline (PBS) Dulbeccos (without calcium or magnesium, pH 7.13) (Gibco BRL, Life Technologies, Paisley, UK)
- Fixative: 0.2% formalin saline solution

**Method**

Because the concentration of the anti-IgM-FITC isotype control antibody was 0.2 mg/ml, and the concentration of PAC1 was 0.025 mg/ml, 2.5 μl of the anti-IgM-FITC isotype control was diluted 1:8 with 17.5 μl of PBS Dulbeccos solution for use in the assay. 5 μl of the diluted anti-IgM-FITC isotype control antibody was added to the control tube and 5 μl of PAC1 to the test tube. 40 μl of PBS Dulbeccos solution was then added to each tube and the monoclonal antibody-buffer mixture was gently agitated and covered until ready to use. 5 μl of citrate anticoagulated whole blood was aliquoted into the control and test polypropylene tubes within 5 minutes of venepuncture. The samples were incubated at room temperature for 20 minutes, and then, 0.5 ml of fixative was added to each tube. The samples were analysed on the flow cytometer within two hours of fixation. 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 % PAC1 binding was then measured in FL1 (Figures 2.3.6 I and 2.3.6 II).
Figure 2.3.6 I: Scatterplot of log FS versus log SS from a PAC1 test sample.

Figure 2.3.6 II: Histogram of cell count versus log fluorescence detected in FL1 from the same sample; flow cytometric analysis showed that 10.4% of platelets in the region of interest in this patient sample exhibited PAC1 binding.
2.3.5 Leucocyte-platelet aggregates

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); (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). 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, 2000); (Furman et al, 2001), (Michelson et al, 2001). For these reasons, flow cytometry was used to measure the percentage of leucocytes-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)
- Anti-CD42b-PE mouse monoclonal antibody (IgG\textsubscript{1}) (Immunotech, Beckman Coulter, Marseille, France)
- Anti-CD45-RPE-Cy5 (Dako, Glostrup, Denmark)
- HBS
- 10 x Hanks Balanced Saline Solution (HBSS) without calcium, magnesium or phenol red (Gibco BRL, Life Technologies, Paisley, UK)
- Distilled H\textsubscript{2}O
- 10\% formaldehyde solution – 0.25 ml of 40\% formaldehyde solution diluted 1:4 with 0.75 ml of distilled H\textsubscript{2}O
- Diluent fixative – made up of 0.5 ml of 10\% formaldehyde solution, 0.6 ml of 10 x HBSS and 0.9 ml of distilled H\textsubscript{2}O.

**Method**

Because the concentration of the anti-IgG\textsubscript{1}-PE isotype control antibody was 6.25 \textmu g/ml, and the concentration of the anti-CD42b-PE monoclonal antibody was 1.56 \textmu g/ml, 5 \textmu l of the anti-IgG\textsubscript{1}-PE control antibody was diluted 1:4 with 15 \textmu l of HBS before use in the assay. The method used was based on one that had been established in our laboratory (Joseph *et al*, 2001). Initially, 5 \textmu l of diluted anti-IgG\textsubscript{1}-PE control antibody was aliquoted into the isotype control sample tube and 5 \textmu l of anti-CD42b-PE antibody into the test sample tube. 5 \textmu l of anti-CD45-RPE-Cy5 antibody (a pan-leucocyte marker), followed by 65 \textmu l of HBS were then added to both tubes, the samples were gently mixed, and covered until use. Within five minutes of venepuncture, 25 \textmu l 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 \textmu l 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 (Figure 2.3.7 I). 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 (Figure 2.3.7 II). 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 (Figures 2.3.7 III, 2.3.7 IV 2.3.7 V). A minimum of 1,000 monocyte events were analysed in each assay.
Figure 2.3.7 I: Only anti-CD45-RPE-Cy5-positive cells (leucocytes) are identified in region 'B' with RPE-Cy5 fluorescence detected in FL4.

Figure 2.3.7 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.
Figures 2.3.7 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.
2.3.6 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 & Penttilä, 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 (Michelson, 1996); (Ault *et al.*, 1992). 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 recently been shown that high concentrations of TO can non-specifically
label dense granules and possibly mitochondrial DNA within platelets, whereas low concentrations of the dye do not result in non-specific labelling (Robinson et al, 1998); (Robinson et al, 2000b); (Robinson et al, 2000a). 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 (Figure 2.3.8 I). 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 (Figure 2.3.8 II). The non-specific fluorescence of the control sample was calculated, and the % of TO-positive (reticulated) platelets in the test sample (Figure 2.3.8 III and Figure 2.3.8 IV) was then measured in FL1 (Figure 2.3.8 V).

**Figure 2.3.8 I: Measurement of the percentage of reticulated platelets in a healthy rested control subject**

![Graph showing the percentage of reticulated platelets over time](image)

**Legend for Figure 2.3.8 I:** Values are percentages and represent the mean of 3 measurements performed on the same blood sample that was kept at room temperature (% RP (room temp)), or in the fridge at 4 °C (% RP (4 °C); The timing on the x-axis refers to the interval between venepuncture and the beginning of sample processing.
Figure 2.3.8 II: Scatterplot of log FS versus 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 2.3.8 III: Scatterplot of log FS versus 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 2.3.8 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 2.3.8 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.
2.4 Platelet Function Analyser - PFA-100® (Dade-Behring, Germany)

2.4.1 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 (Kéényi *et al*, 1999). Platelet aggregometry has been considered to be the 'gold standard' test of platelet function over the past four 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) (see section 2.5 for a more detailed description of platelet aggregometry). However, because aggregometry is usually performed using PRP, platelet function is not 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.

2.4.2 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 flow 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.
Figure 2.4.1: Diagram of the *in vivo* haemostatic process and the PFA-100®

**Legend for Figure 2.4.1: 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 2.4.1 & Figure 2.4.2).
Figure 2.4.2: PFA-100® Cup-capillary system

Legend for Figure 2.4.2: 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); (Harrison et al, 1999); (Fressinaud et al,
1998), and by the platelet count and haematocrit (Harrison et al, 1999).

2.4.3 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 8.9% and 12.3% respectively.

2.4.4 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 group, 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 sample timing on the results obtained. For this reason, all further tests 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).

2.4.5 Review of the literature on the effects of aspirin therapy on the results of the PFA-100®

Several studies have investigated the effects of aspirin therapy on the results obtained with the PFA-100® in healthy controls (table 2.4.1). Kundu et al. reported that the C-EPI closure time was prolonged in seven normal control subjects for at least three days after ingestion of a single 325 mg dose of oral aspirin (Kundu S et al, 1994). The effect of aspirin on the C-APP closure time was not assessed in this small study. A subsequent preliminary study reported that the C-EPI closure time was prolonged in 23/24 subjects
(95.8%) who had ingested 325 mg of aspirin, but the C-ADP closure time was also prolonged in three of the 24 (12.5%) (Mammen et al, 1995). However, the authors did not specify the duration of aspirin therapy involved, or whether the subjects were normal controls or patients with pre-existing vascular disease. Mammen and colleagues later reported the results of a larger study in which 127 healthy controls had PFA-100® testing performed between two and 30 hours after ingestion of 325 mg of aspirin; 0.129 M buffered sodium citrate evacuated tubes were used in this study (Mammen et al, 1998). 94.5% of subjects had prolonged C-EPI closure times on aspirin, but 24.2% of these subjects also had prolonged closure times with the C-ADP cartridge. Harrison et al. found that five out of six (83%) healthy control subjects had prolonged C-EPI closure times two hours after ingesting a single 300 mg dose of aspirin using 0.105 M buffered sodium citrate Vacutainer® tubes (Harrison et al, 1999). In contrast to the other studies, none of the subjects had prolonged C-ADP closure times.

The effect of different aspirin doses on the results of the PFA-100® was investigated in a double-blind, two-way crossover study involving 10 healthy volunteers who were randomly assigned to receive 50 mg or 100 mg of oral aspirin daily for 11 days (Homoncik et al, 2000b). After a washout period of 16 days, the subjects were treated with the alternate dose of aspirin. All subjects received their dose of aspirin on the morning of the test, and PFA-100® analysis was carried out between 30 minutes and four hours after venepuncture throughout the 11 day period using 0.129 M buffered sodium citrate Vacutainer® tubes. The subjects also had serum thromboxane B2 levels measured to “confirm the inhibitory effect of aspirin” on cyclooxygenase activity. The C-ADP closure times were comparable in the two treatment groups and were not prolonged with
aspirin. On the other hand, both aspirin doses prolonged the C-EPI closure time to some degree, and the authors reported the interesting finding that the C-EPI closure times were significantly more prolonged with 100 mg compared with 50 mg of aspirin daily on days 3 and 4 of treatment. In addition, the maximal prolongation of the C-EPI closure time was seen on day 4 with 100 mg of aspirin, but not until day 10 with 50 mg of aspirin daily. In keeping with these results, thromboxane B₂ levels were significantly lower when patients were treated with 100 mg compared with 50 mg of aspirin daily. Although these findings suggest a more pronounced *in vitro* response to 100 mg compared with 50 mg of aspirin daily in healthy volunteers, the results should be interpreted with caution because there was no significant difference in C-EPI closure times between the two treatment groups at any other time point during the study. In summary, these studies suggested that aspirin prolonged the C-EPI closure time in at least 83% of healthy controls, with prolongation of the C-ADP closure time in up to 24%.

From a practical viewpoint, the PFA-100® has the potential to be useful for both monitoring and predicting the *in vitro* response to treatment in patients on long-term aspirin therapy. Because the response to aspirin may differ between patients and healthy controls, a number of studies have investigated the utility of the device in the clinical setting (table 2.4.1). A recent study performed on a diverse group of patients at a Tertiary Care Centre reported that only 68.2% of patients who were taking aspirin for a variety of clinical conditions had prolonged C-EPI closure times using a 0.129 M citrate blood collection system (Ortel *et al*, 2000). However, the dose of aspirin involved was not specified, and compliance was not assessed in a standardised fashion. von Pape *et al.* also reported that only 79% of patients who had discontinued long term treatment with 100
mg of aspirin daily within the preceding 24 hours had a C-EPI closure time > 170 s using 0.106 M sodium citrate Sarstedt Monovette tubes (von Pape et al, 2000). However, the authors did not report the percentage of patients who had C-EPI closure times above the upper limit of their established laboratory reference range of 150 s, and compliance was not formally assessed in this study either. The authors also stated that “an unexpected high number of patients” had prolonged C-ADP closure times above the laboratory normal reference range of 120 s.

Two recent studies have investigated the inhibition of platelet function with aspirin using the PFA-100® in patients with cardiovascular disease. In the first study, C-ADP and C-EPI closure times were measured in 48 patients admitted to hospital for diagnosis or treatment of coronary artery disease (Feuring et al, 1999). The proportion of patients in the study group with stable or unstable coronary artery disease was not stated. All patients were reportedly taking 100 mg of aspirin daily for at least seven days, and blood was collected into Sarstedt Monovette tubes containing 0.106 M unbuffered citrate; the samples were analysed within three hours of venepuncture. In contrast to the published results in healthy controls on aspirin (see above), only 31% of patients had prolonged C-EPI closure times above the upper range of normal for controls. Although not clearly stated in the report, a graphical representation of the data suggested that three patients (6.3%) had prolonged C-ADP closure times. It was concluded that 100 mg of aspirin daily led to insufficient inhibition of platelet aggregation in the majority of patients with coronary artery disease to be detected by the PFA-100®. However, the results of this study cannot be compared with those obtained from healthy controls in other studies, because it has previously been reported that unbuffered sodium citrate is not an ideal
Table 2.4.1 Summary of the relevant literature regarding the effects of aspirin therapy on the results obtained with the PFA-100®.

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>ASA Dose (mg)</th>
<th>Dur of Rx</th>
<th>Time from Last Dose (hrs)</th>
<th>C-ADP (% Prolonged)</th>
<th>C-EPI (% Prolonged)</th>
<th>Reference</th>
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<td>7*</td>
<td>325</td>
<td>1 Dose</td>
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<td>N/S</td>
<td>100</td>
<td>Kundu S et al, 1994</td>
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<tr>
<td>24*</td>
<td>325</td>
<td>N/S</td>
<td>N/S</td>
<td>12.5</td>
<td>95.8</td>
<td>Mammen et al, 1995</td>
</tr>
<tr>
<td>127*</td>
<td>325</td>
<td>1 Dose</td>
<td>2 to 30</td>
<td>24.2</td>
<td>94.5</td>
<td>Mammen et al, 1998</td>
</tr>
<tr>
<td>6*</td>
<td>300</td>
<td>1 Dose</td>
<td>2</td>
<td>0</td>
<td>83</td>
<td>Harrison et al, 1999</td>
</tr>
<tr>
<td>88**</td>
<td>N/S</td>
<td>N/S</td>
<td>N/S</td>
<td>8</td>
<td>68.2</td>
<td>Ortel et al, 2000</td>
</tr>
<tr>
<td>48***</td>
<td>100</td>
<td>≥ 7 days</td>
<td>N/S</td>
<td>6.3†</td>
<td>31</td>
<td>Feuring et al, 1999</td>
</tr>
<tr>
<td>325***</td>
<td>325</td>
<td>≥ 7 days</td>
<td>1 to 24</td>
<td>N/S</td>
<td>90.5</td>
<td>Gum et al, 2001</td>
</tr>
<tr>
<td>31****</td>
<td>300</td>
<td>&gt; 3 months</td>
<td>N/S</td>
<td>N/S†</td>
<td>83.9%</td>
<td>Grau et al, 2003</td>
</tr>
</tbody>
</table>

Legend for Table 2.4.1:

* Healthy control subjects;
** Patients with diverse clinical conditions on aspirin therapy;
*** Patients with coronary artery disease;
**** Patients in the convalescent phase after stroke;
N/S Data not shown or unclear in the published manuscript;
† Percentage calculated from Figure 1 in manuscript by Feuring et al, 1999
†† All stroke patients probably had normal C-ADP closure times on aspirin.
anticoagulant to use with this device. It shortens the C-EPI closure time in comparison
with that seen with other concentrations of citrate, and also significantly increases the
frequency of flow obstructions that may be secondary to abnormal platelet clumping
during the test (Heilmann et al, 1997). Therefore, these results should not be interpreted
as showing in vitro aspirin resistance in 69% of patients with cardiovascular disease. The
results of a larger study involving PFA-100® analysis in 325 patients with stable coronary
artery disease who were on treatment with 325 mg of aspirin daily for the preceding
seven days have also been published (Gum et al, 2001). 0.129 M citrate anticoagulated
blood was used for PFA-100® analysis. 31 patients (9.5%) did not have prolonged C-EPI
closure times above the laboratory normal range and were defined as ‘aspirin-resistant’. It
was interesting to note that only four of these 31 patients (13%) were also ‘aspirin-
resistant’ using optical platelet aggregometry, thus suggesting a cyclooxygenase-
independent mechanism responsible for ‘aspirin-resistance’ on the PFA-100® in the
majority of patients in this study.

Although the phenomenon of ‘aspirin resistance’ is well recognised and widely
discussed in the stroke literature, only one published study has examined the ex vivo
responsiveness to aspirin therapy using the PFA-100® in patients with cerebrovascular
disease (Grau et al, 2003). The results of this small study will be discussed in detail in
section 5.4.

2.5 Platelet aggregometry

2.5.1 General Principle

In order to study the process of platelet to platelet aggregation, Born and Cross developed
the method of optical platelet aggregometry to quantitatively assess the aggregation of blood
platelets in response to various agonists added to the sample in vitro (Born G.V.R. & Cross M.J., 1963). Since that time, optical aggregometry has become the ‘gold standard’ test of platelet function, and until recently, was the only widely available method used to study the effects of various antiplatelet agents on platelet function (Harrison, 2000). Blood is centrifuged gently to obtain platelet-rich plasma (PRP), which is then stirred in a glass cuvette at 37°C between a light source and a photocell in a modified spectrophotometer (Harrison, 2000); (Gum et al, 2001). When an agonist is added to PRP, the platelets aggregate and the transmission of light through the sample increases. The change in light transmission is detected photoelectrically and recorded on a chart over time. The addition of different agonists, such as collagen, ADP and epinephrine, that bind to specific platelet membrane receptors, allows the detection of certain aggregation defects that may be congenital or acquired. Arachidonic acid (sodium arachidonate) can also be added to the sample in vitro to investigate the ‘responsiveness’ to aspirin therapy in vivo. If platelet cyclooxygenase-1 activity has been irreversibly inhibited by aspirin, the addition of sodium arachidonate to PRP will not lead to the formation of thromboxane A$_2$ and platelet aggregation will not occur. On the other hand, if the subject is ‘resistant’ to the antiplatelet effects of aspirin and cyclooxygenase-1 activity has not been blocked, then sodium arachidonate will induce platelet aggregation in vitro. ADP, epinephrine, sodium arachidonate and collagen were chosen as the agonists for the aggregometry experiments that were performed to investigate the response to the various components involved in the PFA-100® test system (chapter 7). The final concentrations of these agonists were selected on the basis of their sensitivity to ASA inhibition, and were amongst those used for diagnosis of platelet function defects in our laboratory.
2.5.2 Equipment & Reagents

*Equipment*

- PAP-4 four channel platelet aggregometer (Bio/Data, USA)
- Sysmex KX-21 analyser for platelet counting (Sysmex U.K. Ltd., Milton Keynes, U.K.)
- Siliconised flat bottom aggregometry test tubes (7.25 x 55 mm) (Bio/Data, USA)
- 5 ml Röhren® polypropylene tubes (Sarstedt®, Germany)
- 1 ml flip-top polypropylene microcentrifuge tubes
- Plastic Pasteur pipettes
- Magnetic stir bars
- Ice bucket and fresh ice

*Reagents*

- NaCl 0.145 mol/l
- Distilled H₂O
- ADP (Sigma Lot 39F7215; Sigma Chemicals, St. Louis, USA): Aliquots of 10 mM stock solution were stored at -70°C in 5 ml Röhren® polypropylene tubes (Sarstedt®, Germany). One aliquot was thawed and stored on ice until use. The stock solution was diluted with 0.145 mol/l NaCl to give a final concentration of 50 μM prior to aggregation.
- Epinephrine (Adrenaline, Sigma Lot 119H3252; Sigma Chemicals, St. Louis, USA): Aliquots of 10 mM stock solution were stored at -70°C in 5 ml Röhren® polypropylene tubes (Sarstedt®, Germany). One aliquot was thawed and stored on ice
until use. The stock solution was diluted with 0.145 mol/l NaCl to give a final concentration of 100 μM prior to aggregation.

- Sodium arachidonate (99% Na salt: Sigma Lot 124H84252; Sigma Chemicals, St. Louis, USA): Aliquots of 20 mM stock solution were stored at -70°C in polypropylene microcentrifuge tubes. One aliquot was thawed and immediately stored on ice until use. The stock solution was diluted with distilled H₂O to give a final concentration of 10 mM prior to aggregation.

- Collagen (Collagen Reagent Horm) and ‘SKF Collagen Reagent Horm’ puffer buffer (Orgaman Teknika Ltd., U.K.). The 1 mg/ml stock solution of collagen and the SKF buffer were stored at 4°C. The collagen was diluted with the buffer to give a final concentration of 10μg/ml prior to aggregation.

2.5.3 Method

0.105 M citrate anticoagulated whole blood was collected, and PRP and platelet poor plasma (PPP) were prepared as described in section 2.2. The PRP and PPP were stored at room temperature in capped 5 ml Röhrren® polypropylene tubes until use. The platelet count in the PRP sample was measured on a Sysmex KX-21 analyser, and the PRP was diluted with autologous PPP to adjust the final platelet count to 200 x 10⁵/L.

The aggregometer was switched on, warmed to 37°C, and the stirring speed adjusted to 1000 rpm. The ‘Mode’ switch was depressed to return the aggregometer to the 'Ready' mode. 300μl of PPP was aliquoted into a cuvette without a stir bar, and 270μl of diluted PRP was pipetted into four cuvettes, each containing a magnetic stir bar. All of the cuvettes were warmed to 37°C in the aggregometer heating block for 3 minutes. The
PPP cuvette was placed in the first channel of the aggregometer, and the channel operation switch depressed again until the 'PPP Set' message was displayed. This set the '100% light transmission (100% aggregation)' baseline for this channel and the process was repeated for channels 2 - 4. The cuvettes containing PRP were then placed in channels 1 - 4, and the operation switch depressed again until the '0% light transmission (0% aggregation)' message was displayed for each channel. After a stable baseline tracing had been obtained, 30 µl of ADP, epinephrine, sodium arachidonate, and collagen were added to cuvettes 1 – 4, respectively. Because each of the reagents were diluted 1:10 when they were added to the cuvette, the final concentration of agonists used were: 5 µM ADP, 10 µM epinephrine, 1 mM sodium arachidonate, and 1 µg/ml of collagen. The aggregation tracing was recorded for 10 minutes, and stopped by depressing the channel operation button once again. The maximum percentage aggregation and the final percentage aggregation were recorded for each agonist. The stir bars were removed using a metal rod, and sterilised and stored in Decon 90 washing solution until further use (Decon Laboratories Ltd., UK).

2.5.4 Quality Control

Preliminary experiments in our laboratory have shown that sodium arachidonate is very unstable at room temperature after freeze-thawing, and will not induce platelet aggregation unless kept on ice until immediately prior to use (data not shown). Because almost all of the samples tested in this study were taken from subjects on aspirin therapy, in whom we expected aggregation to be inhibited, one had to ensure that the agonists were functional if platelet aggregation did not occur in response to one or all of them.
Therefore, a blood sample from a healthy normal subject, who had not taken aspirin or any other antiplatelet agent, was tested in parallel with a study sample at least once per week. On each occasion, the PRP from the healthy volunteer exhibited an expected pattern of aggregation with each of the agonists used.

In addition, a daily quality control check was performed on the Sysmex KX-21 analyser using a stabilised blood sample with a known platelet count (Sysmex Eight-Check™ fixed normal reagent, Sysmex U.K. Ltd., Milton Keynes, U.K.) to ensure that the calculated platelet count in PRP was accurate.

### 2.6 Enzyme Linked Immunosorbent Assays

#### 2.6.1 General Principle

When cells are activated, certain antigens that are expressed on or upregulated to the cell surface can be shed into the circulating blood. The concentration of the antigen in peripheral blood can be used as a marker of cellular activation and can be quantified using a variety of techniques, including sandwich Enzyme Linked Immunosorbent Assay (ELISA) (Bitsch et al, 1998). During an ELISA assay, an antibody that is specific for the antigen in question is bound to the wells of a microtitre plate. When the blood sample is added to the plate, the bound antibody ‘captures’ the antigen present in the sample. After washing off the excess sample that has not bound to the plate, another ‘detecting’ antibody that is conjugated to an enzyme such as horseradish peroxidase (HRP) is added to the plate. Subsequently, a substrate solution containing e.g. tetramethylbenzidine is added to the plate, and the reaction is stopped after a fixed period of time. The HRP bound to the detecting antibody cleaves the tetramethylbenzidine to produce a chromogenic product, and the colour change is then measured using a spectrophotometer.
The degree of colour change is proportional to the amount of detecting antibody bound to
the antigen, and by using suitable standards of known concentration, the amount of
antigen in the sample can then be quantified.

2.6.2 Quality Control

A quality control (QC) plasma provided with each of the commercial kits was
reconstituted according to the manufacturer’s instructions, and diluted 1:20 as per the test
samples (see below). The QC plasma was run on each plate, and the value obtained from
the standard curve was multiplied by the dilution factor to ensure that the result was
within the reference range stated by the manufacturer. All of the test sample assays were
performed in duplicate, and a coefficient of variation between duplicate tests of < 10%
was considered acceptable.

2.6.3 Soluble P-selectin

Principle

The selectins mediate some of the adhesive interactions of platelets, leucocytes and
endothelial cells. During the process of platelet activation, soluble P-selectin (sP-selectin)
is secreted directly into the plasma after the fusion of platelet α-granules with the platelet
surface membrane (Frijns et al, 1997). In addition, another isoform of platelet P-selectin
is first expressed on the cell surface after activation, before being shed into the circulation
by proteolysis (Frijns et al, 1997). Some sP-selectin is also secreted from Weibel-Palade
bodies during the process of endothelial cell activation (McEver et al, 1989); (Bonfanti et
al, 1989); (Fijnheer et al, 1997), although it has been suggested that endothelial cells do
not shed P-selectin into circulating blood unless endothelial cell destruction occurs (Pernerstorfer *et al.*, 2001). Therefore, the levels of sP-selectin in circulating blood can also be used as a measure of the degree of platelet, and to a lesser degree, endothelial cell activation, and can be quantified using a sandwich ELISA immunoassay.

**Reagents**

All reagents were obtained from R&D Systems as part of a Parameter® human soluble P-selectin immunoassay kit (R&D systems, Abingdon, U.K.):

- 96 well microtitre plate coated with a murine monoclonal antibody to human sP-selectin
- sP-selectin standards – six vials of lyophilised recombinant human sP-selectin, with blue dye and preservative (the concentrations of the standards were stated on the vials)
- Sample diluent – buffered protein base, with blue dye and preservative
- sP-selectin conjugate concentrate - sheep polyclonal antibody to recombinant human sP-selectin, conjugated to horseradish peroxidase (HRP) in buffer, with preservative
- HRP-Conjugate diluent, with red dye and preservative
- sP-selectin control – 1 vial of lyophilised human serum containing sP-selectin
- Wash buffer concentrate, with preservative
- Tetramethylbenzidine substrate solution
- Stop solution – 11 ml of acid solution

**Method**

Double spun PPP that had been collected and prepared as outlined in section 2.2 was warmed to 37°C. The sP-selectin standards were reconstituted immediately before use.
with 1 ml of deionised water, gently mixed, and allowed to stand at room temperature for at least 10 minutes until completely dissolved. The sP-selectin control was reconstituted with 500 μl of deionised water, gently mixed and allowed to stand at room temperature for at least 10 minutes also. The sP-selectin control sample and the test samples were diluted 1:20 with sample diluent, although occasionally a 1:40 dilution was required; all determinations were performed in duplicate. 100 μl of sP-selectin standard, diluted sP-selectin control or diluted test sample were added to each well of the microtitre plate. Then, 100 μl of diluted anti-sP-selectin conjugate concentrate was added, the wells were gently mixed, and the plates were incubated for 1 hour at room temperature. Each well was then aspirated and washed three times with 300 μl of wash buffer, before adding 100μl of tetramethylbenzidine substrate solution. After a further 15 minute incubation period at room temperature, the reaction was stopped by adding 100 μl of stop solution to each well. Absorbance values were read at 450 nm using an automated plate reader, with a wavelength correction set to either 620 or 650 nm to correct for optical imperfections in the microtitre plate. The mean absorbance values of the sP-selectin standards were used to plot the reference curve from which the concentrations of the test samples were calculated. The values obtained were multiplied by the dilution factor to calculate the test result. The manufacturer has reported an intra-assay CV for this method of 4.9 to 5.6%, with an inter-assay CV of 7.9 to 9.9%.

203
2.6.4 Soluble E-selectin

**Principle**

Endothelial-leucocyte adhesion molecule (E-selectin) is produced exclusively by endothelial cells in response to stimulation by various cytokines (Malik & Lo, 1996). It is expressed mainly on the endothelial cell surface (Pernerstorfer et al, 2001), and is involved in the adhesion of monocytes and neutrophils to the endothelium (Bitsch et al, 1998). Previous studies have reported elevated levels of circulating E-selectin in the acute and convalescent phases after ischaemic stroke (Frijns et al, 1997); (Fassbender et al, 1999). Because E-selectin is ultimately shed into the circulation by proteolytic cleavage, the levels of the adhesive protein can be quantified using a sandwich ELISA immunoassay. Plasma levels of soluble E-selectin (sE-selectin) were measured in parallel with the other markers of platelet activation to determine whether platelet and / or endothelial cell activation occurred in our study population.

**Reagents**

All reagents were obtained from R&D Systems as part of a Parameter® human soluble E-selectin immunoassay kit (R&D systems, Abingdon, U.K.):

- 96 well microtitre plate coated with a murine monoclonal antibody to human soluble E-selectin
- sE-selectin standards – six vials of lyophilised recombinant human sE-selectin with blue dye (the concentrations of the standards were stated on the vials)
- Sample diluent – buffered protein base, with blue dye and preservative
- sE-selectin conjugate concentrate - anti-human sE-selectin antibodies, conjugated to HRP in buffer, with preservative
• HRP-Conjugate diluent, with preservative
• sE-selectin control - 1 vial of lyophilised human serum containing sE-selectin
• Wash buffer concentrate, with preservative
• Tetramethylbenzidine substrate solution
• Stop solution – 11 ml of acid solution

**Method**

Double spun PPP that had been collected and prepared as outlined in section 2.2 was warmed to 37°C. The sE-selectin standards were reconstituted immediately before use with 1 ml of deionised water, allowed to stand at room temperature for at least 10 minutes, and then gently mixed until the contents were completely dissolved. The sE-selectin control was reconstituted with 500 µl of deionised water, allowed to stand at room temperature for at least 10 minutes, and then gently mixed. The sE-selectin control sample and the test samples were diluted 1:20 with sample diluent; all determinations were performed in duplicate. 100 µl of diluted anti sE-selectin conjugate was added to each well of the microtitre plate. Then, 100 µl of soluble E-selectin standard, diluted sE-selectin control or test sample were added and the wells were gently mixed. The plates were then incubated for 1.5 hours at room temperature. Each well was then aspirated and washed six times with 300 µl of wash buffer before adding 100 µl of tetramethylbenzidine substrate solution. After a further 30 minute incubation at room temperature, the reaction was stopped by adding 100 µl of stop solution. Absorbance values were read at 450 nm using an automated plate reader, with a wavelength correction set to either 620 or 650 nm. The mean absorbance values of the soluble E-selectin standards were used to plot the reference curve from which the concentrations of
the test samples were calculated. The values obtained were multiplied by the dilution factor to calculate the test result. The manufacturer has reported an intra-assay CV for this method of 4.7 to 5%, with an inter-assay CV of 5.7 to 8.8%.

2.7 Latex Agglutination Assay for von Willebrand Factor Antigen

2.7.1 von Willebrand Factor

von Willebrand Factor (VWF) is a multimeric plasma glycoprotein that is synthesised in either endothelial cells or megakaryocytes (Levy & Ginsburg, 2001). Endothelial cells secrete VWF constitutively into the circulating blood or into the subendothelial matrix, but they also release VWF stored in Weibel-Palade bodies in response to endothelial cell activation (Ruggeri, 1997). Megakaryocytes store VWF in α-granules, but do not constitutively secrete it. However, activated platelets release VWF from α-granules during the process of platelet degranulation (Ruggeri, 1997). Because VWF antigen levels have been found to be elevated in the acute and convalescent phase after stroke (Catto et al, 1997), and because the results of the PFA-100® are highly dependent on the VWF antigen levels in the sample (Kundu et al, 1995), accurate quantification of these levels was considered important in our study. VWF antigen was measured using an automated latex agglutination assay (STA Liatest® VWF, Diagnostica Stago, Asnières, France) (Veyradier et al, 1998); (Sukhu et al, 2000).

2.7.2 General Principle

The STA Liatest® VWF utilises polyclonal rabbit antihuman VWF antibodies that are covalently bound to latex particles. When a sample containing VWF antigen is added to the latex suspension, the particles bind to the antigen and form aggregates, thus causing a
decrease in light transmission. The degree of reduction in light transmission is proportional to the amount of VWF antigen present in the sample.

2.7.3 Method

The test was adapted and validated at the Haemostasis Research Unit at University College London for use on a Sysmex CA-1500™ fully automated coagulometer (Sysmex U.K. Ltd., Milton Keynes, U.K.). The assay protocol that was programmed into the analyser dispensed 20 µl of double spun PPP (prepared as outlined in section 2.2) and 60 µl of assay buffer into an analyser reaction tube. The sample was incubated for 60 seconds at 37°C prior to the addition of 100 µl of antibody-coated latex particles. The subsequent reaction was monitored kinetically at 575 nm, and the concentrations of VWF antigen in the test samples were derived from a standard curve that had been processed using Immuno Coagulation Reference Plasma (7 to 170 IU/dl) (Baxter AG, Vienna). Samples with VWF antigen levels outside the range of the standard curve were automatically reprocessed at a higher or lower dilution as appropriate (redilution was set to < 30 or > 140 IU/dl), and the results adjusted for the dilution factor used.

2.7.4 Quality Control

Immuno Coagulation Reference Plasma (declared potency 115 IU/dl) and Immuno Coagulation Control A (potency range 48 to 66 IU/dl) were tested as quality control (QC) preparations after every 20 patient samples. Although the number of QC results from this study was too small for meaningful statistical analysis, results from previous studies in our laboratory (n = 30) showed very low coefficients of variation (CVs) for both QC
preparations. The ‘within run’ CVs for the Immuno Coagulation Reference Plasma and the Immuno Coagulation Control A were 1.47% and 1.96%, respectively. The corresponding ‘between run’ CVs for the two QC preparations, where normal and abnormal samples were tested twice daily over a five day period, were 1.88% and 2.08%, respectively. The results of the STA Liatest® VWF correlated very closely with those obtained using a standard ELISA technique over a wide range of VWF antigen concentrations (10 to 700 IU/dl, $R^2 = 0.98$).

2.8 Routine Haematology Investigations

2.8.1 Full Blood Count

For each study subject, 4 ml of blood was drawn into a sterile Vacutainer® tube containing 7.2 mg of freeze-dried $K_2$ EDTA or 0.054 ml of 15% $K_3$ EDTA, and a further 4.5 ml sample was collected into a sterile Vacutainer® tube containing 0.5 ml of 0.105 M buffered sodium citrate, as described in section 2.2. A full blood count was performed in all subjects using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.), and a Sysmex XE-2100 haematology analyser (Sysmex U.K. Ltd., Milton Keynes, U.K.), because the two instruments have been shown to produce different MPV readings. 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 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.

2.8.2. Coagulation Assays

Patients who were receiving warfarin or heparin therapy had their INR and APTT ratios checked in the routine haematology laboratory at UCLH using standard techniques to determine whether they were adequately anticoagulated on treatment.
3. Flow cytometric evaluation of platelet activation in ischaemic stroke and TIA

3.1 Introduction

Platelet activation is a complex process that may involve platelet adhesion to a thrombogenic surface, shape change, secretion of the contents of alpha, dense or lysosomal granules, and platelet to platelet aggregation (Moran & FitzGerald, 1994). Within the past three decades, several studies have suggested that platelets are excessively activated, or hyper-reactive, in the acute (Dougherty, Jr. et al., 1977; Koudstaal et al., 1993); (van Kooten et al., 1994); (Iwamoto et al., 1995); (McConnell et al., 2001); (Uchiyama et al., 1994) and convalescent phases (Iwamoto et al., 1995); (van Kooten et al., 1999) after cerebral ischaemia. However, these conclusions are predominantly based on the results of platelet aggregometry studies in platelet rich plasma (PRP) (Dougherty, Jr. et al., 1977); (Uchiyama et al., 1994), plasma assays of soluble platelet activation markers, e.g. β-thromboglobulin (Iwamoto et al., 1995), or by measuring the urinary metabolites of thromboxane A2 (Koudstaal et al., 1993); (van Kooten et al., 1994); (McConnell et al., 2001); (van Kooten et al., 1999). Platelet aggregometry studies are semiquantitative, and although they may show that platelet 'reactivity' is altered in patients with cerebrovascular disease (CVD), they cannot determine whether the condition directly activates platelets (Michelson, 1996). Plasma assays of platelet activation markers such as β-thromboglobulin and platelet factor 4 involve sample centrifugation and manipulation, and are subject to artifactual \textit{in vitro} platelet activation with the potential loss of important platelet subpopulations (Michelson,
1996). In addition, urinary assays of metabolites of thromboxane A\textsubscript{2} only provide an indirect measure of platelet activation in these subjects.

The sensitive and specific technique of whole blood flow cytometry can be used to quantify the expression of several different surface markers of platelet activation. As outlined earlier, this method has the advantages of allowing analysis of platelets in the physiological milieu of whole blood, and is less susceptible to artifactual in vitro platelet activation and potential loss of platelet subpopulations than methods which use PRP or washed platelets (Michelson, 1996). CD62P is a protein that is expressed on the platelet surface membrane after α-or dense-granule secretion, and it is ultimately shed into the circulation by proteolysis (Frijns et al, 1997). In addition, another isoform of platelet P-selectin, soluble P-selectin (sP-selectin) is secreted directly into the plasma after the fusion of platelet α-granules with the platelet surface membrane (Frijns et al, 1997).

Platelet surface CD62P mediates the adhesion of platelets to leucocytes (de Bruijne-Admiraal et al, 1992) by binding to its predominant receptor on leucocytes called PSGL-1 (Furie et al, 2001). The CD63 antigen is expressed on the platelet surface after the release of lysosomes or dense granules (Grau et al, 1998); (Israels et al, 1992). Platelet activation may lead to a conformational change in the glycoprotein IIb/IIIa receptor (GpIIb/IIIa) that facilitates ligand binding, and PAC1 is an IgM murine monoclonal antibody that does not bind to resting platelets, but appears to bind specifically to the GpIIb/IIIa site exposed during platelet activation (Abrams & Shattil, 1991); (Shattil et al, 1985). Therefore, quantification of PAC1 binding may be used to assess the degree of activation of GpIIb/IIIa on platelets.
Most studies involving whole blood flow cytometric analysis of a small number of platelet activation markers have suggested that platelets are excessively activated in the acute (Grau et al, 1998); (Zeller et al, 1999); (Meiklejohn et al, 2001); (Marquardt et al, 2002); (Garlichs et al, 2003), subacute (Yamazaki et al, 2001); (Marquardt et al, 2002), or convalescent phases (Konstantopoulos et al, 1995); (Grau et al, 1998); (Meiklejohn et al, 2001); (Grau et al, 2003); (Garlichs et al, 2003) after ischaemic stroke or TIA. However, many of these studies can be criticised for their methodology (Konstantopoulos et al, 1995); (Zeller et al, 1999), limited patient numbers (Konstantopoulos et al, 1995); (Grau et al, 1998); (Grau et al, 2003), for not reassessing patients after the acute or subacute phase (Zeller et al, 1999); (Yamazaki et al, 2001); (Marquardt et al, 2002), or for only assessing patients in the convalescent phase after an ischaemic stroke or TIA (Konstantopoulos et al, 1995); (Grau et al, 2003). In addition, the available data have yielded conflicting results regarding the role of platelet activation in different subtypes of ischaemic stroke (Zeller et al, 1999); (Yamazaki et al, 2001); (Marquardt et al, 2002); (Grau et al, 2003). Other studies were either not designed to examine this issue (Meiklejohn et al, 2001); (Garlichs et al, 2003), or the classification of the different stroke subtypes was imprecise (Konstantopoulos et al, 1995). None of the published studies in stroke patients had measured the percentage of circulating monocyte-platelet complexes before the commencement of this thesis. These have been suggested to be a more sensitive indicator of in vivo platelet activation than either an increase in the percentage of neutrophil-platelet aggregates (Michelson et al, 2001), or CD62P expression on platelets in patients with acute myocardial infarction, or in those...
undergoing percutaneous coronary intervention (Michelson et al, 2001); (Michelson et al, 2000); (Furman et al, 2001).

To address some of the limitations of these studies, whole blood flow cytometry was used to quantify the expression of several markers of platelet activation, and leucocyte-platelet complex formation, in both the acute and convalescent phases after an ischaemic stroke or TIA. We also measured the levels of circulating markers of platelet and endothelial cell activation in our study population.

3.2 Methods

3.2.1 Patient Inclusion / Exclusion Criteria

Suitable consecutive inpatients and outpatients were included in this study if they were older than 18 years of age, had experienced an ischaemic stroke or TIA within the preceding four weeks (acute phase), and were likely to be available for follow-up at least three months after symptom onset (convalescent phase). We excluded patients with a history of primary intracerebral haemorrhage, myocardial infarction within the preceding three months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding three months, or if they had systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis.

3.2.2 Clinical Assessment

A detailed history of the presenting symptom(s) was taken, and all patients underwent a detailed general, neurological and vascular examination by the same examiner (DJHM) using a standardized protocol that was designed at the outset of the study. Information
regarding vascular risk factors, smoking status, alcohol intake, and medication use was collected prospectively on all subjects.

Results of routine haematological, coagulation, biochemical and blood glucose testing were collected. Brain CT or MRI, and colour Doppler ultrasound examination of carotid and vertebral arteries was performed in all patients. Patients with carotid stenosis on colour Doppler ultrasound also underwent extracranial magnetic resonance angiography to further quantify the degree of carotid stenosis. If the results of the ultrasound and MRA examinations were discordant, and the patient was a potentially suitable candidate for carotid endarterectomy or endovascular treatment, intra-arterial catheter angiography was carried out to quantify the degree of carotid stenosis. A chest radiograph and an electrocardiograph (ECG) were obtained in all patients, and transthoracic +/- transoesophageal echocardiography was carried out if a cardioembolic cause for the stroke or TIA was suspected, or when other investigations were uninformative. A 24-hour ECG recording was also performed if paroxysmal atrial fibrillation or flutter was suspected clinically. Intracranial MRA was performed in some patients in whom intracranial atherosclerosis was suspected.

3.2.3 Stroke Subtyping

The underlying mechanism responsible for the ischaemic stroke or TIA was categorised as atherothrombotic, lacunar, cardioembolic, or indeterminate using slight modifications of the National Institute of Neurological Disorders and Stroke diagnostic criteria (Whisnant et al, 1990). Atherothrombotic CVD was defined as a stroke or TIA that occurred in the vascular territory supplied by a severe ipsilateral (≥ 70%) carotid stenosis.
or occlusion. Patients with carotid occlusion were only included in this category if no other cause for stroke or TIA was identified. The degree of carotid stenosis was graded using colour Doppler ultrasound imaging criteria that had been used for grading internal carotid artery stenosis in the Carotid and Vertebral Artery Angioplasty Trial (CAVATAS Investigators, 2001). The degree of internal carotid artery stenosis was graded using measurements of the peak systolic velocity (PSV), the end diastolic velocity (EDV), and the ratio of the PSV in the internal carotid artery (PSVICA) to that in the common carotid artery (PSVCCA). Patients were classified as having > 70% carotid stenosis if the PSVICA was greater than 2.1 m/s, the EDVICA was greater than 1.1 m/s, or the ratio of the PSVICA/PSVCCA was greater than 4.0. These criteria are almost identical to recently published criteria, with the exception that a peak systolic velocity of > 2.1 m/s rather than 2.3 m/s was used to identify an ICA stenosis of ≥ 70% (Sidhu & Allan, 1997). This PSV measurement has been shown to have a sensitivity of 94.8% and a specificity of 78.6% in identifying an ICA stenosis of ≥ 70% using the NASCET method of grading ICA stenosis on digital subtraction angiography (Moneta et al, 1993).

Patients were classified as having a lacunar stroke or TIA if they presented with a pure motor hemiparesis, pure sensory stroke, sensorimotor stroke, ataxic hemiparesis, or the dysarthria clumsy hand syndrome (Fisher, 1982); (Whisnant et al, 1990); (Inzitari et al, 2000) in association with an anatomically compatible infarct on CT or MRI < 1.5 cm in maximum diameter or normal neuroimaging findings (Whisnant et al, 1990). They were also included within this category if they had a lacunar infarct visible on CT or MRI in association with an appropriate clinical presentation. The cardioembolic category included patients with stroke or TIA in association with atrial fibrillation or a definite
cardiac source of embolism e.g. a mobile atrial septal aneurysm and a patent foramen ovale with no other cause for embolism identified (Whisnant et al, 1990). The indeterminate category included CVD patients who could not be classified under any of the above categories, or in whom more than one potential cause for stroke or TIA was identified.

3.2.4 Follow-up

Patients who were alive and willing to return for follow-up were subsequently reassessed at least three months after the onset of their stroke or TIA (convalescent phase). In the atherothrombotic subgroup, the convalescent phase follow-up was performed after carotid surgery or endovascular treatment, unless this treatment had been delayed for at least three months after the initial event.

3.2.5 Control subjects

The patient data were compared with those obtained from a group of control subjects who were recruited from the staff at The Haemostasis Research Unit, University College London Hospitals, and from the local population. Spouses of patients and control subjects were also recruited. Control subjects were clinically assessed by a single examiner (DJHM) using a standardised protocol, and they underwent colour Doppler ultrasound imaging of carotid and vertebral arteries. Subjects were excluded from the control group if they had evidence of severe (> 70%) carotid or vertebral artery stenosis. Otherwise, the exclusion criteria were identical to those applied to the patient group. It was expected that the control subjects would have a different vascular risk profile to the patient population.
3.2.6 Compliance with Antithrombotic Therapy

Because the majority of patients in the acute phase were assessed during their inpatient stay, compliance with antiplatelet or anticoagulant therapy was confirmed by checking the inpatient drug chart. All suitable outpatients in the acute and convalescent phase after symptom onset were phoned before their appointment to stress the importance of drug compliance in the week prior to assessment, and compliance in this group was assessed by history taking alone.

3.2.7 Ethical Approval and Consent

The study was approved by the Local Research Ethics Committees of the participating Hospitals. Written informed consent (or written assent by a relative of the patient, where appropriate) was obtained in all cases.

3.2.8 Blood Sampling and Laboratory Tests

All subjects were rested for at least 20 minutes before venepuncture, and blood sampling was performed as outlined in section 2.2.1. The initial EDTA sample was used for measurement of a FBC, including measurement of the MPV using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.). The first 4.5 ml 3.2% citrate anticoagulated blood sample was used for whole blood flow cytometric analysis. Platelets were distinguished from red and white blood cells by their light scatter characteristics (Michelson, 1996), and the cells were confirmed to be platelets using a monoclonal antibody to the GpIbα (CD42b) subunit of the GpIb-IX-V complex (Abrams
& Shattil, 1991); (Ruggeri, 1997); (Clemetson, 1997); (Escolar & White, 2000) (see section 2.3.3). Using the ‘panel set-up’ on the flow cytometer, the degree of platelet activation in the sample was assessed by measuring the percentage expression of CD62P and CD63 (section 2.3.3), and PAC1 binding (section 2.3.4). The percentages of neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes were also calculated as additional markers of platelet activation (Li et al, 1997); (Furman et al, 1998); (Joseph et al, 2001) (section 2.3.5). The next two citrated samples were used to prepare double spun PPP, and were immediately stored at −70 °C for subsequent measurements of the levels of sP-selectin, sE-selectin (section 2.6.3 and 2.6.4), and VWF:Ag (section 2.7.3) to determine whether platelet and / or endothelial cell activation occurred in our study population. A fourth citrate sample was used for measurement of the platelet count and MPV in citrate-anticoagulated whole blood using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.).

### 3.2.9 Statistical Methods

The Mann-Whitney U test was used for comparison of median values, and an unpaired t-test for comparison of mean values between groups. The chi-square test was used for comparison of proportions between groups, where appropriate. Spearman’s rank correlation analysis was performed to examine the relationship between the platelet surface expression of CD62P and (a) the percentage of circulating leucocyte-platelet complexes, and (b) the levels of circulating sP-selectin. Multiple linear regression analysis was used to examine the independent influence of different predictor variables on each of a number of outcome measures of interest, in turn. Logarithmic
transformations were taken before regression analyses, where appropriate, so that assumptions of normality were reasonably well met. All statistical calculations were performed using SPSS-10 for Windows or Microsoft Excel 97 data analysis software. A p value < 0.05 was considered statistically significant.

### 3.3 Results

Seventy-nine patients were assessed in the acute phase (1-27 days) and 70 patients were re-assessed in the convalescent phase (79-725 days) after an ischaemic stroke or TIA. One patient in the convalescent phase was assessed at 79 days after presentation, before undergoing carotid endovascular treatment, and one patient was assessed at 86 days after symptom onset because she was returning to her home Country. All remaining patients were reassessed at least 90 days after their initial presentation, as originally planned. Nine patients did not have repeat laboratory testing during the convalescent phase; four patients had died, three declined further investigations, one patient was too unwell to re-attend, and one had moved to a different Country. The results were compared with those obtained from 27 control subjects.

The clinical details of the study subjects, and the antithrombotic therapy they were receiving are outlined in Tables 3.1a and 3.1b. The mean age of the acute and convalescent CVD patients was significantly higher, and there was a higher proportion of men in the patient population compared with controls. As expected, most vascular risk factors, with the exception of diabetes mellitus, a positive family history of stroke, and a history of smoking, were more common in the acute and convalescent CVD patients compared with controls. However, the median alcohol intake per week was higher in control subjects than patients. The majority of patients were on treatment with variable
doses of aspirin monotherapy between 75 to 300 mg daily at the time of assessment (median daily dose = 150 mg in the acute phase and 75 mg in the convalescent phase). The three control subjects who were on aspirin monotherapy were taking 75 mg, 81 mg, and 150 mg daily, respectively, for at least nine months prior to assessment.
### Table 3.1a: Clinical Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Acute Patients</th>
<th>Convalescent Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 79</td>
<td>N = 70</td>
<td>N = 27</td>
</tr>
<tr>
<td>Mean Age (Years)</td>
<td>67*</td>
<td>66*</td>
<td>57</td>
</tr>
<tr>
<td>Gender (M / F)</td>
<td>44 / 35 (55.7%)*</td>
<td>39 / 31 (56%)*</td>
<td>8 / 19 (29.6%)</td>
</tr>
<tr>
<td>Stroke / TIA Subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atherothrombotic</td>
<td>19 (24%)†</td>
<td>16 (22.9%)†</td>
<td>0</td>
</tr>
<tr>
<td>Lacunar</td>
<td>20 (25.4%)†</td>
<td>19 (27.1%)†</td>
<td>0</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>12 (15.2%)‡</td>
<td>9 (12.9%)‡</td>
<td>0</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>28 (35.4%)‡</td>
<td>26 (37.1%)‡</td>
<td>0</td>
</tr>
<tr>
<td>Prior Stroke / TIA</td>
<td>27 (34.2%)‡</td>
<td>10 (14.3%)‡</td>
<td>0</td>
</tr>
<tr>
<td>IHD</td>
<td>18 (22.8%)*</td>
<td>13 (18.6%)</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>61 (77.2%)*</td>
<td>58 (82.9%)*</td>
<td>10 (37%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>16 (20.3%)</td>
<td>13 (18.6%)</td>
<td>2 (7.4%)</td>
</tr>
<tr>
<td>A Fib / Flutter</td>
<td>11 (13.9%)‡</td>
<td>7 (10%)‡</td>
<td>0</td>
</tr>
<tr>
<td>Fam. Hx. Stroke</td>
<td>29 (36.7%)</td>
<td>25 (35.7%)</td>
<td>11 (40.7%)</td>
</tr>
<tr>
<td>Prior DVT/PE</td>
<td>5 (6.3%)†</td>
<td>5 (7.1%)†</td>
<td>0</td>
</tr>
<tr>
<td>PVD</td>
<td>20 (25.3%)‡</td>
<td>17 (24.3%)‡</td>
<td>0</td>
</tr>
<tr>
<td>Migraine</td>
<td>11 (13.9%)</td>
<td>10 (14.3%)</td>
<td>2 (7.4%)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>21 (26.6%)</td>
<td>13 (18.6%)</td>
<td>7 (25.9%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>30 (38%)</td>
<td>32 (45.7%)</td>
<td>7 (25.9%)</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>28 (35.4%)</td>
<td>24 (34.3%)</td>
<td>13 (48.2%)</td>
</tr>
<tr>
<td>Alcohol Intake (Median Units /Week)</td>
<td>2*</td>
<td>0.25*</td>
<td>10</td>
</tr>
</tbody>
</table>

**Legend for Table 3.1a:** IHD, Ischaemic heart disease; A Fib / Flutter, Atrial fibrillation / flutter; DVT, Deep venous thrombosis; PE, Pulmonary embolism; PVD, History or clinical features consistent with peripheral vascular disease; *, p < 0.05 for comparison of patients with controls using Chi-squared testing (significant values are highlighted in bold); †, Statistical testing not applicable or not performed.
Table 3.1b: Antithrombotic Regimens in the Study Subjects

<table>
<thead>
<tr>
<th>Antithrombotic therapy</th>
<th>Acute Patients N = 79</th>
<th>Convalescent Patients N = 70</th>
<th>Controls N = 27</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA Monotherapy</td>
<td>55</td>
<td>45</td>
<td>3</td>
</tr>
<tr>
<td>DP Monotherapy</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clopidogrel Monotherapy</td>
<td>4</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Warfarin Monotherapy</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Dalteparin Monotherapy</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASA + DP</td>
<td>10</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>ASA + DP + Dalteparin</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>ASA + Dalteparin</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASA + Indomethacin</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASA + Azapropazone</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Diclofenac #</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>None of above</td>
<td>1**</td>
<td>1†</td>
<td>23</td>
</tr>
</tbody>
</table>

Legend for Table 3.1b: ASA, Aspirin; DP, Modified-release dipyridamole 200 mg twice daily; #, 75 mg twice daily for over 1 year; **, One patient in the acute phase had discontinued warfarin therapy 3 days earlier and was tested before commencing clopidogrel; †, One patient had discontinued clopidogrel after carotid endarterectomy.
The median % Gplb expression was similar in the acute (99%) and convalescent patients (99.2%) and the control group (99%), confirming that the majority of cells analysed on the flow cytometer in each group were platelets. The median % platelet surface expression of CD62P was significantly higher in both the acute (p = 0.02) and convalescent (p = 0.002) CVD patients compared with controls (Figure 3.1a). There were no significant differences in the median % platelet surface expression of CD63 between the acute or convalescent patients and controls (Figure 3.1b). However, although the median % PAC1 binding was similar in the acute patients and controls (p = 0.2), PAC1 binding was reduced in the convalescent patient group compared with controls (p = 0.03) (Figure 3.1c). The percentages of neutrophil-platelet or lymphocyte-platelet complexes were not significantly elevated in either acute or convalescent CVD patients (Figure 3.2a and Figure 3.2c). However, the median % of circulating monocyte-platelet complexes was significantly higher in both the acute and convalescent CVD patient group compared with controls (p = 0.02) (Figure 3.2b). The patient with the highest percentage of neutrophil-platelet (23%) and monocyte-platelet (73%) complexes in the acute phase had a lacunar stroke with a history of mild hypertension, hyperlipidaemia and excess alcohol intake. These percentages fell to within the control range during follow-up.

There were no significant differences in median levels of sP-selectin or sE-selectin between the acute or convalescent CVD patients and controls (Figure 3.3 and Figure 3.4). However, the mean VWF:Ag levels were significantly elevated in both the acute and convalescent phases after ischaemic stroke or TIA (p < 0.001) (Figure 3.5).
Figure 3.1a: Comparison of median % CD62P expression between Control subjects (n = 27) and Acute (n = 79) and Convalescent (n = 70) CVD patients

Legend for Figure 3.1a: CVD, cerebrovascular disease; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.1b: Comparison of median % CD63 expression between Control subjects (n = 27) and Acute (n = 79) and Convalescent (n = 70) CVD patients.

Legend for Figure 3.1b: CVD, cerebrovascular disease; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.1c: Comparison of median PAC1 binding between Control subjects (n = 26) and Acute (n = 70) and Convalescent (n = 70) CVD patients

Legend for Figure 3.1c: CVD, cerebrovascular disease; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.2a: Comparison of median % Neutrophil-Platelet Complexes between Control subjects (n = 27) and Acute (n = 77) and Convalescent (n = 68) CVD patients

Legend for Figure 3.2a: CVD, cerebrovascular disease; % NPhil-Plts, % Neutrophil-Platelet Complexes; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.2b: Comparison of median % Monocyte-Platelet Complexes between Control subjects (n = 27) and Acute (n = 77) and Convalescent (n = 68) CVD patients

Legend for Figure 3.2b: CVD, cerebrovascular disease; % Mono-Plts, % Monocyte-Platelet Complexes; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.2c: Comparison of mean % Lymphocyte-Platelet Complexes between Control subjects (n = 27) and Acute (n = 74) and Convalescent (n = 68) CVD patients

Legend for Figure 3.2c: CVD, cerebrovascular disease; % Lym-Plts, % Lymphocyte-Platelet Complexes; Symbols represent individual values; Bold horizontal lines represent mean values.
The mean white cell count (p = 0.001, p = 0.02), including the mean neutrophil (p = 0.001, p = 0.048) and monocyte counts (p = 0.001, p = 0.007) in EDTA-anticoagulated blood were significantly higher in the acute and convalescent CVD group compared with controls. Otherwise, there were no significant differences in any other FBC parameters in either EDTA- or citrate-anticoagulated whole blood between patients and controls (data not shown).

Spearman’s rank correlation analysis revealed a positive correlation between the percentage expression of CD62P and the percentages of both neutrophil-platelet and monocyte-platelet complexes, but not lymphocyte-platelet complexes in the acute CVD patients. In the convalescent CVD group, there was a significant positive correlation between the percentage expression of CD62P and the percentages of all subtypes of circulating leucocyte-platelet complexes (table 3.2). There was no significant relationship between the percentage expression of CD62P and the levels of sP-selectin in either the acute or convalescent phase after ischaemic stroke or TIA (table 3.2).

Certain demographic features and vascular risk factors were more common in patients than control subjects, and could have partly accounted for the significant differences in the percentages of CD62P, monocyte-platelet complexes, and VWF:Ag levels between acute or convalescent CVD patients and controls. Therefore, multiple linear regression analysis was used to examine the influence of age, gender, ischaemic heart disease, hypertension, atrial fibrillation or flutter, previous venous thrombosis and peripheral vascular disease on the results obtained. Having controlled for all of these factors individually, significant differences in the percentage expression of CD62P (p ≤ 0.02), monocyte-platelet complexes (p < 0.04), and VWF:Ag levels (p < 0.001) remained
between the acute CVD patients and controls. However, the mean percentage difference (95% confidence interval) in monocyte-platelet complexes was no longer significantly higher in convalescent CVD patients compared with controls, after adjusting independently for age (21% higher (-0.2% to 46%), p = 0.053), the presence of peripheral vascular disease (12.8% higher (-20% to 58.9%), p = 0.5), or hypertension (21.2% higher (-0.6% to 47.9%), p = 0.06). None of the other independent variables significantly influenced the differences in the percentages of CD62P, monocyte-platelet complexes, or VWF:Ag levels between convalescent CVD patients and controls. Of interest, having controlled for the higher monocyte count in EDTA-anticoagulated whole blood in CVD patients, the percentages of circulating monocyte-platelet complexes remained higher in both the acute (p = 0.02) and convalescent CVD patients (p = 0.047) compared with controls.

We also compared the levels of platelet and/or endothelial activation markers in the different ischaemic stroke subtypes with controls. With the exception of the patient subgroup in the acute phase after an atherothrombotic ischaemic stroke or TIA, at least one marker of platelet activation was significantly increased in both the acute and convalescent phases after symptom onset (Table 3.3). However, there was a non-significant trend towards higher levels of CD62P (p = 0.2), CD63 (p = 0.4), neutrophil-platelet (p = 0.8), monocyte-platelet (p = 0.2) and lymphocyte-platelet complexes (p = 0.5), and s-P selectin levels (p = 0.09) in acute atherothrombotic patients compared with controls. PAC1 binding was decreased in the convalescent phase in the indeterminate subgroup only compared with controls. In keeping with the results in the main study
population, VWF:Ag levels were increased in all subgroups of patients in both the acute and convalescent phases compared with controls (Table 3.3).

**Figure 3.3:** Comparison of median sP-selectin levels between Control subjects (n = 27) and Acute (n = 79) and Convalescent (n = 70) CVD patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Median (ng/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>36</td>
<td>0.09</td>
</tr>
<tr>
<td>Acute CVD</td>
<td>41.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Convalescent CVD</td>
<td>42.4</td>
<td></td>
</tr>
</tbody>
</table>

**Legend for Figure 3.3:** CVD, cerebrovascular disease; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.4: Comparison of median sE-selectin levels between Control subjects (n = 27) and Acute (n = 79) and Convalescent (n = 70) CVD patients

Legend for Figure 3.4: CVD, cerebrovascular disease; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 3.5: Comparison of mean VWF:Ag levels between Control subjects (n = 27) and Acute (n = 79) and Convalescent (n = 70) CVD patients

*Legend for Figure 3.5: CVD, cerebrovascular disease; VWF:Ag, von Willebrand factor antigen level; Symbols represent individual values; Bold horizontal lines represent mean values.*
Table 3.2: Correlation between the percentage expression of CD62P and the circulating levels of neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes, and levels of sP-selectin in the acute and convalescent phases after ischaemic stroke or TIA

<table>
<thead>
<tr>
<th></th>
<th>Acute Phase</th>
<th></th>
<th>Convalescent Phase</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD62P (%)</td>
<td>Spearman's Rho</td>
<td>significance</td>
<td>CD62P (%)</td>
</tr>
<tr>
<td>% NPhil-Plts</td>
<td>0.316</td>
<td>p = 0.005</td>
<td>0.378</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>% Mono-Plts</td>
<td>0.323</td>
<td>p = 0.004</td>
<td>0.382</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>% Lym-Plts</td>
<td>0.175</td>
<td>p = 0.1</td>
<td>0.288</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>sP-selectin (ng / ml)</td>
<td>-0.084</td>
<td>p = 0.5</td>
<td>-0.088</td>
<td>p = 0.5</td>
</tr>
</tbody>
</table>

Legend for Table 3.2: % NPhil-Plts, % Neutrophil-Platelet Complexes; % Mono-Plts, % Monocyte-Platelet Complexes; % Lym-Plts, % Lymphocyte-Platelet Complexes.
Table 3.3: Platelet and endothelial activation markers that were significantly elevated in the different subtypes of ischaemic stroke compared with Control Subjects (n = 27)

<table>
<thead>
<tr>
<th>Stroke Subtype</th>
<th>Acute phase</th>
<th>n</th>
<th>p value vs. Controls</th>
<th>Convalescent Phase</th>
<th>n</th>
<th>p value vs. Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>VWF:Ag</td>
<td>19</td>
<td>0.001</td>
<td>CD62P</td>
<td>16</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VWF:Ag</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Lacunar</td>
<td>CD62P</td>
<td>20</td>
<td>0.01</td>
<td>CD62P</td>
<td>19</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>VWF:Ag</td>
<td></td>
<td>&lt; 0.001</td>
<td>VWF:Ag</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>sP-selectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>CE</td>
<td>CD63</td>
<td>12</td>
<td>0.02</td>
<td>CD63</td>
<td>9</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Mono-Plts</td>
<td></td>
<td>0.04</td>
<td>VWF:Ag</td>
<td></td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>VWF:Ag</td>
<td></td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indeterminate</td>
<td>Mono-Plts</td>
<td>28</td>
<td>0.02</td>
<td>CD62P</td>
<td>26</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>VWF:Ag</td>
<td></td>
<td>&lt; 0.001</td>
<td>PAC1</td>
<td></td>
<td>0.03*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mono-Plts</td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VWF:Ag</td>
<td></td>
<td>0.002</td>
</tr>
</tbody>
</table>

Legend for Table 3.3: AT, Atherothrombotic Stroke or TIA; Lacunar, Lacunar Stroke or TIA; CE, Cardioembolic Stroke or TIA; Indeterminate, Indeterminate Stroke or TIA; Mono-Plts, % Monocyte-Platelet Complexes; VWF:Ag, von Willebrand factor antigen; * P<0.05, PAC1 levels were lower in the convalescent indeterminate subgroup compared with controls.
3.4 Discussion

We have found evidence of excessive platelet (CD62P) and platelet or endothelial
(VWF:Ag) activation in both the acute and convalescent phases after ischaemic stroke or
TIA compared with controls. The finding of increased CD62P expression in ischaemic
CVD has been previously reported in the acute (Grau et al, 1998); (Zeller et al,
1999; Meiklejohn et al, 2001); (Marquardt et al, 2002); (Garlichs et al, 2003), subacute
(Yamazaki et al, 2001), and convalescent phases after ischaemic stroke or TIA
(Konstantopoulou et al, 1995); (Grau et al, 1998); (Meiklejohn et al, 2001); (Garlichs et
al, 2003). However, the results of this study add to our understanding of the potential
importance of elevated CD62P levels in the pathophysiology of ischaemic CVD. We
have shown that both acute and convalescent CVD patients have elevated levels of
circulating monocyte-platelet complexes, and we have demonstrated a significant positive
correlation between CD62P expression and the levels of monocyte-platelet complexes.
Our data are in agreement with a recent report of increased monocyte-platelet, but not
neutrophil-platelet, complex formation in the hyperacute (within 30 hours) and
convalescent phases (> 3 months later) after ischaemic stroke or TIA (Garlichs et al,
2003). It is uncertain whether the higher levels of CD62P and monocyte-platelet complex
formation represent ‘primary’ differences in platelet activation status between CVD
patients and controls, or whether these changes have arisen as a ‘secondary’ response to
cerebral or ocular ischaemia or infarction. Excessive leukocyte activation could also have
contributed to the higher percentage of leukocyte-platelet complexes in CVD patients,
and we did find higher monocyte counts in EDTA-anticoagulated whole blood in CVD
patients compared with controls. However, as stated earlier, having controlled for the
differences in monocyte counts between groups, the percentages of circulating monocyte-
platelet complexes remained higher in both acute and convalescent CVD patients compared with controls. Although we did not measure leucocyte activation markers in this study, the finding of persistently elevated levels of CD62P and monocyte-platelet complexes in convalescent CVD patients, and the positive correlation between the % CD62P expression and the % monocyte-platelet complexes indicates an ongoing stimulus to platelet activation at least three months after symptom onset. Therefore, these findings are unlikely to represent an acute phase response to ischaemic CVD.

Leucocytes and platelets may have stimulatory or inhibitory influences on one another (Li et al, 2000) and leucocyte-platelet complex formation, mediated by CD62P, is likely to facilitate interaction between the two cell types (Li et al, 2000). This could lead to either leucocyte or platelet activation, and potentially exacerbate the inflammatory/ischaemic cascade in the region of the ischaemic penumbra or infarct zone, and worsen prognosis. In addition, platelets may supply cholesterol to monocytes (Mendelsohn & Loscalzo, 1988), which may then mature into lipid-laden macrophages. These factors may be important in the pathogenesis of infarction, because atherosclerotic plaques that rupture and cause symptoms tend to have a higher lipid content, and larger numbers of macrophages and inflammatory cells than those that do not rupture (Golledge et al, 2000). Furthermore, platelet CD62P has been shown to induce the expression of tissue factor on monocytes (Celi et al, 1994) which could, in turn, lead to activation of the coagulation cascade and increase the risk of thromboembolism. This may partly explain why some patients with ischaemic stroke or TIA have recurrent vascular events despite apparent inhibition of platelet function with aspirin (Helgason et al, 1993). Further work is required to determine whether more potent antithrombotic regimens influence platelet
activation status or monocyte-platelet complex formation in ischaemic CVD, and whether one could predict patients at higher risk of recurrent events using these assays.

The persistent elevation of CD62P levels in our study is not in agreement with a recent report suggesting that CD62P expression falls rapidly to normal levels within the first three months after stroke onset (Marquardt et al., 2002). The disparity between the results of these two studies might reflect the fact that larger numbers of subjects were assessed in our study, and this may have facilitated the detection of subtle differences in CD62P expression between patients and controls. This theory is supported by the fact that the same study group previously reported that CD62P expression was significantly elevated at least three months after ischaemic stroke or TIA compared with controls (Grau et al., 1998). Alternatively, this disparity may reflect differences in flow cytometry methodology between the two studies. We added monoclonal antibodies to the whole blood sample before fixation ('antibody labelling before fixation'), whereas Marquardt et al. carried out fixation of the blood samples before antibody labelling ('fixation before antibody labelling'). 'Fixation before antibody labelling' is known to decrease the binding of activation-dependent monoclonal antibodies compared with 'antibody labelling before fixation' (Michelson et al., 2000).

The median levels of platelet surface CD63 expression, and of circulating sP-selectin or sE-selectin were not significantly higher in acute or convalescent CVD patients compared with controls. However, we might have detected significant differences in the levels of these markers of platelet and endothelial activation between patients and controls if larger numbers of subjects had been studied, because some individual CVD patients had higher levels of these markers than controls (Figure 3.1b,
Figure 3.3 and Figure 3.4). Our results do not support the theory proposed by Marquardt et al. that CD63 is a more sensitive marker of platelet activation than CD62P in the subacute phase after ischaemic stroke (Marquardt et al., 2002).

Although the median percentage PAC1 binding was not significantly reduced in acute CVD patients ($p = 0.2$), there was a significant reduction in median PAC1 binding in convalescent CVD patients compared with controls overall ($p = 0.03$). These results may reflect the fact that PAC1 and fibrinogen inhibit each other’s binding to GpIIb/IIIa (Abrams & Shattil, 1991). In the presence of ongoing platelet activation, with fibrinogened-mediated platelet aggregation and leucocyte-platelet complex formation, it is possible that PAC1 binding might decrease as the number of available GpIIb/IIIa binding sites decreases. Alternatively, the results may be explained by the fact that GpIIb/IIIa receptor activation may occur at a relatively late stage in the platelet activation cascade (Moran & FitzGerald, 1994), or that activation of the GpIIb/IIIa receptor, and hence, PAC1 binding, is a reversible process (Ruf & Patscheke, 1995). Therefore this assay is less likely to identify subtle changes in platelet activation status in the subacute or convalescent phases after ischaemic stroke or TIA, when compared with more robust markers of platelet activation, including CD62P expression or leucocyte-platelet complex formation.

Our data are in agreement with earlier reports of elevated VWF levels in the acute and convalescent phases after ischaemic stroke or TIA (Catto et al., 1997). However, to our knowledge, levels of platelet surface CD62P, monocyte-platelet complexes, and VWF have not been measured simultaneously in a stroke patient population. This is of particular interest because increased VWF levels will enhance platelet adhesion to a thrombogenic surface, and when platelet-monocyte complex formation is increased, this
might facilitate transendothelial migration of monocytes attached to platelets, with exacerbation of the inflammatory and ischaemic cascade, as outlined above.

Almost all acute and convalescent CVD patients were on antiplatelet or anticoagulant therapy (aspirin monotherapy in 70%), whereas only 4/27 (14.8%) controls subjects were taking antiplatelet therapy at the time of assessment. Previous studies have reported that aspirin does not significantly affect the expression of CD62P in control subjects (Chronos et al, 1994) or acute (Meiklejohn et al, 2001) or convalescent stroke patients (Yamazaki et al, 2001). In contrast, preliminary work has suggested that the ADP receptor antagonist, ticlopidine, significantly reduces the expression of CD62P in the convalescent phase after atherothrombotic stroke (Yamazaki et al, 2001), and some of our patients were on clopidogrel. In any case, if antiplatelet therapy decreased the expression of the different platelet and endothelial activation markers in patients with ischaemic stroke or TIA, then the differences between patients and controls may have been underestimated. The finding of excessive platelet +/- endothelial activation, and increased monocyte-platelet complex formation in acute and convalescent CVD patients in this study may partly explain why these patients have a moderately high risk of recurrent vascular events during follow-up despite treatment with antiplatelet or anticoagulant therapy. This emphasises the point that more potent antithrombotic treatment regimens, e.g. combination antiplatelet therapy, might be more effective in CVD patients for secondary prevention of vascular events.

We found that older age, and a higher prevalence of peripheral vascular disease and hypertension influenced the difference in expression of monocyte-platelet complexes between convalescent CVD patients and controls. Further studies are warranted to
compare monocyte-platelet complex formation in patients and controls who have been matched for these demographic and vascular risk factors.

Because we had no pilot data from our laboratory prior to the conclusion of this novel and comprehensive observational analytical study comparing platelet activation status, leucocyte-platelet complex formation, and endothelial activation between CVD patients and controls without a history of CVD, it was not possible to predict the statistical power of our data to identify a statistically significant difference between patients and controls. If one accounts for comparisons between both (i) acute and (ii) convalescent ischaemic CVD patients and controls, and use a p value of < 0.025 to indicate statistical significance for each of the markers measured, the conclusions regarding CD62P, monocyte-platelet complexes, and VWF remain unchanged. However, the reduction in PAC1 binding in convalescent CVD patients compared with controls is no longer statistically significant after adjustment for this dual comparison.

Our data also provide some evidence for ongoing platelet +/- endothelial activation in all subtypes of ischaemic stroke or TIA compared with controls. However, larger studies are needed to confirm these initial findings because of the limited number of patients in each ischaemic CVD subgroup, and because some of the differences between the groups in this small observational study just reached statistical significance.

In conclusion, this study adds to our understanding of the cellular mechanisms involved in the pathogenesis of ischaemic stroke or TIA, and provides evidence for excessive platelet +/- endothelial cell activation in ischaemic CVD patients despite treatment with antithrombotic therapy.
4. Assessment of platelet and endothelial activation in asymptomatic and symptomatic severe carotid stenosis

4.1 Introduction

Atherosclerotic stenosis of the extracranial carotid artery most commonly affects the carotid bifurcation and the proximal 1-2 cm of the internal carotid artery (Leys et al, 1997); (Warlow C.P et al, 1996d). The risk of stroke in the arterial territory supplied by an asymptomatic severely stenosed (≥ 70%) carotid artery is approximately 2% per annum with medical treatment alone (The European Carotid Surgery Trialists Collaborative Group, 1995); (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995).

Approximately 9 to 20% of strokes are caused by thromboembolism from a stenosing atheromatous carotid plaque (Sacco et al, 1989a); (CAVATAS Investigators, 2001). In contrast to asymptomatic severe carotid stenosis, the risk of recurrent ipsilateral stroke in medically treated patients with recently symptomatic severe 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); (McCabe & Brown, 2001). However, the risk of recurrent stroke in symptomatic patients returns towards the asymptomatic level within three years after a transient ischaemic attack (TIA) or stroke, in patients with severe stenosis that is not surgically removed (European Carotid Surgery Trialists' Collaborative Group, 1998).

The mechanisms responsible for this disparity in stroke risk between patients with asymptomatic and symptomatic severe carotid stenosis, and the reasons why the risk of
recurrent events significantly reduces among symptomatic patients over time, are not fully understood. The disparity in risk between the two groups could be secondary to differences in the morphology and/or stability of the atherosclerotic plaque, different degrees of endothelial cell activation, or differences in the thrombogenicity of the circulating blood itself. A plaque may become ‘active’ and more thrombogenic intermittently due to fissuring, ulceration, or rupture of its rigid cap (Warlow C.P et al, 1996d). Patients with symptomatic carotid stenosis tend to have plaques with a thinner fibrous cap, a higher lipid content, and larger numbers of macrophages and inflammatory cells in comparison with plaques from patients with asymptomatic carotid stenosis (Golledge et al, 2000). All of these factors may predispose to plaque rupture, but the actual mechanisms responsible for carotid plaque activation and rupture in the first instance are poorly understood (Golledge et al, 2000). Furthermore, because of plaque ‘healing’, these differences in morphology are unlikely to explain all of the increase in incidence of subsequent events in symptomatic patients for a two to three year period. Endothelial cell activation, with increased expression of adhesion molecules, may be more marked in symptomatic compared with asymptomatic patients. Increased adhesion of leucocytes and platelets to the endothelium overlying and surrounding the plaque could destabilise the plaque and contribute to plaque rupture. In addition, ‘ulceration’ of the plaque could activate platelets by increasing the levels of shear stress (Kroll et al, 1996) and thrombogenic subendothelial components to which platelets are exposed. It is also possible that excessively activated platelets release factors into the microcirculation around the plaque that may be incorporated into the plaque, thus increasing plaque instability in some patients who subsequently become symptomatic.
A number of studies have reported excessive platelet activation or platelet hyper-reactivity in atherothrombotic ischaemic stroke compared with controls (Uchiyama et al, 1994); (Konstantopoulos et al, 1995); (Zeller et al, 1999); (Yamazaki et al, 2001). However, to our knowledge, none of the published studies have compared platelet activation status in patients with asymptomatic and symptomatic severe carotid stenosis. Information regarding platelet activation status in these two groups of patients could improve our understanding of the mechanisms responsible for the higher risk of symptoms in symptomatic compared with asymptomatic patients, and could lead to improved strategies for stroke prevention.

Platelet activation may be assessed *in vivo* by measuring substances released from activated platelets into the plasma and urine. These substances include platelet factor 4, β-thromboglobulin, and metabolites of thromboxane A₂, but technical problems related to sample collection and processing have limited their clinical use (Abrams & Shattil, 1991). We used the sensitive and specific technique of whole blood flow cytometry to investigate whether there were differences in platelet activation status between patients with asymptomatic and symptomatic severe carotid stenosis. This method has the advantages of allowing analysis of platelets in the physiological milieu of whole blood, and is less susceptible to artifactual *in vitro* platelet activation and potential loss of platelet subpopulations than methods which use PRP or washed platelets (Michelson, 1996). We also compared the levels of circulating markers of platelet and endothelial cell activation between the two groups of patients. We hypothesised that we would find excessive platelet activation in patients with recently symptomatic compared with asymptomatic severe carotid stenosis.
4.2 Methods

4.2.1 Patient Recruitment

As part of an observational analytical study, suitable consecutive inpatients with symptomatic severe carotid stenosis were recruited from the inpatient population in the Acute Brain Injury Unit at the National Hospital for Neurology and Neurosurgery, University College London Hospitals, and Atkinson Morley’s Hospital, London. Suitable outpatients with asymptomatic or symptomatic severe carotid stenosis were recruited from the Stroke Clinic at the National Hospital for Neurology and Neurosurgery, London, and from the Vascular Clinics at University College London Hospitals and Atkinson Morley’s Hospital, London.

4.2.2 Clinical Assessment

A detailed history of the presenting symptom(s) was taken, including the method of detection of carotid stenosis in the asymptomatic group, and all patients underwent a detailed general, neurological and vascular examination by the same examiner (DJHM) using a standardised protocol that was designed at the outset of the study. Information regarding stroke risk factors (previous stroke or TIA, ischaemic heart disease, hypertension, diabetes mellitus, peripheral vascular disease, atrial fibrillation or flutter, family history of stroke, history of migraine or prior venous thrombosis), smoking status (non-smoker, ex-smoker or current smoker) and alcohol intake was collected prospectively on all subjects. Information regarding medication intake, including antiplatelet and antithrombotic therapy, was recorded.
4.2.3 Symptomatic Severe Carotid Stenosis: Inclusion / Exclusion criteria

Patients were included in this group if they were older than 18 years of age, had experienced an ischaemic stroke or TIA that occurred in the vascular territory supplied by a severe ipsilateral (≥ 70%) carotid stenosis or occlusion within the preceding four weeks (acute phase), and were likely to be available for follow-up at least three months after symptom onset (convalescent phase). Patients with carotid occlusion were only included in this category if no other cause for stroke or TIA was identified. Brain CT or MRI, and colour Doppler ultrasound examination of carotid and vertebral arteries were performed in all patients. Patients were classified as having > 70% carotid stenosis using the Doppler ultrasound criteria outlined in section 3.2.3. Patients with carotid stenosis on colour Doppler ultrasound also underwent extracranial magnetic resonance angiography to further quantify the degree of carotid stenosis. If the results of the ultrasound and MRA examinations were discordant, and the patient was a potentially suitable candidate for carotid endarterectomy or endovascular treatment, intra-arterial catheter angiography was carried out to definitively quantify the degree of carotid stenosis.

Patients were excluded if they had a history of primary intracerebral haemorrhage, myocardial infarction within the preceding three months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding three months, or if they had systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis.

4.2.4 Asymptomatic Severe Carotid Stenosis: Inclusion / Exclusion Criteria

Patients were defined as having asymptomatic severe carotid stenosis if they had evidence of severe (≥ 70%) stenosis of the internal or common carotid artery noted on
colour Doppler ultrasound that was not associated with symptoms of stroke or TIA in that or another vascular territory within the preceding three year period. Some of these patients also had an extracranial MRA to quantify the degree of carotid stenosis noted on colour Doppler ultrasound. One patient in this category had asymptomatic severe carotid restenosis noted on follow-up colour Doppler ultrasound examination 5 years after undergoing balloon angioplasty without stenting for asymptomatic severe atherosclerotic carotid stenosis. Eight patients had a history of contralateral symptomatic severe carotid stenosis requiring carotid endarterectomy or endovascular treatment in the past. However, all of these patients were free of symptoms of stroke or TIA for at least the preceding three years, and had < 70% stenosis of the treated carotid artery on follow-up colour Doppler ultrasound. The exclusion criteria were identical to those applied to the symptomatic group.

4.2.5 Compliance with Antithrombotic Therapy

Because the majority of symptomatic patients in the acute phase were assessed during their inpatient stay, compliance with antiplatelet or antithrombotic therapy was confirmed by checking the inpatient drug chart. All outpatients in each group were phoned before their appointment to stress the importance of drug compliance in the week prior to assessment, and compliance in this group was assessed by history taking alone.
4.2.6 Ethical Approval and Consent / Assent

The study was approved by the Local Research Ethics Committees of the participating Hospitals. Written informed consent (or written assent by a relative of the patient, where appropriate) was obtained in all cases.

4.2.7 Blood Sampling and Laboratory Tests

The markers of platelet and endothelial activation that were measured in this study were the same as those tested in chapter 3. Briefly, all subjects were rested for at least 20 minutes before venepuncture, and blood sampling was performed as outlined in section 2.2.1. The initial EDTA sample was used for measurement of a FBC, including measurement of the MPV using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.). The first 4.5 ml 3.2% citrate anticoagulated blood sample was used for whole blood flow cytometric analysis. Platelets were distinguished from red and white blood cells by their characteristic pattern of forward scatter and side scatter (Michelson, 1996), and the cells were confirmed to be platelets using a monoclonal antibody to the GpIba (CD42b) subunit of the GpIb-IX-V complex (Abrams & Shattil, 1991); (Ruggeri, 1997); (Clemetson, 1997); (Escolar & White, 2000) (see section 2.3.3). Using the 'panel set-up' on the flow cytometer, the degree of platelet activation in the sample was assessed by measuring the percentage expression of CD62P and CD63 (section 2.3.3), and PAC1 binding (section 2.3.4). The percentages of neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes were also calculated as additional markers of platelet activation (Li et al, 1997); (Furman et al, 1998); (Joseph et al, 2001) (section 2.3.5). The next two citrated samples were used
to prepare double spun PPP, and were immediately stored at -70 °C for subsequent measurements of the levels of sP-selectin, sE-selectin (section 2.6.3 and 2.6.4), and VWF:Ag (section 2.7.3) to determine whether platelet and/or endothelial cell activation occurred in our study population. A fourth citrate sample was used for measurement of the platelet count and MPV in citrate-anticoagulated whole blood using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.).

4.2.8 Statistical Methods

The Mann-Whitney U test was used for comparison of median values between groups when the data were not-normally distributed in either group, and an unpaired t-test for comparison of mean values between groups when the data were normally distributed. The chi-square test was used for comparison of proportions between groups, where appropriate. Multiple linear regression analysis was used to examine the independent influence of different predictor variables on each of a number of outcome measures of interest, in turn. Logarithmic transformations were taken before regression analyses, where appropriate, so that assumptions of normality were reasonably well met. All statistical calculations were performed using SPSS-10 for Windows or Microsoft Excel 97 data analysis software.

4.3 Results

Nineteen symptomatic severe carotid stenosis patients were assessed in the acute phase (0-21 days) after an ischaemic stroke or TIA. Sixteen of these symptomatic patients were reassessed in the convalescent phase (79-365 days). Eight of these patients were reassessed at least three months after successful carotid endarterectomy or endovascular
treatment, four patients were reassessed in the convalescent phase before carotid endarterectomy or endovascular treatment, two patients had carotid occlusion from the outset, and two patients were deemed unsuitable for surgery or endovascular treatment. One patient in the convalescent phase was assessed at 79 days after presentation, before undergoing carotid endovascular treatment, and one patient was assessed at 86 days after symptom onset because she was returning to her home country. All remaining patients were reassessed at least 90 days after their initial presentation, as originally planned. Three patients did not have repeat laboratory testing during the convalescent phase; one patient had died, one had moved to a different Country, and one patient declined further investigations. The results were compared with those obtained from 16 patients with asymptomatic severe carotid stenosis.

The clinical characteristics of the patients are outlined in Table 4.1. Although there was a non-significant trend towards a higher proportion of men in the asymptomatic compared with the symptomatic group, there were no significant differences in vascular risk factor profiles between the two groups (p > 0.05 for comparison of acute or convalescent symptomatic vs. asymptomatic patients, chi-square test). The median ICA stenosis severity on colour Doppler ultrasound in the symptomatic patients was 80 to 95% in the acute phase and 70 to 79% in the convalescent phase. The median ICA stenosis was 80 to 95% in the asymptomatic group. The majority of symptomatic and asymptomatic patients were on treatment with aspirin. Symptomatic patients were on variable doses of aspirin between 75 to 300 mg daily at the time of assessment (median daily dose = 150 mg in the acute phase and 75 mg in the convalescent phase).
Table 4.1: Clinical Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>ASCS N = 16</th>
<th>Acute SSCS N = 19</th>
<th>Convalescent SSCS N = 16</th>
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<tbody>
<tr>
<td>Mean Age (Years)</td>
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<td>69</td>
<td>68</td>
</tr>
<tr>
<td>Gender (M / F)</td>
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<td>9 / 10</td>
<td>8 / 8</td>
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<td><strong>Antithrombotic therapy</strong></td>
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<tr>
<td>ASA Monotherapy</td>
<td>14</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>ASA + DP</td>
<td>2</td>
<td>5</td>
<td>3</td>
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<tr>
<td>ASA + DP + Dalteparin</td>
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<td>1</td>
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<tr>
<td>ASA + Dalteparin</td>
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<td>1</td>
<td>0</td>
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<tr>
<td>ASA + Azaproprazone</td>
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<tr>
<td>Warfarin</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>None of above</td>
<td>0</td>
<td>0</td>
<td>1†</td>
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<tr>
<td>Prior Stroke / TIA</td>
<td>10 (62.5 %)</td>
<td>12 (63.2 %)</td>
<td>9 (56.3 %)</td>
</tr>
<tr>
<td>IHD</td>
<td>9 (56.3 %)</td>
<td>8 (42.1 %)</td>
<td>6 (37.5 %)</td>
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<tr>
<td>Hypertension</td>
<td>14 (87.5 %)</td>
<td>17 (89.5 %)</td>
<td>16 (100 %)</td>
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<tr>
<td>Diabetes Mellitus</td>
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<td>3 (15.8 %)</td>
<td>2 (12.5 %)</td>
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<tr>
<td>PVD</td>
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<td>6 (31.6 %)</td>
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<td>0</td>
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<td>Fam. Hx. Stroke</td>
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<td>7 (36.8 %)</td>
<td>5 (31.3 %)</td>
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<td>Migraine</td>
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</tr>
<tr>
<td>Current</td>
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<td>4 (21.1 %)</td>
<td>2 (12.5 %)</td>
</tr>
<tr>
<td>Ex-smoker</td>
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<td>Non-smoker</td>
<td>5 (31.3 %)</td>
<td>7 (36.8 %)</td>
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</tr>
<tr>
<td>Alcohol Intake (Median Units / Week)</td>
<td>1</td>
<td>0.5</td>
<td>0</td>
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</tbody>
</table>

Legend for Table 4.1: ASCS, Asymptomatic severe carotid stenosis; SSCS, Symptomatic severe carotid stenosis; ASA, Aspirin; DP, Modified-release dipyridamole 200 mg twice daily; IHD, Ischaemic heart disease; A Fib / Flutter, Atrial fibrillation / flutter. †, One patient had discontinued clopidogrel after carotid endarterectomy.
Asymptomatic patients were on variable doses of aspirin between 37.5 to 300 mg daily (median daily dose = 150 mg). However, combination antiplatelet therapy with aspirin and modified-release dipyridamole (200 mg twice daily), and treatment with clopidogrel (75 mg daily), or anticoagulation were more commonly used in the symptomatic group (see below).

The mean platelet count in EDTA-anticoagulated blood was significantly higher in the acute (275 x 10^9/L, p = 0.007) and convalescent symptomatic group (266 x 10^9/L, p = 0.04) compared with the asymptomatic group (223 x 10^9/L). The mean platelet count in citrate-anticoagulated blood was also higher in the acute (231 x 10^9/L, p = 0.05) and convalescent symptomatic group (239 x 10^9/L, p = 0.02) compared with the asymptomatic group (193 x 10^9/L). The platelet count was lower in citrate- than EDTA-anticoagulated whole blood, because the volume of citrate in the Vacutainer tubes dilutes the sample by 10% compared with the EDTA Vacutainer tube. Otherwise, there were no significant differences in any other FBC parameters, including the absolute neutrophil, monocyte, or lymphocyte counts in EDTA, and the MPV in EDTA or citrate, between the symptomatic and asymptomatic patients (data not shown).

The median % GpIb expression was similar in the acute (99.1%) and convalescent symptomatic patients (99.2%) and in the asymptomatic severe carotid stenosis patients (99.3%), confirming that the majority of cells analysed on the flow cytometer in each group were platelets. There were no significant differences overall in the median % platelet surface expression of CD62P and CD63, or PAC1 binding, between the acute or convalescent symptomatic patients and the asymptomatic severe carotid stenosis patients (Figure 4.1, Figure 4.2, and Figure 4.3). However, the % expression of CD62P and CD63

253
was higher in some symptomatic patients compared with the asymptomatic group (Figures 4.1 and Figure 4.2).

The median % of circulating neutrophil-platelet complexes (p = 0.004, Figure 4.4a), monocyte-platelet complexes (p = 0.046, Figure 4.4b), and lymphocyte-platelet complexes (p = 0.02, Figure 4.4c) were significantly higher in the acute symptomatic patients compared with the asymptomatic patient group. In the convalescent phase, the median % of circulating neutrophil-platelet (p = 0.07, Figure 4.4a), monocyte-platelet (p = 0.3, Figure 4.4b) and lymphocyte platelet complexes (p = 0.8, Figure 4.4c) in the symptomatic group had dropped to similar levels to those found in the asymptomatic group.

There were no significant differences in mean levels of sP-selectin, or median levels of sE-selectin between the acute or convalescent symptomatic severe carotid stenosis patients and the asymptomatic patients (Figure 4.5 and Figure 4.6). The mean VWF antigen levels were also similar in the acute or convalescent symptomatic and asymptomatic patients (Figure 4.7).

We investigated whether the higher platelet count in citrate-anticoagulated whole blood accounted for the significant differences in the percentages of circulating leucocyte-platelet complexes between the acute symptomatic and asymptomatic severe carotid stenosis patients. Multiple linear regression analysis was carried out to control for the influence of the different platelet counts in the two groups of patients on the results obtained. The unadjusted mean % of circulating neutrophil-platelet complexes was an estimated 28% higher (95% confidence interval: 9.4 to 49% higher) in the acute symptomatic compared with the asymptomatic patients (p = 0.003, corresponding closely
to the results from the Mann Whitney U test). After adjusting for the platelet count in citrate-anticoagulated whole blood, the mean % of circulating neutrophil-platelet complexes remained higher in the acute symptomatic compared with the asymptomatic group (estimated mean difference 20%, 95% confidence interval: 2.2 to 42% higher, p = 0.03). However, the unadjusted mean percentages of circulating monocyte-platelet complexes (estimated mean difference 26%, 95% confidence interval: 2.8% lower to 63.4% higher, p = 0.09) and lymphocyte-platelet complexes (estimated mean difference 14%, 95% confidence interval: 0.6% lower to 31% higher, p = 0.06) were not significantly different between the acute symptomatic and asymptomatic groups, although both results were significant using the more robust Mann Whitney U test. The adjusted mean percentages of monocyte-platelet (p = 0.08) and lymphocyte-platelet complexes (p = 0.08) were not significantly different between the two groups.

The use of different antiplatelet and anticoagulant regimens (Table 4.1) may also have influenced the observed differences between the acute symptomatic and asymptomatic groups. Therefore, we compared the data obtained from the subgroup of symptomatic and asymptomatic severe carotid stenosis patients who were on treatment with aspirin only. Overall, the results were similar to those obtained from the main patient group. The mean % of neutrophil-platelet complexes (2.7 vs. 2.2%, p = 0.03) and the median % monocyte-platelet complexes (5.4 vs. 4.4%, p = 0.03) were significantly higher in the acute symptomatic (n = 11) compared with the asymptomatic patients on aspirin only (n = 14). However, the median % lymphocyte-platelet complexes was not significantly higher in the acute symptomatic compared with the asymptomatic patients on aspirin only (2.3 vs. 2.1% p = 0.2). The platelet counts in EDTA- (p = 0.02) and
citrate-anticoagulated whole blood (p = 0.04) were significantly higher in the acute symptomatic compared with the asymptomatic severe carotid stenosis patients on aspirin only. Otherwise, there were no significant differences in any of the parameters measured between the acute or convalescent symptomatic and asymptomatic severe carotid stenosis patients on aspirin.
Figure 4.1: Comparison of median % CD62P expression between ASCS patients (n = 15) and Acute (n = 19) and Convalescent (n = 16) SSCS patients

Legend for Figure 4.1: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 4.2: Comparison of median % CD63 expression between ASCS patients (n = 16) and Acute (n = 19) and Convalescent (n = 16) SSCS patients

Legend for Figure 4.2: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 4.3: Comparison of median % PAC1 binding between ASCS patients (n = 15) and Acute (n = 17) and Convalescent (n = 16) SSCS patients

Legend for Figure 4.3: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 4.4a: Comparison of median % Neutrophil-Platelet Complexes between ASCS patients (n = 16) and Acute (n = 18) and Convalescent (n = 15) SSCS patients

Legend for Figure 4.4a: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles; % NPhil-Plts, % Neutrophil-Platelet Complexes.
Figure 4.4b: Comparison of median % Monocyte-Platelet Complexes between ASCS patients (n = 16) and Acute (n = 18) and Convalescent (n = 15) SSCS patients

Legend for Figure 4.4b: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles; % Mono-Plts, % Monocyte-Platelet Complexes.
Figure 4.4c: Comparison of median % Lymphocyte-Platelet Complexes between ASCS patients (n = 15) and Acute (n = 16) and Convalescent (n = 15) SSCS patients

Legend for Figure 4.4c: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles; % Lym-Plts, % Lymphocyte-Platelet Complexes.
Figure 4.5: Comparison of mean plasma levels of sP-selectin between ASCS patients (n = 16) and Acute (n = 19) and Convalescent (n = 16) SSCS patients

Legend for Figure 4.5: ASCS, Asymptomatic severe carotid stenosis; SSCS, Symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent mean values.

Mean = 45.8
Mean = 43.4
Mean = 41.4
p = 0.7
p = 0.4
Figure 4.6: Comparison of median plasma levels of sE-selectin between ASCS patients (n = 16) and Acute (n = 19) and Convalescent (n = 16) SSCS patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Median (ng/ml)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCS</td>
<td>34.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Acute SSCS</td>
<td>33.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Convalescent SSCS</td>
<td>37.2</td>
<td></td>
</tr>
</tbody>
</table>

Legend for Figure 4.6: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; Symbols represent individual values; Bold horizontal lines represent median values; Vertical bars represent the 5th to 95th percentiles.
Figure 4.7: Comparison of mean plasma levels of VWF:Ag between ASCS patients (n = 16) and Acute (n = 19) and Convalescent (n = 16) SSCS patients

Legend for Figure 4.7: ASCS, Asymptomatic severe carotid stenosis; SSCS, symptomatic severe carotid stenosis; VWF:Ag, von Willebrand factor antigen levels; Symbols represent individual values; Bold horizontal lines represent mean values.
4.4 Discussion

We found an increase in the mean platelet count in both acute and convalescent symptomatic compared with asymptomatic severe carotid stenosis patients. Furthermore, for the first time, we have demonstrated that patients with symptomatic severe carotid stenosis have excessive leucocyte-platelet complex formation in the acute phase after an ischaemic stroke or TIA compared with patients with asymptomatic severe carotid stenosis.

Previous studies have shown that the platelet count may be increased (Numminen et al, 1996), decreased (O’Malley et al, 1995), or remain unchanged (Butterworth & Bath, 1998) in the acute phase after cerebral infarction compared with controls. However, to our knowledge, an elevated platelet count has not been reported in acute symptomatic vs. asymptomatic severe carotid stenosis patients.

There were no significant differences in CD62P expression between the acute symptomatic and asymptomatic patients. However, this is not incompatible with our findings of increased leukocyte-platelet complex formation in the acute symptomatic group because CD62P may be 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 ischemic heart disease have suggested that an increase in the percentage of monocyte-platelet complexes may be a more sensitive indicator of in vivo platelet activation than either an increase in the percentage of neutrophil-platelet complexes (Michelson et al, 2001) or CD62P expression on platelets (Michelson et al, 2000);
(Michelson et al, 2001); (Furman et al, 2001). Our results are consistent with this hypothesis, because the absolute percentages of monocyte-platelet complexes were higher than the absolute percentages of neutrophil-platelet complexes in individual patients (Figures 2a and 2b).

The higher percentage of circulating leukocyte-platelet complexes in acute symptomatic compared with asymptomatic patients may represent ‘primary’ differences in platelet activation status between the two groups. These findings could also be ‘secondary’ to carotid plaque activation in the symptomatic group, with subsequent platelet activation and leukocyte-platelet complex formation, or may have arisen as an acute phase response to cerebral or ocular ischemia or infarction. It is also possible that excessive leukocyte-platelet complex formation represents both a ‘primary’ and ‘secondary’ phenomenon in the acute symptomatic group. Alternatively, excessive leukocyte activation may have contributed to the higher percentage of leukocyte-platelet complexes in the acute symptomatic group (Joseph et al, 2001; Akopov et al, 1996). However, the finding of an increased platelet count in both the acute and convalescent symptomatic compared with the asymptomatic patients suggests that there is an ongoing stimulus to platelet production, and possibly platelet activation, in the symptomatic group. To investigate the importance of leukocyte-platelet complexes in the pathogenesis of stroke or TIA, additional prospective studies are required to determine whether asymptomatic or symptomatic severe carotid stenosis patients with excessive leukocyte-platelet complex formation are at higher risk of subsequent stroke or TIA than patients with lower percentages of circulating complexes. If this is the case, more potent antithrombotic therapy in these high risk patients (Zhao et al, 2001), or better selection of
patients for carotid endarterectomy or endovascular treatment might facilitate more effective primary or secondary stroke prevention.

Increased leukocyte-platelet complex formation may play a role in the pathogenesis of TIA or stroke in patients with recently symptomatic severe carotid stenosis. Platelets and leukocytes may have stimulatory influences on one another, and leukocyte-platelet complex formation is likely to facilitate interaction between the two cell types (Li et al, 2000); (Grau et al, 1994). When a stenosing atherosclerotic plaque ruptures, platelet adhesion to the plaque contents may stimulate further platelet activation and neutrophil-platelet complex formation. This may, in turn, amplify platelet secretion and aggregation in the thrombogenic milieu of the plaque (Li et al, 2000) and further increase the risk of platelet thromboembolism. Furthermore, neutrophil-platelet complex formation may promote neutrophil activation, with the release of inflammatory cytokines (Li et al, 2000) that could attract other leukocytes to the ruptured plaque. The unadjusted percentage of monocyte-platelet complexes was also higher in acute symptomatic than asymptomatic patients, and circulating monocyte-platelet complexes were also elevated in acute symptomatic compared with asymptomatic patients on aspirin only. These data are of interest because platelets may supply cholesterol to monocytes (Mendelsohn & Loscalzo, 1988), which may then mature into lipid-laden macrophages. This may be important in the pathogenesis of ischemic infarction, because atherosclerotic plaques that rupture and cause symptoms have a higher lipid content, and larger numbers of macrophages and inflammatory cells than those that do not rupture.(Golledge et al, 2000)

Furthermore, platelet CD62P has been shown to induce the expression of tissue factor on monocytes (Celi et al, 1994), which could, in turn, lead to activation of the coagulation
pathway and increase the risk of thromboembolism. Further studies in larger groups of subjects are required to confirm our initial findings, because some of the differences between the groups in this small observational study just reached statistical significance.

Further work is required to determine whether one can identify asymptomatic and symptomatic severe carotid stenosis patients at high risk of thromboembolism, using a combination of techniques such as flow cytometry to detect excessive platelet activation \textit{ex vivo} despite treatment with aspirin therapy, and e.g. transcranial Doppler imaging to quantify the embolic load. If this proves feasible, more potent antiplatelet or anticoagulant therapy in high risk patients, or better selection of patients for carotid endarterectomy or endovascular treatment might facilitate more effective primary or secondary stroke prevention. Zhao et al. have recently suggested that combination antiplatelet therapy with dipyridamole and an ADP receptor antagonist, or a combination of aspirin, dipyridamole, and an ADP receptor antagonist may reduce monocyte-platelet and neutrophil-platelet aggregate formation \textit{in vitro} (Zhao et al, 2001). Because aspirin fails to prevent a high rate of recurrent stroke (26 % over two years) in medically treated patients with recently symptomatic severe carotid stenosis, (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991), the use of alternative antiplatelet regimens in these patients deserves further study.

We did not find an increase in the median percentage CD63 expression in acute symptomatic patients compared with asymptomatic severe carotid stenosis patients overall. This may reflect the fact that CD63 is not a sensitive marker for identifying differences in platelet activation status between patients with symptomatic and asymptomatic severe carotid stenosis. Alternatively, the small number of patients
assessed in this study may have contributed to a type II error, and we might have detected significant differences in CD63 levels between the two groups of patients if larger numbers of subjects were studied. The latter theory is supported by the finding of higher levels of CD63 in some acute and convalescent symptomatic patients compared with the levels seen in the asymptomatic group. In addition, there was no significant difference in PAC1 binding between the acute symptomatic and asymptomatic patients. PAC1 binding occurs at a relatively late stage in the platelet activation process, when the GpIIb/IIIa receptor is activated. Therefore, this marker is less likely to identify subtle differences in platelet activation status between two groups of patients with atherosclerosis than the other markers expressed after platelet degranulation. Furthermore, we did not find any significant differences in the circulating levels of sP-selectin, sE-selectin or VWF antigen levels between the acute symptomatic and asymptomatic patients. One could interpret these data as showing no difference in the degree of endothelial cell activation between acute symptomatic and asymptomatic patients. However, one should be cautious in interpreting these data, and larger studies are warranted to re-examine the usefulness of these plasma markers in detecting differences in platelet and endothelial activation status between patients with symptomatic and asymptomatic severe carotid stenosis.

There were no significant differences in any of the markers of platelet or endothelial activation between the convalescent symptomatic and asymptomatic severe carotid stenosis patients. This may, in part, be secondary to resolution of the acute phase response in the three month period after the initial stroke or TIA, in combination with some degree of plaque healing before repeat blood sampling was performed. However, platelets may be activated by high shear stress caused by increased blood velocity and
turbulence as blood flows past an arterial stenosis (Kroll et al, 1996). Consequently, successful surgical removal of, or endovascular treatment of the stenosing atheromatous plaque in 8/16 (50%) symptomatic patients before retesting during the convalescent phase undoubtedly reduced the degree of shear-induced platelet activation in these patients compared with the acute setting. Therefore, it is not surprising that the differences between the symptomatic and asymptomatic groups were no longer significant at this stage, and it is reassuring that surgical or endovascular treatment was associated with a reduction in the percentage of leucocyte-platelet complexes in the symptomatic group during follow-up.

This study adds to our understanding of the possible underlying mechanisms responsible for the higher risk of stroke or TIA in patients with recently symptomatic compared with asymptomatic severe carotid stenosis. Further work is required to examine the effects of different antiplatelet regimens on platelet activation ex vivo in patients with severe carotid stenosis, and to determine whether flow cytometric techniques will be useful for risk stratification in this patient population.
5. Measurement of the antiplatelet effects of low or medium dose oral aspirin in ischaemic stroke or TIA using the PFA-100®

5.1 Introduction

Aspirin is the most commonly prescribed antiplatelet agent for the secondary prevention of vascular events in patients presenting with ischaemic stroke or TIA (cerebrovascular disease (CVD)). Aspirin selectively and irreversibly inhibits the cyclooxygenase 1-mediated breakdown of arachidonic acid (Harker, 1998), thus inhibiting the subsequent formation of thromboxane A₂ (a potent platelet aggregator and vasoconstrictor). However, 160 to 300 mg of aspirin daily, administered within 48 hours of an acute ischaemic stroke, reduces the relative risk of recurrent ischaemic stroke in the first 14 to 28 days by only 24 to 28% (International Stroke Trial Collaborative Group, 1997); (CAST (Chinese Acute Stroke Trial) Collaborative Group, 1997). In addition, long-term secondary prevention with 30 to 1500 mg of aspirin daily reduces the relative risk of subsequent vascular events by only 13 to 18% in this patient population (Diener et al, 1996); (Algra & van Gijn, 1996). Therefore, the majority of these patients are not protected from further vascular events with aspirin.

There is no universally accepted definition of what constitutes ‘aspirin resistance’, and the term is often interpreted differently by basic scientists and clinicians. At a cellular level, aspirin resistance could refer to incomplete inhibition, or lack of inhibition of platelet cyclooxygenase-1 ex vivo in patients on aspirin therapy. Cyclooxygenase
function can be assessed by measuring the levels of thromboxane A\textsubscript{2} metabolites in plasma or urine, but technical problems related to sample collection and processing have limited the clinical usefulness of these assays (Abrams & Shattil, 1991). The degree of platelet aggregation in response to \textit{in vitro} stimulation by arachidonic acid is also frequently used to assess cyclooxygenase inhibition by aspirin. However, platelet aggregometry is usually performed in PRP, rather than in the physiological milieu of whole blood, and the assays are semiquantitative and subject to artifactual \textit{in vitro} platelet activation during sample collection and processing (Michelson, 1996). Moreover, there is no standard reference range for arachidonic acid-induced platelet aggregation in patients on aspirin that would allow one to consistently classify these patients as either aspirin resistant or aspirin responsive \textit{ex vivo} (Helgason \textit{et al}, 1993); (Gum \textit{et al}, 2001).

In the clinical setting, aspirin resistance might be defined as the recurrence of vascular events during follow-up in patients taking aspirin. We prefer to describe these patients as ‘aspirin failures’ (Chyatte & Chen, 1990); (Bornstein \textit{et al}, 1994) in the absence of laboratory assessment of platelet function, because platelets can be activated via cyclooxygenase-independent pathways. Furthermore, recurrent events may occur in subjects who are ‘responsive’ to the antiplatelet effects of aspirin \textit{ex vivo} using platelet aggregometry (Helgason \textit{et al}, 1993). From a practical clinical viewpoint, we consider patients to be aspirin resistant if they have excessive platelet activation or hyper-reactivity \textit{ex vivo} despite treatment with aspirin.

Most studies that examined the issue of aspirin resistance in ischaemic stroke or TIA have employed platelet aggregometry using PRP rather than whole blood (Helgason \textit{et al}, 1993); (Helgason \textit{et al}, 1994); (Chamorro \textit{et al}, 1999a). Using platelet
aggregometry, the incidence of aspirin resistance in this patient population ranged from 2.7 to 25.5% (Helgason et al, 1993); (Helgason et al, 1994). If one could identify patients with ex vivo aspirin resistance with a simple and reliable laboratory test on whole blood, then perhaps one could improve secondary prevention by treating these individuals with an alternate antiplatelet regimen.

The platelet function analyser (PFA-100®) (Dade-Behring, Germany) is a cartridge-based test system that measures platelet function in whole blood in response to biochemical stimulation and moderately high shear stress (Kundu et al, 1995). The usefulness of this device in monitoring the response to antiplatelet therapy in ischaemic CVD has only been evaluated in one small pilot study in patients in the convalescent phase after ischaemic stroke (Grau et al, 2003).

We investigated whether the PFA-100 could detect platelet hyper-reactivity in patients with ischaemic CVD despite treatment with 75 to 300 mg of aspirin daily. We also assessed the ex vivo responsiveness to aspirin therapy in this population, and investigated whether ischaemic CVD patients with aspirin resistance could be identified with this assay. We investigated whether the ex vivo responsiveness to aspirin therapy varied according to the underlying mechanism responsible for the ischaemic stroke or TIA. In addition, we measured von Willebrand factor antigen (VWF:Ag) levels in the different subject groups because the results of the PFA-100 are influenced by the levels of functional von Willebrand Factor (VWF) in the circulating blood (Kundu et al, 1995); (Harrison et al, 1999); (Fressinaud et al, 1998), and increased levels of VWF have been reported in patients with ischaemic CVD (Catto et al, 1997).
5.2 Methods

5.2.1 Patient Inclusion / Exclusion criteria

Suitable consecutive inpatients were recruited from the inpatient population in the Acute Brain Injury Unit at the National Hospital for Neurology and Neurosurgery, University College London Hospitals, and Atkinson Morley’s Hospital, London. Suitable outpatients were recruited from the Stroke Clinic at the National Hospital for Neurology and Neurosurgery, London. Patients were included in this study if they were older than 18 years of age, had experienced an ischaemic stroke or TIA within the preceding four weeks (acute phase), were receiving or due to receive aspirin therapy, and were likely to be available for follow-up at least three months after symptom onset (convalescent phase). We excluded patients who were on treatment with another antiplatelet agent or a non-steroidal anti-inflammatory drug in combination with aspirin, or those who were receiving heparin or warfarin. Patients were also excluded if they had a history of primary intracerebral haemorrhage, myocardial infarction within the preceding three months, ongoing unstable angina, unstable symptomatic peripheral vascular disease, major surgery or systemic haemorrhage within the preceding three months, or if they had systemic vasculitis, underlying neoplasia, or a known bleeding or clotting diathesis.

5.2.2 Clinical Assessment

A detailed history of the presenting symptom(s) was taken, and all patients underwent a detailed general, neurological and vascular examination by the same examiner (DJHM) using a standardised protocol that was designed at the outset of the study. Information regarding vascular risk factors (previous stroke or TIA, ischaemic heart disease, hypertension, diabetes mellitus, peripheral vascular disease, atrial fibrillation or flutter,
hyperlipidaemia, family history of stroke, history of migraine or prior venous thrombosis), smoking status (non-smoker, ex-smoker or current smoker) and alcohol intake was collected prospectively on all subjects. Information regarding medication intake, including statin use, was recorded.

Results of routine haematological, coagulation, biochemical and blood glucose testing were collected. Brain CT or MRI, and colour Doppler ultrasound examination of carotid and vertebral arteries was performed in all patients. Patients with carotid stenosis on colour Doppler ultrasound also underwent extracranial magnetic resonance angiography to further quantify the degree of carotid stenosis. If the results of the ultrasound and MRA examinations were discordant, and the patient was a potentially suitable candidate for carotid endarterectomy or endovascular treatment, intra-arterial catheter angiography was carried out to quantify the degree of carotid stenosis. A chest radiograph and an electrocardiograph (ECG) were obtained in all patients, and transthoracic +/- transoesophageal echocardiography was carried out if a cardioembolic cause for the stroke or TIA was suspected, or when other investigations were uninformative. A 24-hour ECG recording was also performed if paroxysmal atrial fibrillation or flutter was suspected clinically. Intracranial MRA was performed in some patients in whom intracranial atherosclerosis was suspected.

5.2.3 Stroke subtyping

The underlying mechanism responsible for the ischaemic stroke or TIA was categorised as atherothrombotic, lacunar, cardioembolic, or indeterminate, as described in section 3.2.3.
5.2.4 Follow-up

Patients who were alive and willing to return for follow-up were subsequently reassessed at least three months after the onset of their ischaemic stroke or TIA (convalescent phase). In the atherothrombotic subgroup, the convalescent phase follow-up was performed after carotid surgery or endovascular treatment, unless this treatment had been delayed for at least 3 months after the initial event.

5.2.5 Control subjects

The patient data were compared with those obtained from a group of control subjects who were recruited from the staff at The Haemostasis Research Unit, University College London Hospitals, and from the local population. Spouses of patients and control subjects were also recruited. Control subjects were also clinically assessed by a single examiner (DJHM) using a standardised protocol, and they underwent colour Doppler ultrasound imaging of carotid and vertebral arteries. Subjects were excluded from the control group if they had evidence of severe (> 70%) carotid or vertebral artery stenosis, or if they were receiving antiplatelet or anticoagulant therapy. Otherwise, the exclusion criteria were identical to those applied to the patient group. It was expected that the control subjects would have a different vascular risk profile to the patient population.

5.2.6 Compliance

Because the majority of patients in the acute phase were assessed during their inpatient stay, compliance with aspirin therapy was confirmed by checking the inpatient drug chart. All suitable outpatients in the acute and convalescent phase after symptom onset
were phoned before their appointment to stress the importance of drug compliance in the week prior to assessment, and compliance in this group was assessed by history taking alone.

5.2.7 Ethical approval and consent
The study was approved by the Local Research Ethics Committees of the participating Hospitals. Written informed consent (or written assent by a relative of the patient, where appropriate) was obtained in all cases.

5.2.8 Blood Sampling and laboratory tests
All subjects were rested for at least 20 minutes before venepuncture to standardise conditions and to minimise platelet activation in vivo, and blood sampling was performed as outlined in section 2.2.1. The initial EDTA sample was used for measurement of a FBC, including measurement of the MPV using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.).

Four further 4.5 ml samples were collected into sterile Vacutainer tubes containing 0.5 ml of 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 4.5 ml blood sample was used for measurement of platelet function in citrate-anticoagulated whole blood using the PFA-100 between 2 and 2 ½ hours after venepuncture, as described in section 2.4.2 (see Figure 2.4.1 and Figure 2.4.2). All samples were tested using both the C-ADP and C-EPI cartridges. As outlined previously, 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 were recorded as “test time exceeded”. We
arbitrarily defined these closure times as 301 s. 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. Studies in normal controls have suggested that a single dose of
aspirin (300 to 325 mg) prolongs the C-EPI closure time in 83% - 100% of healthy
controls (Kundu S et al, 1994), (Mammen et al, 1995); (Mammen et al, 1998); (Harrison
et al, 1999), with prolongation of the C-ADP closure time in up to 24% (Mammen et al,
1998). If one defines aspirin-resistance on the PFA-100 as the lack of prolongation of the
C-EPI closure time with aspirin, up to 27% of control subjects may be aspirin-resistant
using this device.

The next two citrated samples were used to prepare double spun PPP, and were
immediately stored at -70 °C for subsequent measurements of the levels of VWF:Ag
(section 2.7.3) to determine whether platelet and / or endothelial cell activation occurred
in our study population. A fourth citrate sample was used for measurement of the platelet
count and MPV in citrate-anticoagulated whole blood using a GEN-S haematology
analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.).
5.2.9 Statistical Methods

To establish the normal range of PFA-100 closure times for the control subjects who were not on antiplatelet or anticoagulant therapy, we calculated the median (5\textsuperscript{th} to 95\textsuperscript{th} percentile) closure time for the C-ADP cartridge (data not normally distributed), and the mean (+/- 2 standard deviations) closure time for the C-EPI cartridge. Because the maximum closure time recorded by the PFA-100 is 300 seconds, and because some patients on aspirin had C-EPI closure times exceeding 300 seconds, we assumed that the C-EPI closure time data in patients did not conform to a normal distribution. Based on data from previous studies outlined above, patients who did not have prolonged C-EPI closure times on aspirin therapy were defined as having \textit{ex vivo} aspirin-resistance in this study. We used the Mann-Whitney U test for comparison of median values between groups when the data were not-normally distributed in either group, and an unpaired t-test for comparison of mean values between groups when the data were normally distributed. Chi-squared testing was used for comparison of proportions between groups, where appropriate. Spearman’s rank correlation analysis was performed to examine the relationship between the VWF:Ag levels in platelet rich plasma and the PFA-100 closure times. All statistical calculations were performed using SPSS-10 for Windows or Microsoft Excel 97 data analysis software.

5.3 Results

Fifty-five patients in the acute phase (1-27 days) and 45 patients in the convalescent phase (90-725 days) after an ischaemic stroke or TIA were included in this study. Two patients in the acute phase and one patient in the convalescent phase were assessed twice (on different aspirin doses), and therefore data from 57 samples in the acute phase and 46
samples in the convalescent phase were used for analysis. Eight patients who did not meet all the inclusion criteria in the acute phase were included in the convalescent CVD
Table 5.1: Clinical Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Acute Patients</th>
<th>Convalescent Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 55</td>
<td>N = 45</td>
<td>N = 23</td>
</tr>
<tr>
<td>Mean Age (Years)</td>
<td>67*</td>
<td>67*</td>
<td>57</td>
</tr>
<tr>
<td>Gender (M / F)</td>
<td>29/26 (52.7%)*</td>
<td>26/19 (57.8%)*</td>
<td>7/16 (30.4%)</td>
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<tr>
<td>Stroke / TIA Subtype</td>
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<td><em>Atherothrombotic</em></td>
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<td>8 (17.8%)*</td>
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<tr>
<td><em>Lacunar</em></td>
<td>17 (30.9%)*</td>
<td>16 (35.6%)*</td>
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<tr>
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<td><em>Indeterminate</em></td>
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<td>16 (35.6%)*</td>
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<td>11 (24.4%)*</td>
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<tr>
<td>IHD</td>
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<td>7 (15.6%)*</td>
<td>0 (0%)</td>
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<td>Hypertension</td>
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<td>34 (75.6%)*</td>
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<td>Diabetes Mellitus</td>
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<td>8 (17.8%)</td>
<td>2 (8.7%)</td>
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<td>A Fib / Flutter</td>
<td>8 (14.5%)*</td>
<td>3 (6.7%)*</td>
<td>0 (0%)</td>
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<tr>
<td>Known Hyperlipidaemia</td>
<td>30 (54.5%)*</td>
<td>26 (57.8%)*</td>
<td>4 (17.4%)</td>
</tr>
<tr>
<td>Statin Rx.</td>
<td>19 (34.5%)*</td>
<td>21 (46.7%)*</td>
<td>1 (4.3%)</td>
</tr>
<tr>
<td>Fam. Hx. Stroke</td>
<td>19 (34.5%)</td>
<td>20 (44.4%)</td>
<td>10 (43.5%)</td>
</tr>
<tr>
<td>Prior VT</td>
<td>3 (5.5%)*</td>
<td>2 (4.4%)*</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Migraine</td>
<td>7 (12.7%)</td>
<td>5 (11.1%)</td>
<td>2 (8.7%)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>18 (32.7%)</td>
<td>9 (20%)</td>
<td>7 (30.4%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>17 (30.9%)</td>
<td>22 (48.9%)*</td>
<td>5 (21.7%)</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>20 (36.4%)</td>
<td>14 (31.1%)</td>
<td>11 (47.8%)</td>
</tr>
<tr>
<td>Alcohol Intake</td>
<td>2*</td>
<td>0*</td>
<td>14</td>
</tr>
</tbody>
</table>

(Median Units / Week)

Legend for Table 5.1: *, p < 0.05 for comparison of patients with controls using Chi-square testing (significant values are highlighted in bold); †, statistical testing not applicable or not performed; VT, Venous thrombosis
group because they subsequently met all of the inclusion criteria during follow-up. All remaining convalescent phase patients had been included in the acute CVD group. The results were compared with those obtained from 23 control subjects who were not on antiplatelet or anticoagulant therapy.

The clinical details of the study subjects are outlined in Table 5.1. The mean age of the acute and convalescent CVD patients (67 years) was significantly higher than that of controls (57 years). Hypertension and a definite diagnosis of hyperlipidaemia was more common in the acute and convalescent CVD patients compared with controls, and the proportion of ex-smokers was higher in the convalescent patient group compared with controls. However, the median alcohol intake per week was higher in control subjects than patients. Otherwise, there was no significant difference in vascular risk factor profiles between patients and controls.

Patients were on treatment with variable doses of aspirin between 75 to 300 mg daily at the time of assessment (median daily dose = 150 mg in the acute phase and 75 mg in the convalescent phase). The median duration of aspirin therapy before venepuncture was 10 days in the acute patient group and 120 days in the convalescent group. The mean interval between ingestion of the last dose of aspirin and blood sampling was 7.5 hours and 6.4 hours in the acute and convalescent groups, respectively.

The normal range of C-ADP closure times in control subjects in this study was 60 - 223 s (median 90 s). This is wider than the reference range previously published by our group in a population of younger healthy controls (mean 80.17 s, range 58-102 s (mean +/- 2 standard deviations)) because one female control subject had a C-ADP closure time of 243 s (Harrison et al, 1999). Although it is possible that this subject had a mild
undiagnosed platelet function disorder, she was included in our calculations because she satisfied all of the inclusion and exclusion criteria, and denied ingestion of aspirin therapy before testing. The intra-assay coefficient of variation (CV) for the C-ADP closure time, based on data from 10 healthy controls subjects in our laboratory, was 8.8%. There was a non-significant trend towards shortening of the median C-ADP closure time in the acute (80 s) and convalescent CVD patients (88 s) compared with controls (90 s), (p = 0.07, and p = 0.8, respectively) (Figure 5.1). Four patients (7%) in the acute phase had shortening of the C-ADP closure time below the lower range of normal for controls who were not on aspirin, suggesting some degree of platelet hyper-reactivity in these subjects despite being treated with aspirin therapy. None of the patients in the convalescent phase had shortened C-ADP closure times compared with controls.

The normal range of C-EPI closure times in the control group was 64 - 164 s (mean 114 s, median 117 s). This is also wider than the reference range previously published by our group in a population of younger healthy controls (mean 103.6 s, range 81-126 s [mean +/- 2 standard deviations]) (Harrison et al, 1999). The intra-assay coefficient of variation (CV) for the C-EPI closure time in our laboratory was 12.3%. The median C-EPI closure time was significantly prolonged in the acute (138 s vs.117 s, p = 0.009) and convalescent (172 s vs.117 s, p < 0.001) CVD patients on 75 to 300 mg of aspirin daily compared with controls (Figure 5.2). However, only 40% of patients in the acute phase and 57% of patients in the convalescent phase had a prolonged C-EPI closure time on aspirin therapy. Therefore, 60% of patients in the acute phase and 43% of patients in the convalescent phase exhibited ex vivo aspirin-resistance on treatment with 75 to 300 mg of aspirin daily using the PFA-100. The median C-ADP closure times were

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significantly shorter in the aspirin resistant compared with the aspirin responsive patients in the acute (73 vs. 94 s, \( p < 0.001 \)) and convalescent (81 vs. 91 s, \( p = 0.008 \)) phases. Only two patients who were aspirin-resistant and one who was aspirin-responsive had a further stroke or TIA on aspirin monotherapy (75 mg daily) during our follow-up period (median 140 days). Therefore, we could not draw any firm conclusions about the usefulness of this device in identifying patients at high risk of stroke recurrence on aspirin during follow-up.

We took the opportunity to measure the C-ADP and C-EPI closure times in six patients in the acute phase after ischaemic stroke or TIA (a) when they had been off aspirin therapy for at least 7 days and (b) when they were on treatment with aspirin for at least three days. The closure times off and on aspirin therapy are recorded in Table 5.2. Although treatment with aspirin increased the C-EPI closure time to some degree in all patients, four of the six patients did not exhibit prolongation the C-EPI closure time above the upper range of normal for controls who were not on aspirin.

We also investigated whether the \textit{ex vivo} response to aspirin therapy varied according to the underlying mechanism responsible for the ischaemic stroke or TIA. In comparison with control subjects who were not on aspirin (117 s), the median C-EPI closure time was significantly prolonged in the acute lacunar (144 s, \( p = 0.02 \)) and cardioembolic patients (161 s, \( p = 0.01 \)), but not in the acute atherothrombotic (119 s) or indeterminate (130 s) CVD patients on aspirin (Figure 5.3a). Overall, 7/11 (64%) atherothrombotic, 10/18 (56%) lacunar, 5/10 (50%) cardioembolic, and 12/18 (67%) indeterminate patients were aspirin-resistant in the acute phase after stroke or TIA.
Figure 5.1: Comparison of median C-ADP closure times in Control subjects not on antiplatelet or anticoagulant therapy (n = 23) with Acute (n = 57) or Convalescent (n = 46) Stroke or TIA patients on ASA

Legend for Figure 5.1: Symbols represent individual values; Horizontal bars represent median closure times; ASA, aspirin
Figure 5.2: Comparison of median C-EPI closure times in Control subjects not on antiplatelet or anticoagulant therapy (n = 23) with Acute (n = 57) or Convalescent (n = 46) Stroke or TIA patients on ASA

Legend for Figure 5.2: Symbols represent individual values; Horizontal bars represent median closure times; ASA, aspirin.
Table 5.2: C-ADP and C-EPI closure times (s) in response to aspirin therapy in six patients in the acute phase after ischaemic stroke or TIA (ASA doses are given in parentheses). The data are plotted in graphical form below the table.

<table>
<thead>
<tr>
<th></th>
<th>Patient 1 (300 mg)</th>
<th>Patient 2 (300 mg)</th>
<th>Patient 3 (150 mg)</th>
<th>Patient 4 (75 mg)</th>
<th>Patient 5 (75 mg)</th>
<th>Patient 6 (300 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-ADP Pre-ASA</td>
<td>81</td>
<td>65</td>
<td>77</td>
<td>76</td>
<td>78</td>
<td>81</td>
</tr>
<tr>
<td>C-ADP Post-ASA</td>
<td>84</td>
<td>69</td>
<td>73</td>
<td>91</td>
<td>72</td>
<td>93</td>
</tr>
<tr>
<td>C-EPI Pre-ASA</td>
<td>88</td>
<td>77</td>
<td>85</td>
<td>87</td>
<td>93</td>
<td>86</td>
</tr>
<tr>
<td>C-EPI Post-ASA</td>
<td>253</td>
<td>168</td>
<td>139</td>
<td>112</td>
<td>103</td>
<td>122</td>
</tr>
<tr>
<td>Baseline vWG:Ag</td>
<td>156</td>
<td>178</td>
<td>175</td>
<td>232</td>
<td>187</td>
<td>220</td>
</tr>
<tr>
<td>Baseline (IU/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Graphical representation of closure times](image-url)

Patient Treatment Status

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In the convalescent phase, the median C-EPI closure time was significantly prolonged in the atherothrombotic (188 s, p < 0.001), lacunar (165 s, p < 0.001), and indeterminate (175 s, p < 0.001) CVD subgroups on aspirin compared with controls who were not on aspirin (117 s) (Figure 5.3b). The treatment status of the atherothrombotic patients in the convalescent phase deserves specific mention. Two samples were tested before carotid endarterectomy, one sample was tested when the patient had subsequently developed a carotid occlusion, and six samples were tested at least three months after carotid endarterectomy (n = 5) or stenting (n = 1). Although there was a non-significant prolongation of the median C-EPI closure time in the cardioembolic subgroup (153 s, p = 0.06), these results should be interpreted with caution because there were only five cardioembolic stroke patients on aspirin therapy during follow-up (Figure 5.3b). Overall, 1/9 (11.1%) atherothrombotic, 8/16 (50%) lacunar, 3/5 (60%) cardioembolic, and 8/17 (47.1%) indeterminate CVD patients were aspirin-resistant in the convalescent phase.

The mean VWF:Ag level was significantly elevated in the acute (p < 0.001) and convalescent (p < 0.001) ischaemic CVD patients compared with controls (Figure 5.4). Spearman's rank correlation analysis showed that there was a significant inverse relationship between the plasma levels of VWF:Ag and the C-ADP closure times in control subjects and acute CVD patients, but not in the convalescent CVD patients (Table 5.3). There was also a significant inverse relationship between the plasma levels of VWF:Ag and the C-EPI closure times in control subjects and in both acute and convalescent stroke patients (Table 5.4). In addition, post-hoc analysis of the PFA-100 data revealed that the mean plasma levels of VWF:Ag were significantly higher in aspirin-resistant compared with aspirin-responsive patients in the acute phase (253.6 vs.
169.4 lU/dl, p = 0.001, 2-tailed t-test), but not in the convalescent phase (219.1 vs. 187.1 lU/dl, p = 0.2, 2-tailed t-test) after ischaemic stroke or TIA.

There was no significant difference in haematocrit in EDTA, or platelet count or MPV in EDTA or citrate between the acute or convalescent stroke or TIA patients and controls (data not shown).

5.4 Discussion
This is the first study to examine the ex vivo responsiveness to aspirin therapy in the acute and convalescent phases after ischaemic stroke or TIA using the PFA-100. We have identified some patients with apparent platelet hyper-reactivity in the acute phase after ischaemic stroke or TIA despite being treated with 75 to 300 mg of aspirin daily. In addition, 60% of patients in the acute phase and 43% of patients in the convalescent phase after ischaemic stroke or TIA were resistant to the antiplatelet effects of aspirin ex vivo. Although all six patients who were tested before and after treatment with aspirin had some prolongation of the C-EPI closure time with aspirin, the increase in the C-EPI closure time in at least four of these patients was less than that which had been predicted from published data in control subjects. It is uncertain whether the degree of prolongation of the C-EPI closure times with aspirin therapy observed in these patients is sufficient to protect against recurrent vascular events. One important clinical question that also needs to be addressed in future studies is whether aspirin-resistant patients are at higher risk of stroke recurrence than aspirin-responsive patients during long-term follow-up. This was not one of the objectives of this study, and the limited number of outcome events during our follow-up period did not allow further exploration of this issue.
Figure 5.3a: Comparison of Median C-EPI closure times in Controls subjects not on antiplatelet or anticoagulant therapy with Acute Stroke or TIA subtypes on aspirin.

Legend for Figure 5.3a: Symbols represent individual values; AT, Acute Atherothrombotic Stroke or TIA (n = 11); Lacunar, Acute Lacunar Stroke or TIA (n = 18); CE, Acute Cardioembolic Stroke or TIA (n = 10); Indeterminate, Acute Indeterminate Stroke or TIA (n = 18); Horizontal bars represent median closure times.
Figure 5.3b: Comparison of Median C-EPI closure times in Controls subjects not on antiplatelet or anticoagulant therapy with Convalescent Stroke or TIA subtypes on aspirin

Legend for Figure 5.3b: Symbols represent individual values; AT, Convalescent Atherothrombotic Stroke / TIA (n = 9); Lacunar, Convalescent Lacunar Stroke or TIA (n = 16); CE, Convalescent Cardioembolic Stroke or TIA (n = 5); Indeterminate, Convalescent Indeterminate Stroke or TIA (n = 16); Horizontal bars represent median closure times.
Figure 5.4: Comparison of mean VWF:Ag levels in Control Subjects not on antiplatelet or anticoagulant therapy (n = 23) with Acute (n = 57) or Convalescent (n = 46) Stroke or TIA Patients on ASA

Legend for Figure 5.4: Symbols represent individual values; VWF:Ag, von Willebrand factor antigen; Horizontal bars represent mean VWF:Ag levels.
Table 5.3: Correlation between the plasma levels of VWF:Ag and the C-ADP closure times recorded in the Control subjects who were not on aspirin or antiplatelet therapy and in the Acute and Convalescent Stroke patients on ASA

<table>
<thead>
<tr>
<th></th>
<th>Spearman's rho</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls Not on ASA</td>
<td>-0.52</td>
<td>0.01</td>
</tr>
<tr>
<td>Acute Patients on ASA</td>
<td>-0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Convalescent Patients on ASA</td>
<td>-0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 5.4: Correlation between the plasma levels of VWF:Ag and the C-EPI closure times recorded in the Control subjects who were not on aspirin or antiplatelet therapy and in the Acute and Convalescent Stroke patients on ASA

<table>
<thead>
<tr>
<th></th>
<th>Spearman's rho</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls Not on ASA</td>
<td>-0.61</td>
<td>0.002</td>
</tr>
<tr>
<td>Acute Patients on ASA</td>
<td>-0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Convalescent Patients on ASA</td>
<td>-0.39</td>
<td>0.008</td>
</tr>
</tbody>
</table>
It should be noted that this study was designed to assess the profile of aspirin responsiveness in a population of ischaemic stroke or TIA patients with typical vascular risk factor profiles compared with controls without a history of ischaemic cerebrovascular disease. As a consequence, some vascular risk factors were more common in patients than control subjects and these may have contributed to aspirin resistance in the patient population. However, because one could not assume that the C-EPI closure times were normally distributed, regression analysis could not be performed to examine the influence of these independent variables on the results of the PFA-100. In addition, one of the limitations of this study is that we did not assess the ex vivo responsiveness to varying doses of aspirin therapy in controls subjects, and further studies are warranted to examine this issue.

Grau et al. recently published the results of a case–crossover study on 31 patients in the convalescent phase after a lacunar or ‘atherothrombotic’ ischaemic stroke who were on treatment with aspirin (100 to 300 mg daily) (Grau et al, 2003). Patients were treated with 75 mg of clopidogrel daily, or a combination of clopidogrel and 300 mg of aspirin daily for four weeks at a time. The expression of CD62P and CD63 were measured using whole blood flow cytometry and the method of ‘fixation before antibody labelling’ (see section 3.4). PFA-100 analysis was carried out using 3.2% buffered sodium citrate samples. In this study, 5 of 31 patients (16.1%) did not have prolonged C-EPI closure times on aspirin, and could be defined as being aspirin resistant using the PFA-100. In addition, the authors reported the interesting finding that the combination of aspirin and clopidogrel significantly prolonged the C-ADP closure time compared with treatment with monotherapy with aspirin (p = 0.0009) or clopidogrel (p = 0.007). Overall,
the C-ADP closure time was prolonged in 2/31 patients on clopidogrel (6.5%), but in 8/29 patients (27.6%) on aspirin and clopidogrel. The C-ADP closure time was similar on treatment with aspirin compared with clopidogrel (p = 0.51).

A number of studies have also investigated the utility of the device in other clinical settings. One study of a diverse group of patients at a tertiary care centre showed that only 68.2% of patients who were taking aspirin for a variety of clinical conditions had prolonged C-EPI closure times using a 0.129 M citrate blood collection system (Ortel et al, 2000). However, the dose of aspirin involved was not specified, and compliance was not assessed in a standardised fashion. von Pape et al. also reported that only 79% of patients who had discontinued long term treatment with 100 mg of aspirin daily within the preceding 24 hours had a C-EPI closure time > 170 s using 0.106 M sodium citrate Sarstedt Monovette tubes (von Pape et al, 2000). However, the authors did not report the percentage of patients who had C-EPI closure times above the upper limit of their established laboratory reference range of 150 s, and compliance was not formally assessed in this study either. The authors also stated that “an unexpected high number of patients” had prolonged C-ADP closure times above the laboratory normal reference range of 120 s.

Two recent studies have investigated the inhibition of platelet function with aspirin using the PFA-100 in patients with cardiovascular disease. In the first study, C-ADP and C-EPI closure times were measured in 48 patients admitted to hospital for diagnosis or treatment of coronary artery disease (Feuring et al, 1999). The proportion of
patients in the study group with stable or unstable coronary artery disease was not stated. All patients were reportedly taking 100 mg of aspirin daily for at least seven days, and blood was collected into Sarstedt Monovette tubes containing 0.106 M unbuffered citrate; the samples were analysed within three hours of venepuncture. In contrast to the published results in healthy controls on aspirin (see above), only 31% of patients had prolonged C-EPI closure times above the upper range of normal for controls. Although not clearly stated in the report, a graphical representation of the data suggested that three patients (6.3%) had prolonged C-ADP closure times. It was concluded that 100 mg of aspirin daily led to insufficient inhibition of platelet aggregation in the majority of patients with coronary artery disease to be detected by the PFA-100. However, the results of this study cannot be compared with those obtained from healthy controls in other studies, because it has previously been reported that unbuffered sodium citrate is not an ideal anticoagulant to use with this device. It shortens the C-EPI closure time in comparison with that seen with other concentrations of citrate, and also significantly increases the frequency of flow obstructions that may be secondary to abnormal platelet clumping during the test (Heilmann et al, 1997). Therefore, these results should not be interpreted as showing ex vivo aspirin resistance in 69% of patients with cardiovascular disease.

The results of a larger study involving PFA-100 analysis in 325 patients with stable coronary artery disease who were on treatment with 325 mg of aspirin daily for the preceding seven days have also been published (Gum et al, 2001). 0.129 M citrate anticoagulated blood was used for PFA-100 analysis. 31 patients (9.5%) did not have prolonged C-EPI closure times above the laboratory normal range and were defined as
aspirin-resistant. It was interesting to note that only four of these 31 patients (13%) were also aspirin-resistant using optical platelet aggregometry, thus suggesting that cyclooxygenase-independent mechanisms were responsible for aspirin-resistance on the PFA-100 in the majority of patients in this study.

The potential mechanisms responsible for aspirin resistance on the PFA-100 can be divided into those that are cyclooxygenase-dependent and those that are independent of the cyclooxygenase pathway.

**Cyclooxygenase-dependent mechanisms of aspirin resistance**

Cyclooxygenase-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 (Schrör, 1997). However, its expression can also be regulated by certain stimuli (FitzGerald & Patrono, 2001). In contrast, cyclooxygenase-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 (Schrör, 1997); (Weber et al, 1999). It has traditionally been accepted that the only isoform of the enzyme present in platelets is cyclooxygenase-1 (FitzGerald & Patrono, 2001). Although Weber et al. recently published data suggesting that cyclooxygenase-2 is also present in platelets (Weber et al, 1999); (Weber et al, 2002)), this was not confirmed by others (Patrignani et al, 1999); (Reiter et al, 2001). However, more recent immunohistochemistry studies have shown that normal platelets express low levels of cyclooxygenase-2, but that the expression of this isoenzyme is upregulated in patients with increased platelet turnover (Rocca et al, 2002).
Aspirin resistance on the PFA-100 could be secondary to incomplete inhibition of cyclooxygenase-1 with subsequent thromboxane A2 formation despite treatment with 75–300 mg of aspirin daily. Although data from platelet aggregometry studies suggest that patients may have a more pronounced *ex vivo* response to higher doses of aspirin (Helgason *et al.*, 1994; Chamorro *et al.*, 1999a), there are no published studies on the effects of dose escalation on the results of the PFA-100 in ischaemic CVD. However, Homoncik *et al.* recently reported the results of a double-blind, two-way crossover study involving 10 healthy volunteers who were randomly assigned to receive 50 mg or 100 mg of oral aspirin daily for 11 days (Homoncik *et al.*, 2000b). After a washout period of 16 days, the subjects were treated with the alternate dose of aspirin. All subjects received their dose of aspirin on the morning of the test, and PFA-100 analysis was carried out between 30 minutes and four hours after venepuncture throughout the 11 day period using 0.129 M buffered sodium citrate Vacutainer tubes. The subjects also had serum thromboxane B2 levels measured to “confirm the inhibitory effect of aspirin” on cyclooxygenase activity. The C-ADP closure times were comparable in the two treatment groups and were not prolonged with aspirin. On the other hand, both aspirin doses prolonged the C-EPI closure time to some degree, and the authors reported the interesting finding that the C-EPI closure times were significantly more prolonged with 100 mg compared with 50 mg of aspirin daily on days 3 and 4 of treatment. In addition, the maximal prolongation of the C-EPI closure time was seen on day 4 with 100 mg of aspirin, but not until day 10 with 50 mg of aspirin daily. In keeping with these results, thromboxane B2 levels were significantly lower when patients were treated with 100 mg compared with 50 mg of aspirin daily. Although these findings suggest a more
pronounced ex vivo response to 100 mg compared with 50 mg of aspirin daily in healthy volunteers, the results should be interpreted with caution because there was no significant difference in C-EPI closure times between the two treatment groups at any other time point during the study. Further studies are required to examine whether the ex vivo response to aspirin varies as the dose is increased from 75 mg to 150 mg to 300 mg daily in patients with ischaemic stroke or TIA (see chapter 7).

If platelet production and turnover is increased, as might be seen in the setting of acute ischaemic CVD, then newly formed platelets possessing functionally active cyclooxygenase-1 may be able to produce thromboxane A_2 and contribute to aspirin resistance between aspirin doses, even if cyclooxygenase-1 is inhibited in the circulating platelet pool. Furthermore, if platelet cyclooxygenase-2 is upregulated in certain patients with ischaemic stroke or TIA, this could contribute to aspirin-resistance because aspirin is approximately 170-fold less potent at inhibiting cyclooxygenase-2 than cyclooxygenase-1 (Weber et al, 1999); (Reiter et al, 2001). Endothelial cells can rapidly resynthesise cyclooxygenase-1 and may continue to synthesise PGs via cyclooxygenase-2-mediated pathways after administration of aspirin (Harker, 1998). In patients with endothelial damage or atherosclerosis, PGs could be transferred from endothelial cells to platelets that are adherent to the subendothelial matrix (Schrör, 1997). In theory, this could result in enhanced platelet thromboxane A_2 formation by bypassing cyclooxygenase-1 in platelets, and cause aspirin resistance.

**Cyclooxygenase-independent mechanisms of aspirin resistance**

Elevated levels of VWF:Ag will facilitate enhanced platelet adhesion to subendothelial structures (e.g. collagen) and enhanced platelet to platelet aggregation under high shear
stress conditions. We found higher plasma levels of VWF:Ag in both the acute and convalescent phases after ischaemic stroke or TIA compared with controls who were not on aspirin. In addition, aspirin resistant patients had significantly higher levels of VWF:Ag in the acute phase, and a non-significant trend towards higher VWF:Ag levels in the convalescent phase compared with patients who were defined as aspirin responsive. Because of the inverse correlation between the levels of VWF:Ag and both the C-ADP and C-EPI closure times, elevated levels of VWF in ischaemic stroke or TIA are likely to play an important role in mediating aspirin resistance using this device. Because VWF levels may be elevated as part of the acute phase response (Homoncik et al, 2000a), this may have contributed to the higher prevalence of aspirin resistance in the acute phase compared with the convalescent phase after ischaemic stroke or TIA. Only six patients were studied before and after administration of aspirin monotherapy and further studies are required to determine whether higher baseline VWF:Ag levels are associated with a less pronounced ex vivo response to aspirin therapy in ischaemic CVD using the PFA-100.

Fibrinogen is the predominant ligand that binds to the glycoprotein IIb/IIIa receptor to mediate platelet-platelet aggregation (Wagner et al, 1996), and fibrinogen levels have been shown to be elevated in the acute phase after ischaemic stroke or TIA (Lip et al, 2002). Previous studies have suggested that fibrinogen does not significantly influence closure times recorded with the PFA-100 (Kundu et al, 1995); (Harrison et al, 1999). However, it is possible that as platelet plug formation progresses, and the flow rate in the device decreases, that fibrinogen may play an important role in stabilising the platelet aggregate further (Ruggeri, 2000). Consequently, elevated fibrinogen levels may
have a role to play in mediating aspirin resistance on the PFA-100 in patients with CVD, by causing premature C-EPI closure times despite aspirin therapy. We did not measure fibrinogen levels in our patient population, but further work is required to examine this hypothesis.

Certain platelet polymorphisms, especially those that influence platelet surface collagen receptor density (Carlsson et al, 1999), or the function of the glycoprotein IIb/IIIa receptor (Gum et al, 2001); (Cooke et al, 1998), may increase the likelihood of platelet adhesion or aggregation, respectively, and play a role in mediating aspirin resistance using the PFA-100.

Our preliminary data suggested that aspirin resistance was more common in acute atherothrombotic or indeterminate stroke or TIA patients than in patients with lacunar or cardioembolic CVD compared with controls. However, the number of patients in each subgroup was too small to draw any firm conclusions about the impact of stroke or TIA subtype on the prevalence of aspirin resistance after ischemic CVD. Further studies are warranted to re-investigate this issue and to determine whether aspirin resistant patients have a higher risk of stroke recurrence during long-term follow up, because the risk of early stroke recurrence is higher in patients with atherothrombotic CVD (Moroney et al, 1998; Sacco et al, 1989b) than in those with cardioembolic (International Stroke Trial Collaborative Group, 1997) or lacunar CVD. (Moroney et al, 1998); (Sacco et al, 1989b) In the convalescent phase, aspirin resistance was less common in atherothrombotic patients than in the other subgroups compared with controls. However, six of the nine atherothrombotic patients had undergone carotid endarterectomy or stenting before repeat testing, and each of these patients had less than 70% carotid stenosis on follow-up colour.
Doppler ultrasound. Successful surgical removal or stenting of the stenosing plaque in most of these patients, in addition to plaque stabilisation or carotid occlusion in the remainder over time, may explain why the vast majority of these patients subsequently became aspirin-responsive during follow-up. Some of the difference between the acute and convalescent phase results may also be secondary to resolution of the acute phase response over time.

Overall, this study has demonstrated that a large proportion of patients with ischaemic CVD are resistant to the antiplatelet effects of low to medium dose aspirin ex vivo using the PFA-100. The device has the potential to be useful for monitoring the response to aspirin therapy, but further work is required to determine whether the PFA-100 can reliably predict which ischaemic stroke or TIA patients are at high risk of recurrent vascular events during follow-up.
6. Measurement of the percentage of reticulated platelets in the early and late phases after ischaemic stroke or TIA

6.1 Introduction

The results of the flow cytometry experiments presented in chapter 3, along with published data illustrate that platelets are excessively activated in patients with ischaemic stroke or TIA (Grau et al, 1998); (Meiklejohn et al, 2001); (Marquardt et al, 2002); (Zeller et al, 1999); (Yamazaki et al, 2001); (Konstantopoulos et al, 1995); (Grau et al, 2003). Studies using washed, fixed platelets, obtained from PRP, have shown that the percentage of reticulated platelets (% RP) may increase when thrombopoiesis is increased, and may be normal or decreased when platelet production is suppressed (Ault et al, 1992). As outlined in section 2.3.6, measurement of the % RP 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 % RP may not be increased in ischaemic CVD unless the stimulus to platelet activation also promotes thrombopoiesis. The measurement of the % RP may also facilitate a better understanding of the mechanisms that mediate aspirin resistance in some CVD patients, because it has recently been suggested that COX-2 expression is upregulated in newly released (reticulated) platelets (Rocca et al, 2002). Functionally active COX-2 could contribute to thromboxane A2 formation in platelets, despite inhibition of COX-1 by aspirin therapy.

Previous studies have measured the % RP in small numbers of patients in the acute phase after an 'arterial thrombosis' (Rinder et al, 1998). Flow cytometric experiments in patients with thrombotic events in association with transient (n = 6) or
chronic thrombocytosis (n = 14) revealed an increase in the % RP compared with asymptomatic patients with thrombocytosis (n = 23) or normal controls (n = 83) (Rinder et al, 1998). In contrast, the % RP was not increased in 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 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. Two other recent studies have investigated the percentage of circulating reticulated platelets using flow cytometry in patients with ischaemic stroke. Smith et al. reported that the % RP in fixed whole blood was increased in 18 patients within three days of onset of an ischaemic stroke compared with 14 matched controls (12.8 vs. 8.2%, p = 0.044) (Smith et al, 2002). All patients were treated with aspirin, but they were not reassessed in the convalescent phase after symptom onset. Nakamura et al. compared the % RP in washed and fixed platelets obtained from PRP in 68 ischaemic stroke patients with 140 'neurological control' subjects (Nakamura et al, 2002). 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. However, the same patients were not assessed at each time point in this study. The % RP was higher in patients with cardioembolic stroke (n = 17) compared with
atherothrombotic stroke (n = 26), lacunar stroke (n = 25) or controls (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. The authors also suggested that the % RP in the cardioembolic subgroup was higher in the acute (n = 4) compared with the chronic phase (n = 10) after stroke onset (p < 0.05). However, they advised caution in interpreting these results because of the small number of patients studied. In addition, the authors reported the interesting finding that 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, it was 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.

We carried out an observational analytical study to investigate whether the % RP is increased in the early and late phases after an ischaemic stroke or TIA. We analysed the data to determine if the % RP was increased in any particular subtype of ischaemic CVD. In addition, because reticulated platelets are assumed to be larger and more reactive than mature platelets (Harrison et al, 1997), we examined the relationship between the % RP and the MPV in EDTA- and citrate-anticoagulated whole blood. Finally, we performed preliminary investigations to determine whether the % RP varied on different antiplatelet regimens in the early phase after symptom onset, and examined
the impact of aspirin dose escalation on the percentage of reticulated platelets in the convalescent phase after ischaemic stroke or TIA.

6.2 Methods

The same patients and controls who were included in the study entitled 'Flow cytometric evaluation of platelet activation in ischaemic stroke and TIA' were included in this study (see chapter 3). Therefore, the inclusion / exclusion criteria, method of clinical assessment and stroke subtyping, timing of initial and follow-up investigations, and assessment of drug compliance are identical to those outlined in chapter 3, and will not be repeated here. However, the 'acute' phase is referred to as the 'early' phase, and the 'convalescent' phase is referred to as the 'late' phase in this part of the study. Written informed consent, or assent where appropriate, was obtained from all subjects, and these experiments were approved by the Ethics Committees of the participating Hospitals.

Blood sampling was performed as outlined in Chapter 3. The first 4 ml of blood was collected into a sterile Vacutainer tube containing 7.2 mg of K₂ EDTA or 0.054 ml of 15% K₃ EDTA using a 21G Butterfly needle. This sample was used for FBC measurement, including measurement of the MPV using a GEN-S haematology analyser as outlined in section 2.2.1 (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.). The next 4.5 ml citrate-anticoagulated blood sample was used for whole blood flow cytometric analysis as outlined in Chapter 3. The methodology used to prepare the samples for measurement of the % GpIb binding (section 2.3.3) and the % RP (section 2.3.6) has been described previously. Three further samples were collected into 3.2% citrate anticoagulated Vacutainer tubes, and for the purpose of this study, the fourth citrated sample was used for measurement of the MPV and platelet count using a GEN-S
haematology analyser. Of note, it was planned that the FBC measurements would be performed between 2 and 4 hours after venepuncture to standardise the effect of EDTA-induced platelet swelling on the MPV results (O'Malley et al, 1995).

To determine whether the % RP was influenced by the antiplatelet drug regimens used, assays were repeated in the same patients if their antiplatelet therapy was altered in the first month after symptom onset. To investigate whether the % RP was influenced by the dose of aspirin used, reticulated platelet assays were also carried out in 10 patients in the late phase after an ischaemic stroke or TIA after two weeks treatment with 75, 150 or 300 mg daily, respectively. The characteristics of the patients involved in this pilot study are described in detail in chapter 7.

For flow cytometric analysis, a manual gate was positioned around the platelet cloud, and the platelets were identified by their characteristic pattern of forward scatter and side scatter. 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 (section 2.3.3). 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 test sample fluorescence in FL1 (see section 2.3.6 and Figures 2.38 II - 2.3.8 V).


**Statistical Methods**

Unpaired t-tests were used for comparison of the mean percentages of reticulated platelets between groups, where appropriate. Paired t-tests were used for comparison of the mean percentages of reticulated platelets between the same patients on different aspirin doses, and on different antiplatelet regimens. The Mann Whitney U test was used for comparison of median values between groups when the data were not normally distributed in either group. Pearson’s correlation analysis was performed to examine the relationship between the % RP and the MPV in EDTA- and citrate-anticoagulated whole blood. A p value < 0.05 was considered to be statistically significant. However, when comparing the % RP between the subgroups of late phase CVD patients on different aspirin doses, a p value of < 0.017 was considered significant to account for the multiple comparisons between subgroups. All statistical calculations were performed using SPSS-10 for Windows or Microsoft Excel 97 data analysis software.

**6.3 Results**

As outlined in chapter 3, 79 patients were assessed in the early phase (1-27 days) and 70 patients were re-assessed in the late phase (79-725 days) after an ischaemic stroke or TIA. The results were compared with those obtained from 27 control subjects. The clinical details of the study subjects, and the antithrombotic therapy they were receiving are outlined in Tables 3.1a and 3.1b, and will not be repeated here. The majority of patients were on treatment with variable doses of aspirin monotherapy between 75 to 300 mg daily at the time of assessment (median daily dose = 150 mg in the early phase and 75 mg in the late phase). The three control subjects who were on aspirin monotherapy were
taking 75 mg, 81 mg, and 150 mg daily, respectively, for at least nine months prior to assessment.

There were no significant differences in the % RP between early or late phase CVD patients and controls (Figure 6.1). Further analysis did not reveal any significant differences in the % RP between patients in the acute (0 - 7 days, n = 41) or subacute phases (8-30 days, n = 38) after symptom onset compared with controls (p > 0.3). Moreover, the % RP was not significantly increased in any particular subtype of ischaemic stroke compared with controls (Figure 6.2 and Figure 6.3).

The median time from venepuncture to measurement of the MPV was 172 minutes and 162 minutes in the early and late phase CVD patients, respectively, and 163 minutes in the control group. Only 4 patients in the early phase, 1 patient in the late phase, and 4 control subjects had their FBC measurements performed outside the preplanned time frame of 2 to 4 hours. As outlined in chapter 3, the mean white cell count (p = 0.001, p = 0.02), including the mean neutrophil (p = 0.001, p = 0.048) and monocyte counts (p = 0.001, p = 0.007) in EDTA-anticoagulated blood were significantly higher in the early and late phases after symptom onset compared with controls. Otherwise, there were no significant differences in any other FBC parameters, including the MPV, in either EDTA- or citrate-anticoagulated whole blood between patients and controls (Figure 6.4a and Figure 6.4b).

In the early phase after symptom onset, there was a weak positive correlation between % RP and the MPV in EDTA- (p < 0.001) and citrate-anticoagulated whole blood (p = 0.004) (Figure 6.5a and Figure 6.5b). However, the correlation between the %
RP and the MPV in EDTA ($p = 0.1$) and citrate ($p = 0.09$) was no longer significant in the late phase after ischaemic CVD (Figure 6.6a and Figure 6.6b).

Figure 6.1: Comparison of the mean percentage of reticulated platelets between control subjects ($n = 27$) and early ($n = 79$) and late phase ($n = 70$) ischaemic CVD patients

Legend for Figure 6.1: CVD, cerebrovascular disease; Symbols represent individual values; Horizontal lines represent mean % RP.
Figure 6.2: Comparison of the mean percentage of reticulated platelets between control subjects (n = 27) and Early Phase ischaemic CVD subtypes

<table>
<thead>
<tr>
<th></th>
<th>% RP</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>9.2%</td>
<td>0.8</td>
</tr>
<tr>
<td>AT</td>
<td>8.9%</td>
<td>0.3</td>
</tr>
<tr>
<td>Lac</td>
<td>10.9%</td>
<td>0.3</td>
</tr>
<tr>
<td>CE</td>
<td>11%</td>
<td>0.4</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>10.4%</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Legend for Figure 6.2: CVD, cerebrovascular disease; Symbols represent individual values; AT, Atherothrombotic Stroke or TIA (n = 19); Lac, Lacunar Stroke or TIA (n = 20); CE, Cardioembolic Stroke or TIA (n = 12); Indeterminate, Indeterminate Stroke or TIA (n = 28); Horizontal bars represent mean % RP.
Figure 6.3: Comparison of the mean percentage of reticulated platelets between control subjects (n = 27) and Late Phase ischaemic CVD subtypes

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>AT</th>
<th>Lac</th>
<th>CE</th>
<th>Indeterminate</th>
</tr>
</thead>
<tbody>
<tr>
<td>% RP</td>
<td>9.2%</td>
<td>11.6%</td>
<td>10.1%</td>
<td>10.8%</td>
<td>10.8%</td>
</tr>
<tr>
<td>p</td>
<td>0.1</td>
<td>0.5</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

**Legend for Figure 6.3:** CVD, cerebrovascular disease; Symbols represent individual values; AT, Atherothrombotic Stroke or TIA (n = 16); Lac, Lacunar Stroke or TIA (n = 19); CE, Cardioembolic Stroke or TIA (n = 9); Indeterminate, Indeterminate Stroke or TIA (n = 26); Horizontal bars represent mean % RP.
Figure 6.4a: Comparison of median MPV (fl) in EDTA-anticoagulated blood between controls (n = 27) and early (n = 78) and late phase (n = 69) CVD patients

Legend for Figure 6.4a: CVD, cerebrovascular disease; Symbols represent individual values; Horizontal lines represent the median MPV in EDTA in each subject group; Vertical bars represent the 5th to 95th percentiles.
Figure 6.4b: Comparison of mean MPV (fl) in citrate-anticoagulated blood between controls (n = 27) and early (n = 78) and late phase (n = 68) CVD patients

Legend for Figure 6.4b: CVD, cerebrovascular disease; Symbols represent individual values; Horizontal lines represent the mean MPV in citrate in each subject group; Vertical bars represent the 5th to 95th percentiles.
Figure 6.5a and Figure 6.5b: Correlation between the % RP and MPV in EDTA and citrate in the early phase after ischaemic CVD; Symbols represent individual values

**Figure 6.5a**
Pearson correlation coefficient = 0.39

![Graph showing correlation between MPV and T.O. TEST % for EDTA](image)

**Figure 6.5b**
Pearson correlation coefficient = 0.33

![Graph showing correlation between MPV and T.O. TEST % for citrate](image)
Figure 6.6a and Figure 6.6b: Correlation between the % RP and MPV in EDTA and citrate in the late phase after ischaemic CVD; Symbols represent individual values

**Figure 6.6a**
Pearson correlation coefficient = 0.19

![Figure 6.6a](image1)

**Figure 6.6b**
Pearson correlation coefficient = 0.21

![Figure 6.6b](image2)
Figure 6.7: Comparison of mean % reticulated platelets between Late Phase CVD patients (n = 10) on 75 vs. 150 mg (p = 0.5), 75 vs. 300 mg (p = 0.4), and 150 vs. 300 mg of aspirin daily (p = 0.6)

Legend for Figure 6.7 ASA, aspirin; Symbols represent individual values; Horizontal lines represent the mean % RP on each dose of aspirin.

Seven patients had reticulated platelet measurements performed in the first 20 days after symptom onset both off and on aspirin therapy. The prescribed aspirin dose was 75 mg (n = 2), 150 mg (n = 2), or 300 mg daily (n = 3), and the treatment duration varied between 3 days and more than 1 year. Three of these seven patients were on warfarin after
cessation of aspirin therapy, and their aspirin had been discontinued at least 8 days before repeat blood sampling was performed. The mean % RP was similar when patients were off aspirin (9.1%) compared with when they were receiving aspirin (9.7%, p = 0.6). Eight patients were analysed before and after the addition of 200 mg of modified release dipyridamole BD to their aspirin therapy. The mean % RP was not significantly lower on combination therapy with aspirin and dipyridamole (8.9%) compared with aspirin monotherapy (9.6%, p = 0.7). We also examined the impact of increasing the aspirin dose from 75 mg to 150 mg to 300 mg daily, at two-week intervals, on the results of the reticulated platelet assay in ten patients in the late phase after ischaemic CVD. Although there was a trend towards a reduction in the mean % RP with increasing aspirin doses, the differences between the subgroups did not reach statistical significance (Figure 6.7).

6.4 Discussion
Using whole blood flow cytometry, we did not find a significant increase in the percentage of reticulated platelets in the early or late phases after ischaemic stroke or TIA, or in any ischaemic CVD subgroup, compared with controls. Our results are in keeping with data published previously in a small group of patients in the acute phase after a vascular event in the coronary, cerebral or peripheral vascular tree (Rinder et al, 1998). However, they do not confirm other preliminary reports of an increased percentage of reticulated platelets at different stages after ischaemic stroke (Smith et al, 2002); (Nakamura et al, 2002). The discrepancy between our results and those reported by Smith et al. may reflect the fact that the early phase patients in our study were investigated at various times points in the first 30 days, rather than in the first 3 days after symptom onset (Smith et al, 2002). Because reticulated platelets have a life span in
animal studies of less than 24 hours (Ault & Knowles, 1995), one might expect to find a higher % RP in CVD patients if the assays are performed closer to the time of symptom onset, before a large proportion of reticulated platelets have been removed from the circulation. However, because the p value in the small study by Smith et al. just reached conventional statistical significance (p = 0.044), further studies involving larger groups of subjects are required to confirm their preliminary findings. Nakamura et al. only identified an increase in the % RP in the cardioembolic stroke subgroup, but did not report the comparison of the % RP in the ischaemic stroke population overall with control subjects. Once again, only small numbers of patients with cardioembolic stroke were included in that study (n = 17), and the same reservations regarding interpretation of their data could be applied. Of course, it is also possible that the moderate sample size in our study could have contributed to a type II error, and if larger numbers of patients were studied, we may have identified significant differences between patients and controls. It is also important to note that a different methodology for measuring the % RP was used in our study. We used a low concentration of thiazole orange that has been shown to specifically stain mRNA in reticulated platelets. (Robinson et al., 1998); (Robinson et al., 2000b), (Robinson et al., 2000a). However, it appears that Smith et al. used a higher concentration of thiazole orange (800 μl) that may have contributed to non-specific labelling of platelet dense granules and possibly mitochondrial DNA. In addition, the samples were fixed before the addition of thiazole orange in both of the aforementioned studies (Smith et al., 2002); (Nakamura et al., 2002), and Nakamura et al. used PRP rather than whole blood, whereas, our analyses were performed in unfixed whole blood. These factors could have contributed to differences in the results obtained between studies.
We did not find a significant increase in the MPV in EDTA- or citrate-anticoagulated whole blood in either the early or late phase CVD patients compared with controls. These findings are in contrast to those reported in previous studies of ischaemic stroke (O'Malley et al, 1995); (Butterworth & Bath, 1998), but in agreement with those in a more recent smaller pilot study outlined above (Smith et al, 2002). O'Malley et al. found that the MPV (EDTA) was significantly increased in 58 patients in the first 48 hours after an ischaemic stroke compared with 50 non-stroke controls (p < 0.001) (O'Malley et al, 1995). All analyses were performed at 24 hours after venepuncture, because EDTA-induced platelet swelling is maximal in the first two hours, and increases in the first 24 hours after blood sampling (O'Malley et al, 1995). The MPV did not significantly decrease in 29 patients who were retested at least 6 months after symptom onset compared with the early phase samples. However, the authors did not report the statistical comparison between the late phase patient samples and the control group. Butterworth et al. reported that the mean MPV in EDTA (p = 0.015) and citrate (p = 0.04) was higher in 137 ischaemic stroke patients within 48 hours of symptom onset compared with 65 age-, gender- and race-matched controls (Butterworth & Bath, 1998). Once again, they reported that the mean MPV was not significantly different at the 3 month follow-up visit (n = 99) compared with the acute phase (n = 137), and one assumes that the convalescent MPV value was still significantly higher than that seen in controls. A number of factors may have contributed to the discrepancies between the results of our study and those published by O'Malley et al. and Butterworth et al. The number of patients included in our study was smaller, and the fact that we used a lower concentration of sodium citrate (9:1 blood:citrate) than Butterworth et al. (4:1
blood:citrate) may have influenced our results. In healthy controls, lower concentrations of sodium citrate have been shown to cause some degree of platelet swelling in the first two hours after venepuncture compared with higher concentrations of the anticoagulant (Bath, 1993). However, because this was not one of the objectives of our study, it is not known whether citrate anticoagulant-induced platelet swelling affects patient and control samples equally, and whether this may have confounded the results of our MPV assay. In addition, the differences between the results of the studies may also have been influenced by the fact that we measured the MPV in EDTA at an earlier, but standardised time after venepuncture compared with others (O'Malley et al, 1995), but did not use the equations published by Trowbridge et al to correct for this time delay (Bath, 1993); (Butterworth & Bath, 1998).

We found a weak positive correlation between % RP and the MPV in the early phase after ischaemic CVD. This is consistent with the hypothesis that the MPV increases as the % RP in the sample increases, and that reticulated platelets are larger than more mature ‘non-reticulated’ platelets. The non-significant correlation between these indices in the late phase after symptom onset is not explained, because the mean % RP (Figure 6.1) and the mean MPV in EDTA and citrate (Figures 6.4a and 6.4b) were similar at both stages after symptom onset.

The % RP was not significantly influenced by treatment with low to medium dose aspirin, or by the addition of dipyridamole to the aspirin treatment regimen. These preliminary data provide support for our rationale of combining the results of reticulated platelet assays from CVD patients on variable doses of aspirin between 75 to 300 mg daily in comparison with those obtained from controls. It is important to note that our
results do not confidently exclude an effect of antiplatelet or anticoagulant therapy on the results of our reticulated platelet assay, because of the small number of subjects included in this analysis. The use of antithrombotic therapy in the majority of our patients could have masked any significant differences between the patient and control groups (table 3.1b). However, Smith et al. did find a significant increase in the % RP in acute ischaemic stroke compared with controls, and all of their patients were treated with aspirin therapy. It is important to note that 45% of control subjects in Nakamura’s study were on antiplatelet or anticoagulant therapy (Nakamura et al., 2002). It is possible that these agents could have decreased the % RP to a greater degree in controls than in CVD patients with excessive platelet activation, and accentuated the differences between the two groups of subjects. Therefore, further well designed studies in larger groups of controls and ‘matched’ CVD patients, tested before and after a change in antiplatelet therapy, are required to address this issue.

In conclusion, based on our data and the literature that has been reviewed in chapter 3, one can conclude that measurement of the % RP is a less sensitive marker of platelet activation ex vivo than the expression of CD62P or CD63, or the percentage of circulating leucocyte-platelet complexes. However, studies in larger groups of CVD patients in the acute (within 24 hours) and late phases after symptom onset may improve our understanding of the pathophysiology of ischaemic CVD, and perhaps the phenomenon of aspirin resistance. If a reference standard for the measurement of reticulated platelets is developed, and if the methodology used to quantify the % RP is standardised, this will facilitate comparison of results from different laboratories and would facilitate multicentre collaborative studies.
7. Assessment of platelet activation and function in response to increasing doses of oral aspirin in patients with a history of ischaemic stroke or TIA

7.1 Introduction
As outlined in section 1.6.4, The Dutch TIA Trial showed that 30 mg or 283 mg of aspirin daily were equally effective at preventing non fatal stroke, non fatal MI or vascular death during long-term follow-up in patients with ischaemic cerebrovascular disease (The Dutch TIA Trial Study Group, 1991). A subsequent meta-analysis in patients who had a history of TIA or non disabling stroke showed that the relative reduction in the risk of subsequent vascular events was similar with low dose (< 100 mg daily, 13%) or medium dose aspirin (300 mg daily, 9%) compared with controls (Algra & van Gijn, 1996). The most recent meta-analysis performed by The Antithrombotic Trialists’ Collaboration did not include data on the effects of different aspirin doses on outcome in the subgroup of patients with prior ischaemic stroke or TIA (Antithrombotic Trialists' Collaboration, 2002). However, compared with controls, the proportional reduction in the odds of experiencing a recurrent vascular event was 32% with low dose aspirin (75 - 150 mg daily) and 26% with medium dose aspirin (160 – 325 mg daily) amongst different groups of patients at high risk of vascular events. The benefit of secondary prevention with doses of aspirin < 75 mg daily was less clear (p = 0.05) (Antithrombotic Trialists' Collaboration, 2002). Because of the uncertainty about the efficacy of very low dose aspirin (< 75 mg daily) in patients at high risk of vascular
events overall, it is reasonable to propose that 75 to 300 mg of aspirin daily should be used for long term secondary prevention in patients with ischaemic stroke or TIA. However, it is uncertain whether 75, 150, or 300 mg is the optimal dose for long-term secondary prevention of vascular events in this patient population. Because higher doses of aspirin are associated with more side effects than lower doses of the drug (The Dutch TIA Trial Study Group, 1991), (Farrell et al, 1991), it would be useful to have some laboratory data to guide clinical practice and prescription habits.

The data presented in chapter 5 on the measurement of the antiplatelet effects of aspirin using the PFA-100® in ischaemic cerebrovascular disease (see chapter 5) included patients on different doses of aspirin between 75 to 300 mg daily. Although the findings by Homoncik et al. suggested a more pronounced ex vivo response to 100 mg compared with 50 mg of aspirin daily using the PFA-100® in healthy volunteers (Homoncik et al, 2000b), the results should be interpreted with caution for reasons outlined previously (see chapter 5). To our knowledge, there are only preliminary published data on the effects of different aspirin doses on the results of the PFA-100® in ischaemic CVD (Grau et al, 2003). To address this issue, we carried out a pilot study to determine whether the ex vivo response to aspirin therapy varied with incremental doses of the drug. We also measured platelet aggregation in response to the various chemical agonists used in the PFA-100 test system, namely, ADP, epinephrine, and collagen, in addition to sodium arachidonate-induced platelet aggregation. Furthermore, whole blood flow cytometry was used to determine whether the expression of a number of platelet activation markers varied with different doses of aspirin.
7.2 Methods

7.2.1 Patient Inclusion / Exclusion Criteria

Ten patients in the convalescent phase (at least three months after symptom onset) after an ischaemic stroke or TIA were recruited for this study. Nine patients had participated in the observational analytical study entitled 'Measurement of the antiplatelet effects of low or medium dose oral aspirin in ischaemic stroke or TIA using the PFA-100®' (see chapter 5). These patients fulfilled all the requirements for inclusion/exclusion in that study, and were on treatment with aspirin monotherapy (75 to 300 mg daily) for long-term secondary prevention of vascular events. One patient was recruited in the convalescent phase after a left central retinal infarction of indeterminate aetiology, and he also fulfilled the same inclusion/exclusion criteria outlined in chapter 5. All patients underwent a detailed clinical assessment at the beginning of the study, and the underlying mechanism responsible for the ischaemic stroke or TIA was determined using the criteria laid down in chapter 5.

7.2.2 Patient treatment protocol

All patients were treated with 75 mg, 150 mg, and subsequently 300 mg of soluble aspirin daily for two weeks at a time. The same investigator dispensed the aspirin tablets at each study visit (DJHM) to ensure that the same formulation of aspirin was administered to all patients throughout the study. A clinical assessment, and blood sampling and analysis were performed at the end of each two week treatment period (see below). When the study was completed, patients recommenced the original dose of aspirin they had been taking for long-term secondary prevention.
7.2.3 Blood sampling and laboratory tests

All subjects were rested for at least 20 minutes before venepuncture, and blood sampling was performed as outlined in section 2.2.1. The initial EDTA sample was used for measurement of FBC, including measurement of the MPV using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.). The first 4.5 ml 3.2% citrate anticoagulated blood sample was used for measurement of C-ADP and C-EPI closure times in whole blood using the PFA-100® within 2 and 2 ½ hours after venepuncture (see sections 2.4.2 and 5.2.8), and for whole blood flow cytometric analysis (see section 2.3.3). The percentage expression of Gplb, CD62P and CD63 (section 2.3.3), PAC1 binding (section 2.3.4), and the percentage of neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes (section 2.3.5) were calculated as outlined previously. The next two citrated samples were used to prepare double spun PPP, and were immediately stored at -70 °C for subsequent measurements of the levels of sP-selectin, sE-selectin (sections 2.6.3 and 2.6.4), and VWF:Ag (section 2.7.3). The fourth citrate sample was used for measurement of the platelet count and MPV in citrate-anticoagulated whole blood using a GEN-S haematology analyser (Beckman Coulter United Kingdom Ltd., High Wycombe, U.K.). Three additional 4.5 ml samples were drawn into 0.105 M sodium citrate Vacutainer® tubes to prepare PRP and PPP for platelet aggregometry studies, as outlined in section 2.5.3. The final concentration of agonists used in the platelet aggregometry experiments were 5 μM ADP, 10 μM epinephrine, 1 mM sodium arachidonate, and 1 μg/ml of collagen. The maximum percentage aggregation and the final percentage aggregation were recorded for each of the four agonists. However, only the data regarding the maximum percentage aggregation were
used for statistical comparisons between groups. It is important to note that the maximum aggregation values generated by the PAP-4 instrument incorporate the changes in light transmission caused by addition of the reagent (the ‘reagent jump’) and true platelet aggregation. Because epinephrine- and collagen-induced platelet aggregation are primarily dependent on intact cyclooxygenase function, and sodium arachidonate is entirely dependent on cyclooxygenase to induce aggregation, we predicted that the responses to these agonists would be markedly inhibited in aspirin-responsive subjects (Helgason et al, 1993). However, because ADP-induced platelet aggregation is only partially dependent on intact cyclooxygenase function, we did not expect the same degree of inhibition of platelet aggregation in aspirin responsive subjects with this agonist.

7.2.4 PFA-100® Control Data

The PFA-100® data obtained from the patients in this study on varying doses of aspirin were compared with the PFA-100® data collected from the 23 control subjects of similar age who participated in the study on the ‘Measurement of the antiplatelet effects of low or medium dose oral aspirin in ischaemic stroke or TIA using the PFA-100®’ (chapter 5). As outlined previously, these control subjects had no history of CVD, were not on antiplatelet or anticoagulant therapy, and underwent colour Doppler ultrasound examination of carotid and vertebral arteries to rule out severe (> 70%) carotid or vertebral stenosis.

7.2.5 Platelet Aggregometry Control Data and Quality Control

Each stroke patient acted as his / her own control, because the patient’s own PPP was used to set the aggregometer to 100% aggregation, and the patient’s PRP was used to set
the baseline aggregation to 0% (section 2.5.3). However, we also performed aggregometry studies on six control subjects (age range: 45 - 72 years old) who had no prior history of stroke or TIA, to establish the range of aggregation responses to each of the agonists used in our laboratory. Five of these control subjects were not on aspirin therapy, whereas one control subject was taking 75 mg of aspirin daily for primary prevention of vascular events. Five control subjects were tested before receiving aspirin to ensure that the agonists used were inducing the expected degree of platelet aggregation, and retesting was planned for all six controls after two weeks treatment with 75, 150 and 300 mg of aspirin daily, respectively. In addition, a blood sample from a healthy laboratory volunteer, who had not taken aspirin or any other antiplatelet agent in the preceding 10 days, was tested in parallel with a study sample at least once per week to assess the reliability of the results obtained in the CVD patients on aspirin. On each occasion, the PRP from the healthy volunteer exhibited an expected pattern of aggregation with each of the agonists used (data not shown).

7.2.6 Compliance

Patients and controls were strongly encouraged to fully comply with their treatment regimen at each study visit. The aspirin tablets were stored in a blister pack to facilitate adherence to the study protocol, and compliance was assessed at each visit by pill counting and / or history taking.
7.2.7 Statistical Methods

The normal range of PFA-100\textsuperscript{®} closure times in 23 control subjects who were not on antiplatelet or anticoagulant therapy was established by calculating the median (5\textsuperscript{th} to 95\textsuperscript{th} percentile) closure time for the C-ADP cartridge, and the mean (+/- 2 standard deviations) closure time for the C-EPI cartridge (chapter 5). Because the maximum closure time recorded by the PFA-100\textsuperscript{®} is 300 seconds, and because some patients on aspirin had C-EPI closure times exceeding 300 seconds, we assumed that the C-EPI closure time data in patients did not conform to a normal distribution. As outlined in chapter 5, patients who did not have prolonged C-EPI closure times on aspirin therapy were defined as having \textit{ex vivo} aspirin-resistance in this study. We used the Mann-Whitney U test for comparison of median C-ADP and C-EPI closure times between the patient and control groups.

For the aggregometry data, we calculated the maximum platelet aggregation in response to each of the agonists used in the control group, both off and on aspirin therapy. For descriptive purposes, CVD patients were defined as being 'aspirin-resistant' \textit{using platelet aggregometry} if their maximum percentage platelet aggregation exceeded the maximum percentage aggregation observed in control subjects on any dose of aspirin.

In addition, for the PFA-100, flow cytometry, sP-selectin, sE-selectin, VWF:Ag assays, and platelet aggregometry data, we compared the results obtained from the patient subgroups on the following dosing regimens:

- 75 mg daily vs. 150 mg daily,
- 75 mg daily vs. 300 mg daily
- 150 mg daily vs. 300 mg daily.
A paired t-test was used for comparison of mean values between groups when the data were normally distributed, and the Wilcoxon signed rank test for comparison of median values between groups when the data were not normally distributed in either group. To adjust for the multiple comparisons between groups, a p value < 0.01 was considered significant for the PFA-100 data, and a p value of < 0.017 was considered significant for all other data. All statistical calculations were performed using SPSS-10 for Windows or Microsoft Excel 97 data analysis software.

7.2.8 Ethical approval and consent

The study was approved by the Local Research Ethics Committees at The National Hospital for Neurology and Neurosurgery and UCLH. Written informed consent was obtained from all participants.

7.3 Results

The mean age of the 10 convalescent CVD patients included in this study was 64 years, with a mean age of 57 years in both the 23 control subjects involved in the PFA study (chapter 5), and the six control subjects involved in the aggregometry study. The underlying mechanism responsible for the ischaemic stroke or TIA in the 10 convalescent CVD patients was determined to be lacunar in four patients and indeterminate in five patients (the patient with the left central retinal infarction was categorised as having an ischaemic stroke of indeterminate aetiology). The remaining patient was assessed two years after successful right carotid endarterectomy for symptomatic 80% right carotid stenosis associated with atherothrombotic TIAs; he had been free of symptoms since the time of surgery.
All stroke patients fully complied with their treatment regimen throughout the study period. One control subject missed one 75 mg tablet during the two week treatment period. One of the controls had GI intolerance on 300 mg of aspirin daily, and discontinued aspirin before being retested on this dose. Otherwise, all control subjects fully complied with the treatment regimen throughout the study period. Therefore, data from six controls on 75mg and 150 mg of aspirin daily, and five controls on 300 mg of aspirin daily were included in the analysis.

**PFA-100**

The normal range of C-ADP closure times in control subjects was 60 - 223 s (median 90 s). There were no significant differences in median C-ADP closure times between control subjects who were not on aspirin or antiplatelet therapy and stroke patients on 75 mg, 150 mg or 300 mg daily (Figure 7.1). One patient on 75 mg, and a different patient on 150 mg of aspirin daily had a shortened C-ADP closure time below the lower range of normal for controls, suggesting some degree of platelet hyper-reactivity in these two patients despite treatment with aspirin therapy. There were no significant differences in median C-ADP closure times between stroke patients on 75 and 150 mg, 75 mg and 300 mg, and 150 and 300 mg of aspirin daily (p > 0.1 for all comparisons).

The normal range of C-EPI closure times in the control group was 64 - 164 s (mean 114 s, median 117 s). Compared with control subjects, the median C-EPI closure
Figure 7.1: Comparison of median C-ADP closure times in Control subjects who were not on ASA (n = 23) with Convalescent Stroke / TIA patients on treatment with 75 mg (n = 10), 150 mg (n = 10), or 300 mg (n = 10) of aspirin daily for 2 weeks.

Legend for Figure 7.1: Symbols represent individual values; Horizontal lines represent the median C-ADP Closure time in each group.

time was significantly prolonged in patients on each dose of aspirin (75 mg, 150 mg and 300 mg daily) (Figure 7.2). However, five of the ten (50%) convalescent CVD patients involved in this pilot study exhibited ex vivo aspirin resistance using the PFA-100, regardless of the dose of aspirin used. There was no evidence of heterogeneity of effect.
between dosing regimens (p > 0.4 for comparisons of median C-EPI closure times between stroke patients on 75 and 150 mg, 75 and 300 mg, and 150 and 300 mg of aspirin daily). In addition, only two patients exhibited a consistent trend towards greater prolongation of the C-EPI closure time as the aspirin dose was increased from 75 mg to 150 mg to 300 mg daily (Figure 7.3). It was also of interest that two patients who were aspirin-responsive ex vivo on 75 mg daily were aspirin-resistant on 300 mg daily (Figure 7.3).

**Flow cytometry**

The mean % expression of CD62P and CD63, and the mean % PAC1 binding was similar in stroke patients on 75 mg, 150 mg and 300 mg daily (p > 0.09 for comparisons between stroke patients on 75 and 150 mg, 75 mg and 300 mg, and 150 and 300 mg of aspirin daily) (Figure 7.4, Figure 7.5, Figure 7.6). The mean % of circulating neutrophil-platelet and monocyte-platelet complexes, and the median % lymphocyte-platelet complexes were also similar in patients on each dose of aspirin (p > 0.1 for all comparisons between stroke patients on 75 and 150 mg, 75 mg and 300 mg, and 150 and 300 mg of aspirin daily) (Figure 7.7, Figure 7.8, Figure 7.9).

**sP-selectin, sE-selectin and VWF:Ag data**

There were no significant differences in the levels of sP-selectin, sE-selectin or VWF:Ag between stroke patients on 75 and 150 mg, 75 mg and 300 mg, and 150 and 300 mg of aspirin daily (p > 0.5 for all comparisons, data not shown).
Figure 7.2: Comparison of median C-EPI closure times in Control subjects who were not on ASA (n = 23) with Convalescent Stroke / TIA patients on treatment with 75 mg (n = 10), 150 mg (n = 10), or 300 mg (n = 10) of aspirin daily for 2 weeks.

Legend for Figure 7.2: Symbols represent individual values; Dotted line represents the upper range of normal for control subjects not on ASA or anticoagulant therapy.
Figure 7.3: C-EPI closure times in individual CVD patients on 75 mg, 150 mg, and 300 mg of aspirin daily

Legend for Figure 7.3: Only patient 2 and patient 3 exhibited a consistent trend towards greater prolongation of the C-EPI closure time with increasing doses of aspirin
Figure 7.4. Comparison of mean percentage CD62P expression between stroke patients on 75 and 150 mg (p = 0.3), 75 mg and 300 mg (p = 0.3), and 150 and 300 mg of aspirin daily (p = 0.9)

Legend for Figure 7.4: Symbols represent individual values; Horizontal lines represent mean % expression of CD62P on each dose of aspirin
Figure 7.5. Comparison of mean percentage CD63 expression between stroke patients on 75 and 150 mg (p = 0.6), 75 mg and 300 mg (p = 0.97), and 150 and 300 mg of aspirin daily (p = 0.6)

Legend for Figure 7.5: Symbols represent individual values; Horizontal lines represent mean % expression of CD63 on each dose of aspirin
Figure 7.6. Comparison of mean percentage PAC1 binding between stroke patients on 75 and 150 mg (p = 0.4), 75 mg and 300 mg (p = 0.09), and 150 and 300 mg of aspirin daily (p = 0.1)

Legend for Figure 7.6: Symbols represent individual values; Horizontal lines represent mean % PAC1 binding on each dose of aspirin
Figure 7.7. Comparison of mean percentage circulating Neutrophil-Platelet Complexes between stroke patients on 75 and 150 mg (p = 0.4), 75 mg and 300 mg (p = 0.4), and 150 and 300 mg of aspirin daily (p = 0.9)

Legend for Figure 7.7: Symbols represent individual values; NPhil-Plts %, % of neutrophils bound to platelets; Horizontal lines represent the mean % of circulating neutrophil-platelet complexes on each dose of aspirin.
Figure 7.8. Comparison of mean percentage circulating Monocyte-Platelet Complexes between stroke patients on 75 and 150 mg (p = 0.2), 75 mg and 300 mg (p = 0.6), and 150 and 300 mg of aspirin daily (p = 0.5)

Legend for Figure 7.8: Symbols represent individual values; Mono-Plts %, % of monocytes bound to platelets; Horizontal lines represent the mean % of circulating monocyte-platelet complexes on each dose of aspirin.
Figure 7.9. Comparison of median percentage circulating Lymphocyte-Platelet Complexes between stroke patients on 75 and 150 mg (p = 0.1), 75 mg and 300 mg (p = 0.6), and 150 and 300 mg of aspirin daily (p = 0.3)

Legend for Figure 7.9: Symbols represent individual values; Lym-Plts %, % of lymphocytes bound to platelets; Horizontal lines represent the median % of circulating Lymphocyte-Platelet Complexes on each dose of aspirin; Vertical bars represent the 5th to 95th percentiles.
Aggregometry

The maximum percentage aggregation in the control group in response to the different platelet agonists is illustrated in table 7.1. Overall, aspirin resistance on platelet aggregometry could be defined as ADP-induced platelet aggregation of > 87%, epinephrine-induced platelet aggregation of >79%, sodium arachidonate induced platelet aggregation of > 21%, and collagen-induced platelet aggregation of ≥ 100% (Table 7.1). The platelet aggregometry results obtained from CVD patients on 75, 150, and 300 mg of aspirin daily are shown in Figure 7.9, Figure 7.10, and Figure 7.11. None of the patients were defined as aspirin resistant by ADP-induced, sodium arachidonate-induced, or collagen-induced platelet aggregometry. Two CVD patients on 75 mg of aspirin daily were aspirin resistant in response to 10 µM epinephrine, but both of these patients were defined as aspirin responsive on platelet aggregometry when the dose was increased to either 150 mg or 300 mg of aspirin daily. Using the PFA-100, one of these patients was also aspirin resistant, but the other was aspirin responsive on 75 mg of aspirin daily. Overall, of the 30 patient samples tested using the PFA-100, 15 (50%) samples could be classified as aspirin resistant, whereas only one of these samples was classified as aspirin resistant using optical platelet aggregometry (6.7%).

There were no significant differences in the maximum percentage aggregation in response to 5 µM ADP, 10 µM epinephrine or 1 mM sodium arachidonate between CVD patients on 75 mg, 150 mg or 300 mg of aspirin daily (Figure 7.10, Figure 7.11, and Figure 7.12). Although there was a trend towards a reduction in the maximum percentage collagen-induced platelet aggregation as the dose of aspirin was increased above 75 mg
daily, the differences between the groups did not reach statistical significance after adjusting the p value to account for multiple comparisons (Figure 7.13).

Table 7.1: Maximum Percentage Aggregation in response to 5 μM ADP, 10 μM Epinephrine (EPl), 1 mM Sodium Arachidonate (NaAA), and 1 μg/ml of collagen in Control subjects

<table>
<thead>
<tr>
<th></th>
<th>5 μM ADP</th>
<th>10 μM EPI</th>
<th>1mM NaAA</th>
<th>1 μg/ml Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls not on ASA (n = 5)</td>
<td>85 (82-89)</td>
<td>90 (78-102)*</td>
<td>85 (79-91)</td>
<td>87 (78-97)</td>
</tr>
<tr>
<td>Controls + 75 mg ASA (n = 6)</td>
<td>65 (56-87)</td>
<td>34 (23-45)</td>
<td>12 (6.0-18)</td>
<td>31 (15-47)</td>
</tr>
<tr>
<td>Controls + 150 mg ASA (n = 6)</td>
<td>69 (54-79)</td>
<td>34 (12-56)</td>
<td>11 (6.0-16)</td>
<td>30 (-0.4-60)*</td>
</tr>
<tr>
<td>Controls + 300 mg ASA (n = 5)</td>
<td>63 (43-72)</td>
<td>40 (0.6-79)</td>
<td>13 (6.0-21)</td>
<td>37.8 (-24-110)*</td>
</tr>
</tbody>
</table>

Legend for Table 7.1: Median maximum aggregation (5th – 95th percentile) for ADP; Mean (+/- 2 standard deviations) for EPl, NaAA, and Collagen; values are percentages; *, values outside of range of 0 – 100% because of small sample size, minimum % aggregation taken as 0%; maximum % aggregation taken as 100%.
Figure 7.10: Comparison of median maximum percentage aggregation in response to 5 μM ADP between stroke patients on 75 and 150 mg (p = 0.4), 75 mg and 300 mg (p = 0.6), and 150 and 300 mg of aspirin daily (p = 0.5)

Legend for Figure 7.10: Symbols represent individual values; %, Maximum percentage aggregation in response to 5 μm ADP; Horizontal lines represent the median maximum percentage aggregation to ADP on each dose of aspirin; Vertical bars represent the 5th to 95th percentiles.
Figure 7.11: Comparison of mean maximum percentage aggregation in response to 10 μM epinephrine between stroke patients on 75 and 150 mg (p = 0.2), 75 mg and 300 mg (p = 0.4), and 150 and 300 mg of aspirin daily (p = 0.7)

Legend for Figure 7.11: Symbols represent individual values; %, Maximum percentage aggregation in response to 10 μM epinephrine; Horizontal lines represent the mean maximum percentage aggregation to epinephrine on each dose of aspirin.
Figure 7.12: Comparison of mean maximum percentage aggregation in response to 1 mM sodium arachidonate between stroke patients on 75 and 150 mg (p = 0.4), 75 and 300 mg (p = 0.5), and 150 and 300 mg of aspirin daily (p = 0.9)

Legend for Figure 7.12: Symbols represent individual values; %, Maximum percentage aggregation in response to 1mM sodium arachidonate; Horizontal lines represent the mean maximum percentage aggregation to sodium arachidonate on each dose of aspirin.
Figure 7.13: Comparison of mean maximum percentage aggregation in response to 1 μg/ml of collagen between stroke patients on 75 and 150 mg (p = 0.04), 75 and 300 mg (p = 0.07), and 150 and 300 mg of aspirin daily (p = 0.9)

Legend for Figure 7.13: Symbols represent individual values; %, Maximum percentage aggregation in response to 1 μg/ml of collagen; Horizontal lines represent the mean maximum percentage aggregation to collagen on each dose of aspirin.
7.4 Discussion

In this pilot study using the PFA-100, we did not find any convincing evidence that the ex vivo inhibition of platelet adhesion and aggregation in whole blood in a group of patients with CVD was more pronounced as the aspirin dose was increased from 75 mg to 150 mg to 300 mg daily. Five of our ten patients remained ‘aspirin resistant’ on each dose of aspirin. Only two patients exhibited a consistent trend towards greater prolongation of the C-EPI closure time, whereas other patients had a variable response to increasing aspirin doses using the PFA-100.

Two CVD patients on 75 mg of aspirin daily were defined as being aspirin resistant using epinephrine-induced aggregometry in PRP, and both patients were aspirin responsive on higher doses of the drug. All other patients in our study who were defined as aspirin resistant on the PFA-100 were aspirin-responsive using platelet aggregometry in response to ADP, epinephrine, sodium arachidonate, and collagen. However, there was a non-significant trend towards greater inhibition of collagen-induced platelet aggregation in PRP with 150 or 300 mg of aspirin daily compared with 75 mg daily (Figure 7.13). These findings are in keeping with those of Tohgi et al. who reported a dose-dependent increase in the inhibition of collagen- and low dose ADP-induced platelet aggregation in the convalescent phase after ischaemic stroke as the dose was increased from 40 mg (n = 19) to 320 mg daily (n = 18) (Tohgi et al, 1992). In addition, the serum thromboxane B\(_2\) levels and the urinary concentrations of 11-dehydro-thromboxane B\(_2\) decreased with increasing aspirin doses. Overall, there was poor concordance between the results obtained using the PFA-100 and platelet aggregometry in PRP; 15/30 samples were classified as aspirin resistant using the PFA-100, whereas only one of these 15 samples was classified as aspirin resistant using epinephrine-induced platelet aggregation...
aggregometry. A similar discordance between the results obtained using the PFA-100 and optical platelet aggregometry was observed in patients with ischaemic heart disease (Gum et al, 2001). It is interesting that one patient who was aspirin resistant using both the PFA-100 and epinephrine-induced platelet aggregometry, was aspirin responsive using sodium arachidonate-induced platelet aggregation in this study. It is possible that epinephrine-induced platelet aggregation is more sensitive than sodium arachidonate-induced platelet aggregation at identifying patients who are aspirin resistant on the PFA-100. The fact that sodium arachidonate-induced platelet aggregation was inhibited to a significant degree in all of our patients supports the hypothesis that cyclooxygenase-independent mechanisms are important in mediating aspirin resistance using the PFA-100 (see Chapter 5). Because the PFA-100 measures platelet function in whole blood at moderately high shear rates, this assay should more closely mimic in vivo platelet aggregation in patients with atherosclerosis in comparison with platelet aggregometry in PRP that is performed at very low shear rates. Therefore, the PFA-100 should be more sensitive at identifying clinically relevant ex vivo platelet hyper-reactivity and aspirin resistance than platelet aggregometry. Although data from platelet aggregometry studies suggest that patients may have a more pronounced ex vivo response to higher doses of aspirin (Helgason et al, 1994); (Chamorro et al, 1999a), only preliminary data were available before this study on the effects of different aspirin doses on the results of the PFA-100 in ischaemic CVD (Grau et al, 2003).

There were no significant differences in the expression of the different flow cytometric markers of platelet activation or leucocyte-platelet complex formation, or soluble markers of platelet + / - endothelial activation between CVD patients on 75, 150
and 300 mg daily. As outlined earlier (chapter 3), previous studies have reported that aspirin does not significantly affect the expression of CD62P in control subjects (Chronos et al, 1994) or acute (Meiklejohn et al, 2001) or convalescent stroke patients (Yamazaki et al, 2001). Our data are consistent with these findings, and with those from a more recent study in which the expression of CD62P and CD63 were similar in patients on 300 mg of aspirin daily (n = 26) compared with treatment with < 300 mg daily (n = 5) (p > 0.3) (Grau et al, 2003). However, to the best of our knowledge, other studies have not simultaneously examined all of these markers in the same patient population in response to increasing doses of aspirin. These findings support the rationale of combining data from CVD patients on 75 to 300 mg of aspirin daily, when comparing the results of these assays between CVD patients and control subjects (see chapter 3).

In conclusion, the results of this small pilot study suggest that the ex vivo responsiveness to aspirin is similar with low to medium dose therapy in a group of patients in the convalescent phase after ischaemic stroke or TIA. However, if one combines the available data from clinical studies and meta-analyses (The Dutch TIA Trial Study Group, 1991); (Algra & van Gijn, 1996); (Antithrombotic Trialists' Collaboration, 2002) with the results of our preliminary platelet aggregometry and PFA-100 experiments, it is reasonable to suggest that CVD patients should be treated with 150 mg of aspirin daily for long-term secondary prevention of vascular events. This suggestion is in keeping with the results of another recent study on the effects of aspirin dose escalation in patients with ischaemic stroke (Gan et al, 2002). Gan et al. carried out platelet aggregometry experiments in PRP in response to stimulation with 2 µM ADP and 2 µg/ml collagen in 16 patients after a non-cardioembolic ischaemic stroke. Platelet
aggregometry was performed before commencing aspirin, and repeated after treatment with incremental doses of aspirin (40 mg, 80 mg, 160 mg, 325 mg, 650 mg and 1300 mg daily) for two weeks at a time. Compared with the baseline results obtained before commencing aspirin therapy, significant inhibition of platelet aggregation was observed with aspirin doses greater than 80 mg daily. However, 80 mg to 1300 mg of aspirin daily inhibited platelet aggregation to a similar degree, without any convincing evidence of significantly greater inhibition of platelet function in PRP with incremental doses of the drug above 80 mg daily. Further long-term clinical and laboratory studies in larger groups of subjects are warranted to re-examine this important clinical issue, and to determine whether laboratory data can facilitate choosing an appropriate dose of aspirin in individual patients with ischaemic CVD. In addition, further work is required to determine whether patients who are defined as being aspirin resistant on the PFA-100 on 300 mg of aspirin daily would have more pronounced inhibition of platelet function with higher aspirin doses.
8. Summary and Future Work

The different studies described in this thesis investigated several important aspects of platelet activation and function in ischaemic stroke, TIA and asymptomatic severe carotid stenosis. Full blood count parameters were measured in all subjects, and the sensitive and specific technique of whole blood flow cytometry was used to quantify the expression of a comprehensive panel of platelet activation markers, and the percentage of leucocyte-platelet complexes in CVD patients and controls. Furthermore, soluble markers of platelet +/- endothelial activation were studied in all subject groups. We used a relatively novel platelet function analyser, the PFA-100®, to assess the \textit{ex vivo} response to antiplatelet therapy in these patients. We performed preliminary experiments to investigate whether the \textit{ex vivo} response to aspirin varied with incremental doses of the drug, and compared the results of the PFA-100® with those obtained from optical platelet aggregometry experiments. We also investigated the impact of alterations in the aspirin dose, and of the antiplatelet regimen used, on the expression of the platelet activation markers studied. Although the potential relevance of the results of these experiments has been discussed in detail in each chapter, the following section summarises these results, and outlines some topics that warrant further investigation in future studies.

\textit{Platelet degranulation and monocyte-platelet complex formation are increased in the acute and convalescent phases after ischaemic stroke or TIA}

In Chapter 3, we have found evidence of excessive platelet +/- endothelial activation (elevated CD62P and VWF:Ag levels) in both the acute and convalescent phases after
ischaemic stroke or TIA compared with controls without a history of CVD. These findings contribute to the literature on this subject, and raise the possibility of ‘primary’ differences in platelet activation status between ischaemic CVD patients and controls. These differences may precede symptom onset and may contribute to the pathogenesis of ischaemic events in this patient population. Alternatively, these differences may be ‘secondary’ to exposure to ongoing stimuli to platelet activation after an ischaemic stroke or TIA. The finding of elevated CD62P levels, in conjunction with increased levels of circulating monocyte-platelet complexes ex vivo in ischaemic CVD supports the hypothesis that CD62P is important in mediating monocyte-platelet complex formation in ischaemic CVD. The finding of excessive platelet +/- endothelial activation on antiplatelet or anticoagulant therapy may also partly explain why these patients have a moderately high risk of recurrent vascular events during follow-up despite antithrombotic treatment. Our results emphasise the need for more potent antithrombotic treatment regimens in ischaemic CVD patients for secondary prevention of vascular events.

Certain factors were shown to influence the differences in the expression of monocyte-platelet complexes between patients and controls. Further studies are warranted to compare the percentage of circulating leucocyte-platelet complexes in CVD patients and controls who have been matched for age, and the presence of peripheral vascular disease and hypertension. Furthermore, in contrast to some previous studies, our preliminary data suggest that excessive platelet +/- endothelial activation occurs in all subtypes of ischaemic stroke or TIA, including lacunar stroke, compared with controls. Larger studies are needed to confirm these findings because of the limited number of patients in each ischaemic CVD subgroup. In addition, further work in larger groups of
subjects is required to determine whether the expression of certain platelet activation markers, and the percentage of leucocyte-platelet complexes, can be influenced by changes in the prescribed antithrombotic treatment regimen.

**Increased platelet count and leucocyte-platelet complex formation in patients with recently symptomatic compared with asymptomatic severe carotid stenosis**

In chapter 4, we have shown that there was a significant increase in both the mean platelet count and the percentage of neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes in patients with acute symptomatic compared with asymptomatic severe carotid stenosis. A number of factors may have influenced our results, including the higher platelet count in the acute symptomatic group, and the use of different antithrombotic regimens in the asymptomatic and symptomatic groups. However, having adjusted for differences in platelet counts between groups, the percentage of circulating neutrophil-platelet complexes remained higher in the acute symptomatic compared with asymptomatic severe carotid stenosis patients. Furthermore, having limited our analysis to those patients in each group who were on treatment with aspirin monotherapy, the percentages of neutrophil-platelet and monocyte-platelet complexes were higher in patients with recently symptomatic compared with asymptomatic severe carotid stenosis. 8/16 (50%) symptomatic patients who were studied in the convalescent phase after symptom onset had undergone successful carotid endarterectomy or endovascular treatment before reassessment. Therefore, it was not surprising that the differences between the asymptomatic and symptomatic groups were not statistically significant at this stage.
It is difficult to determine whether the findings represent primary or secondary differences in platelet activation status between patients with asymptomatic and acute symptomatic severe carotid stenosis, or a combination of both processes. The higher mean platelet count in both the acute and convalescent symptomatic patients compared with the asymptomatic severe carotid stenosis patients suggests that there is an ongoing stimulus to excessive platelet production, and possibly platelet activation, in the symptomatic group. As stated in Chapter 4, further studies in larger groups of subjects are required to confirm our initial findings, because some of the differences between the groups in this small observational study just reached statistical significance. One would need to do a prospective study in asymptomatic severe carotid stenosis patients to determine whether patients who subsequently develop symptoms have higher baseline levels of platelet activation, or leucocyte-platelet complex formation, than patients who remain asymptomatic during long term follow-up. If one could identify asymptomatic patients with excessive platelet activation who experience cerebrovascular symptoms during follow-up, then perhaps one could target these patients with more potent antiplatelet regimens, or perhaps treat these individuals with carotid endarterectomy or endovascular treatment to reduce this risk.

Detection of platelet hyper-reactivity and ex vivo ‘aspirin resistance’ in patients with ischaemic stroke and TIA using the PFA-100®

There is a substantial body of evidence to support the conclusion that antiplatelet therapy is mildly to moderately effective at reducing the risk of recurrent vascular events in patients with ischaemic CVD. However, because traditional tests of platelet function,
such as platelet aggregometry, have several limitations and do not necessarily reflect
platelet function in the physiological milieu of whole blood, the response to antiplatelet
therapy \textit{ex vivo} is not routinely measured. This is in contrast to patients on anticoagulant
therapy, in whom treatment is tailored to suit the individual patient. Until recently,
reliable user-friendly devices to assess platelet function in the physiological milieu of
whole blood were not available. The data presented in Chapter 5 outline our initial
experience with the PFA-100\textsuperscript{®} in assessing the \textit{ex vivo} response to aspirin therapy in
ischaemic CVD. These data suggest that some CVD patients have apparent platelet
hyper-reactivity in the acute phase after ischaemic stroke or TIA despite being treated
with 75 to 300 mg of aspirin daily. In addition, the majority (60\%) of patients in the acute
phase and a significant minority (43\%) of patients in the convalescent phase after
ischaemic stroke or TIA were resistant to the antiplatelet effects of aspirin \textit{ex vivo} using
the PFA-100\textsuperscript{®}. We have also shown that elevated levels of VWF in ischaemic stroke or
TIA are likely to play an important role in mediating aspirin resistance using this device.
The clinical relevance of these findings is uncertain, but further studies are warranted to
determine whether aspirin-resistant patients are at higher risk of stroke recurrence than
aspirin-responsive patients during long-term follow-up. Our study had a number of
limitations, and further work is required to compare the results obtained from ischaemic
CVD patients with those obtained from vascular risk factor-matched controls. In addition,
most published studies in control subjects examined the \textit{ex vivo} response to a single dose
of aspirin using the PFA-100\textsuperscript{®}. Further experiments are required to examine the response
to incremental doses of aspirin in control subjects to confirm that the majority of controls

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are aspirin-responsive on low to medium dose therapy, and to facilitate direct comparison of results obtained from controls with those obtained from ischaemic CVD patients.

**The percentage of circulating reticulated platelets is not increased in the early or late phases after ischaemic stroke or TIA**

Using whole blood flow cytometry, we did not find a significant increase in the percentage of reticulated platelets in the early or late phases after ischaemic stroke or TIA, or in any ischaemic CVD subgroup, compared with controls (chapter 6). These data are in keeping with those published previously in a small group of patients in the acute phase after a coronary, cerebral or peripheral vascular event, but do not confirm other preliminary reports of an increased percentage of reticulated platelets at different stages after ischaemic stroke. The discrepancy between our results and those reported by others may reflect differences in the timing of blood sampling after symptom onset, differences in sample size, or differences in methodology used to measure the percentage of reticulated platelets between studies. It is acknowledged that the moderate sample size in our study could have contributed to a type II error, and if larger numbers of patients were studied, one might have identified significant differences between patients and controls. Our data suggest that measurement of the % RP is a less sensitive marker of platelet activation *ex vivo* than the expression of CD62P or CD63, or the percentage of circulating leucocyte-platelet complexes. Nevertheless, larger studies are warranted to investigate whether the % RP can be used as a marker of increased platelet production and/or turnover in the hyperacute phase (within 24 hours) after an ischaemic stroke or TIA compared with healthy controls. Because reticulated platelets may have higher levels of
COX-2, measurement of the % RP may facilitate a better understanding of the mechanisms responsible for aspirin resistance in the hyperacute phase after ischaemic stroke or TIA. From a methodological viewpoint, there is sufficient evidence from previous studies to support the use of low concentrations of thiazole orange, as used in this thesis, in reticulated platelet assays to overcome the problem of non-specific labelling of platelet dense granules and possibly mitochondrial DNA. This issue should be taken into account when planning further studies measuring the % RP in patients or controls. One of the main limitations of all reticulated platelet assays that have been published to date is the lack of a reference standard. Consequently, the percentage of reticulated platelets in normal controls is arbitrarily defined, and the quoted normal range varies markedly between studies, thus hindering direct comparison of data obtained from different studies. In view of the observed platelet lifespan of 8 to 11 days, approximately 10% of platelets are turned over on a daily basis, and therefore, one would expect approximately 10% of platelets to be 'reticulated' in healthy control samples. Until a commercially available 'reticulated platelet reference standard' becomes available, rigorous and regular comparisons between different batches of thiazole orange should be performed during any ongoing study to ensure that a new batch of the reagent provides similar results to an existing one. Although one must be meticulous in ensuring that different batches of reagents provide similar and reproducible results in any longitudinal flow cytometry study, this principle is particularly important with commercially available thiazole orange. The experience in our laboratory suggests that this reagent is unstable after approximately three months of use.
We did not find a significant increase in the MPV in EDTA- or citrate-anticoagulated whole blood in either the early or late phase CVD patients compared with controls. Published studies have yielded conflicting results regarding MPV values in ischaemic CVD compared with controls. The discrepancy between the results of our study and those reported by Butterworth et al. (Butterworth & Bath, 1998) may reflect differences in the concentrations of citrate anticoagulant used, and differences in timing between venepuncture and sample analysis in the two studies. The interesting finding of a weak positive correlation between % RP and the MPV in the early phase after ischaemic CVD supports the hypothesis that reticulated platelets are larger than more mature ‘non-reticulated’ platelets. However, larger studies are needed to confirm these initial findings, and to investigate whether antiplatelet agents or anticoagulant therapy influence the expression of the % of RP in either the acute or convalescent phases after symptom onset.

The ex vivo inhibition of shear-induced platelet adhesion and aggregation is not reliably dose-dependent on treatment with between 75 and 300 mg of aspirin daily

Our pilot PFA-100® data (chapter 7) did not provide any convincing evidence that the ex vivo inhibition of shear-induced platelet adhesion and aggregation in whole blood was more pronounced as the aspirin dose was increased from 75 mg to 150 mg to 300 mg daily in a group of patients in the convalescent phase after ischaemic CVD. Two patients exhibited a consistent trend towards greater prolongation of the C-EPI closure time, whereas other patients had a variable response to increasing aspirin doses using the PFA-100®.
Cyclooxygenase-independent mechanisms are important in mediating ‘aspirin resistance’ using the PFA-100®

Two patients who were aspirin resistant on 75 mg daily using epinephrine-induced platelet aggregometry, were aspirin responsive on higher doses of the drug, and there was a non-significant trend towards greater inhibition of collagen-induced platelet aggregation with 150 or 300 mg compared with 75 mg of aspirin daily. Overall, there was poor concordance between the results obtained using the PFA-100® and platelet aggregometry in PRP, and our data raises the possibility that epinephrine-induced platelet aggregation is more sensitive than sodium arachidonate-induced platelet aggregation at identifying patients who are aspirin resistant on the PFA-100®. The fact that sodium arachidonate-induced platelet aggregation was inhibited to a significant degree in all of our patients supports the hypothesis that cyclooxygenase-independent mechanisms are important in mediating aspirin resistance using the PFA-100®. These data are in keeping with the results presented in Chapter 5, where it was suggested that high VWF levels in ischaemic CVD may play an important role in mediating aspirin resistance using the PFA-100®. Because VWF plays a crucial role in platelet adhesion and aggregation under conditions of high shear stress, further studies are required to investigate whether combination antiplatelet therapy can prolong the C-EPI closure time in patients who are resistant to aspirin monotherapy. In the interim, if one decides to use aspirin monotherapy for secondary prevention of vascular events in patients with ischaemic CVD, it is reasonable to prescribe 150 mg daily. One challenge for the future is to determine whether one can use a reliable and automated laboratory test, such as the PFA-100®, or a
modified form of this device, to choose an appropriate dose, and to monitor the response to aspirin therapy in individuals with ischaemic CVD.
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