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

A Thesis submitted to University of Dublin, Trinity College 2012

for examination for a PhD in Neurological Studies

by

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Declaration

I designed all of the studies described in this thesis with the advice and supervision of my colleagues listed in the acknowledgements section. I recruited and clinically assessed all of the control subjects and patients studied in this thesis. I performed venepuncture on all of the study subjects, prepared the samples for analysis, carried out the flow cytometry experiments, PFA-100® assays, VerifyNow® assays and FBC measurements and prepared the platelet poor plasma for further analysis. I performed transcranial Doppler ultrasound on all control subjects and patients with assistance from some of my colleagues acknowledged below. I assisted in performing colour Doppler ultrasound and MRI of carotid arteries on patients recruited to this study. I carried out some of the ELISA assays, but was assisted with these experiments by my colleagues acknowledged below. I entered all of the data onto a database and performed the statistical analysis of the data with the help and supervision of Dr. Dominick J.H. McCabe.

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Summary

Aims: The purpose of this thesis was to comprehensively assess the levels of platelet activation, platelet function and platelet turnover in patients with moderate or severe asymptomatic or symptomatic carotid artery stenosis, and to perform preliminary experiments to investigate coagulation system potential and endothelial activation in these patients. We performed transcranial Doppler ultrasound to assess for the presence of cerebral microembolic signals (MES), and colour Doppler ultrasound of carotid plaques in these patients. We also performed a pilot study of platelet reactivity using the PFA-100® and VerifyNow® analysers in patients in the convalescent phase following TIA or ischaemic stroke.

Methods: An observational study, with longitudinal follow-up in the symptomatic cohort, was performed to assess the above parameters in patients with moderate or severe (≥50%) asymptomatic *vs.* early (≤4 weeks) and late phase symptomatic (≥3 months after TIA or stroke) carotid stenosis. Platelet activation and turnover were quantified with whole blood flow cytometry to assess the 'unstimulated expression' of platelet activation markers (CD62P and CD63), leucocyte-platelet complex formation, and the percentage of circulating reticulated platelets. Inhibition of platelet function at moderately high shear stress was assessed with the platelet function analyser (PFA-100® collagen- epinephrine [C-EPI] and collagen-ADP [C-ADP] cartridges). Coagulation system / thrombin generation potential was assessed, and endothelial activation was quantified with von Willebrand factor and von Willebrand factor propeptide levels in platelet poor plasma in a subgroup of our patients. Transcranial Doppler ultrasound was performed to assess for the presence of cerebral MES in both study groups. Colour Doppler ultrasound was performed to characterise carotid plaques into more unstable echolucent *vs.* more stable echogenic plaques.

Results: There was no difference in unstimulated platelet surface CD62P or CD63 expression between asymptomatic and symptomatic carotid stenosis patients, but the median % lymphocyte-platelet complexes was higher in early symptomatic than asymptomatic patients (2.8 vs. 2.4%, p=0.001). There were no differences in the prevalence of high on-treatment platelet reactivity (HTPR) in asymptomatic versus early symptomatic, late symptomatic or late symptomatic post-intervention carotid stenosis groups as a whole ($p \geq 0.13$). HTPR on the C-EPI cartridge was more prevalent in early symptomatic versus late symptomatic carotid stenosis patients (63% vs. 34%; $p = 0.017$),

including patients on aspirin monotherapy ($p = 0.016$), or on aspirin alone or in or combination with other antiplatelet agents ($p = 0.01$). There were no significant differences in % circulating reticulated platelets (%RP) between asymptomatic and symptomatic carotid stenosis patients at any stage after symptom onset ($p \geq 0.7$), or between early and late phase symptomatic patients ($p \geq 0.82$). Peak thrombin ($p = 0.01$) and endogenous thrombin potential (ETP) ($p = 0.047$) were higher in early symptomatic than asymptomatic patients. VWF:AgII levels were higher in early ($p < 0.001$), late ($p = 0.01$) and late post-intervention ($p = 0.038$) symptomatic than asymptomatic patients (8.9mg/mL). 32% of early ($p = 0.02$) and 19% of late phase symptomatic ($p = 0.2$) patients versus 12% of asymptomatic patients were MES positive. Early symptomatic MES negative patients had higher percentage lymphocyte-platelet complexes than asymptomatic MES negative patients ($p = 0.0085$). There were no significant differences in measurements of plaque echolucency between early symptomatic and asymptomatic groups, in terms of Gray-Weale Scores ≤ 2 versus > 3. In a pilot study on the VerifyNow, 'aspirin HTPR' was identified in 8% patients on aspirin-dipyridamole combination therapy ($\geq$ 550 Aspirin Reaction Units on the Aspirin cartridge). 44% had HTPR on clopidogrel ($\geq$ 194 $P2Y_{12}$ reaction units on the $P2Y_{12}$ cartridge).

Conclusions: Quantification of platelet surface activation marker expression with unstimulated whole blood flow cytometry is not likely to be useful for identifying patients with carotid stenosis who will benefit from intervention. However, measurement of lymphocyte-platelet complexes may be informative in this regard. Further longitudinal studies are warranted to determine whether HTPR in early versus late symptomatic carotid stenosis patients, and increased thrombin generation potential and endothelial activation in early symptomatic versus asymptomatic patients contribute to the higher risk of subsequent TIA or stroke in recently symptomatic patients. MES were more common in early symptomatic than asymptomatic carotid stenosis patients. Based on these data, a larger, longitudinal study assessing the clinical predictive value of tests of platelet activation, platelet function, coagulation system potential and endothelial activation, in conjunction with multimodal carotid plaque imaging and MES detection in patients with carotid stenosis has been designed by our research group.

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Abbreviations

ACA	Anterior Cerebral Artery
ACAS	Asymptomatic Carotid Atherosclerosis Study
ACE Trial	Aspirin and Carotid Endarterectomy Trial
ACES Trial	Asymptomatic Carotid Emboli Study
ACST	Asymptomatic Carotid Surgery Trial
ADAMTS-13	A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif no 13
AMP	Adenosine Monophosphate
ARR	Absolute Risk Reducation
ARU	Aspirin Reaction Unit
ATP	Adenosine Triphosphate
βTG	β-thromboglobulin
C-ADP	Collagen-ADP
cAMP	Adenosine 3′, 5′-cyclic monophosphate
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CASANOVA	Carotid Artery Stenosis with Asymptomatic Narrowing: Operation Versus Aspirin
CAST	Chinese Acute Stroke Trial
CAVATAS	Carotid and Vertebral Artery Transluminal Angioplasty Study
CCA	Common Carotid Artery
CD	Cluster Differentiation
CDUS	Colour Doppler Ultrasound

CEA	Carotid Endarterectomy
C-EPI	Collagen-Epinephrine
χ^2	Chi-Squared statistic
CI	Confidence Interval
cGMP	Guanosine 3′, 5′-cyclic monophosphate
CNR	Contrast to Noise Ratio
COX	Cyclooxygenase
CPD	Cerebral Protection Device
CREST	Carotid Revascularization Endarterectomy *vs.* Stenting Trial
CT	Computerised Tomography
CTA	Computerised Tomography Angiography
CVD	Cerebrovascular Disease
CVE	Cerebrovascular Event
Cy5	Cyanine 5
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
DP	Dipyridamole
DSA	Digital Subtraction Angiography
DWI	Diffusion Weighted Imaging
EAFT	European Atrial Fibrillation Trial
ECA	External Carotid Artery
ECST	European Carotid Surgery Trial
EDV	End Diastolic Velocity
ELISA	Enzyme Linked Immunosorbent Assay
E-selectin	Endothelial-leucocyte adhesion molecule

ESPS-2	Second European Stroke Prevention Study
ETP	Endogenous Thrombin Potential
EVA-3S	Endarterectomy *vs.* Angioplasty in patients with Symptomatic Severe carotid Stenosis
FBC	Full Blood Count
FITC	Fluorescein Isothiocyanate
FISS	Fraxiparin in Stroke Study
FISS bis	Fraxiparine in Ischaemic Stroke Study
FLAIR	FLuid Attenuation Inversion Recovery
5-HT	Serotonin
FS	Forward Scatter
G Proteins	Guanine Nucleotide Binding Regulatory Proteins
Gp	Glycoprotein
GSM	Gray Scale Median
GTP	Guanosine Triphosphate
HAEST	Heparin in Acute Embolic Stroke Trial
HBS	HEPES buffered saline
HBSS	Hanks Balanced Saline Solution
HTPR	High On-Treatment Platelet Reactivity
HR	Hazard Ratio
HRP	Horseradish Peroxidase
HS	Haemorrhagic Stroke
H_2O	Water
ICA	Internal Carotid Artery
ICAROS	Imaging in Carotid Angioplasty and Risk of Stroke

ICH	Intracerebral Haemorrhage
ICSS	International Carotid Stenting Study
IP_3	Inositol 1,4,5-triphosphate
Iso-TRAP	iso- thrombin receptor activating peptide
IST	International Stroke Trial
ITT	Intention to Treat
κ	Kappa statistic
LAMP	Lysosomal-Associated Membrane Proteins
LMWH	Low Molecular Weight Heparin
LR / NC	Lipid Rich / Necrotic Core
Mac-1	Macrophage 1 antigen
MAST-I	Multicentre Acute Stroke Trial-Italy
MCA	Middle Cerebral Artery
MCL	Multi-tube Carousel Loader
MCP-1	Monocyte Chemotactic Protein-1
MES	Microembolic Signal
MFI	Mean Fluorescence Intensity
MI	Myocardial Infarction
MMP-9	Matrix Metalloproteinase-9
MPV	Mean Platelet Volume
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRS	modified Rankin Scale
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NSAID	Non-steroidal Anti-inflammatory Drug

NVAF	Non-valvular Atrial Fibrillation
OCS	Open Canalicular System
OR	Odds Ratio
PAD	Peripheral Arterial Disease
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor-1
PARs	Protease Activated Receptors
PAU	Platelet Aggregation Unit
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PDW	Platelet Distribution Width
PE	Phycoerythrin
PECAM	Platelet Endothelial Cell Adhesion Molecule Platelet Endothelial Cell Adhesion Molecule
PF-4	Platelet Factor 4
PFA-100®	Platelet Function Analyser-100, Dade-Behring, Germany
PG	Prostaglandin
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-biphosphate
PLAT	Progetto Lombardo Atero-Trombosi Study
PMT	Photomultiplier Tube
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PRU	P2Y12 Reaction Unit
PS	Phosphatidylserine

PSGL-1	P-selectin glycoprotein ligand-1
PSV	Peak Systolic Velocity
PTE	Phosphatidylethanolamine
RGD	Arginine-Glycine-Aspartic Acid
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
RP	Reticulated platelets
RPFA	Ultegra-Rapid Platelet Function Analyser
RPE	R-phycoerythrin
RRR	Relative Risk Reduction
RS	Recurrent Ischaemic Stroke
SALT	Swedish Aspirin Low-dose Trial
SAPPHIRE	Stenting and Angioplasty with Protection in Patients at High Risk for Endarterectomy Trial
SLAM	Signalling Lymphocyte Activation Molecule
SNR	Signal to Noise Ratio
SPACE	Stent protected Percutaneous Angioplasty of the Carotid artery *vs.* Endarterectomy Trial
SPIRIT	Stroke Prevention in Reversible Ischaemia Trial
SS	Side Scatter
T	Tesla
TAT	Thrombin-antithrombin complex
TCD	Transcranial Doppler
TEG	Thrombelastography Platelet Mapping
TIA	Transient Ischaemic Attack

TNF-α	Tumour Necrosis Factor-α
TO	Thiazole Orange
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
TOF	Time of Flight
tPA	tissue-type Plasminogen Activator
TxA_2	Thromboxane A_2
VA309	Veterans Affairs Trial
VWF:Ag	von Willebrand factor
VWF:Ag II	von Willebrand factor propeptide
WARSS	Warfarin-Aspirin Recurrent Stroke Study

1. General Introduction

1.1. Overview of Transient Ischaemic Attack (TIA) and Ischaemic Stroke

1.1.1. Definition of TIA and stroke

The most commonly quoted definition of stroke is: "A neurological deficit of sudden onset; with focal rather than global neurological dysfunction; with symptoms lasting more than 24 hours or resulting in death before 24 hours; and in which, after adequate investigation, symptoms are presumed to be of a non-traumatic vascular origin". (Bamford, 1992) The aetiology of stroke may be complicated however, and more so in some groups than in others.(Kristensen *et al*, 1997) In his original paper, Bamford was attempting to introduce a rational definition and classification scheme which would be important for guiding investigations, treatment and informing prognosis. Such an approach excludes some of the rarer causes of stroke, which by their aetiology, fall outside the relatively rigid standard definition. For example, cervical artery dissection, can be related to neck trauma, and stroke secondary to cervical dissection was the leading cause of stroke in one large young cohort study.(Kristensen *et al*, 1997) Patients presenting with subarachnoid haemorrhage may present with a severe headache and meningism without focal or global neurological dysfunction. Stroke can occasionally cause global neurological dysfunction from the outset, e.g. in patients with extensive subarachnoid haemorrhage or bilateral cerebral hemispheric or brain stem involvement. The definition does not include neurological dysfunction caused by subdural or extradural haemorrhage,(Klijn *et al*, 1997;Whisnant, 1990) traumatic intracranial contusional haemorrhage, and some authors also exclude cerebral infarction or haemorrhage caused by an infection or a tumour.(Warlow, 1996) These decisions are

practical, because the prognosis and treatment of these types of neurological disorders are often very different from that of an ischaemic stroke which fits well into the standard definition. Therefore, one needs to be flexible in including these cases within the definition of stroke, as deemed appropriate. For the purpose of this thesis, Bamford's definition of ischaemic stroke was employed.

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

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

It may well be proposed that one should dual classify TIAs according to (a) the clinical definition, as outlined above, to allow one to consistently perform international epidemiological studies in different countries, and (b) a 'tissue based definition', as proposed by others if imaging data are available in the neurovascular stroke centre in question.

For the purpose of this thesis, the European Stroke Organisation definition of stroke and TIA were used, unless explicitly stated in the text.

1.1.2. Epidemiology of stroke (emphasis on Ischaemic Stroke)

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

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

The majority of patients survive their first ischaemic stroke with 95% alive at seven days, 80-90% alive at 30 days, and 77% alive at one year,(Feigin *et al*, 2009;Warlow, 1996) 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, 1998) Recurrence may occur 'early' or 'late' after the index stroke, and the reported rate of recurrence varies depending on the aetiology of the initial stroke, the duration of follow up, and the study design.(Rothwell *et al*, 2005;Feigin *et al*, 2009)

Overall, early recurrence occurs in 1.4% to 18.5% of patients in the first month after ischaemic stroke.(Petty *et al*, 2000) The risk of recurrent ipsilateral stroke in medically treated patients with severe (> 70%) symptomatic extracranial carotid stenosis is particularly high in the first year, at approximately 18%, with an overall risk of 26% over two years.(North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991)

In summary, acute cerebrovascular events are common, and can be debilitating or fatal. However, the opportunities that exist to modify risk factors, provide acute and long-term

rehabilitation, enhance secondary prevention and improve long term prognosis in patients who survive their first or subsequent cerebrovascular event cannot be underestimated.

1.1.3. Aetiology of TIAs and ischaemic stroke

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

In the following section, I will outline the underlying pathogenic mechanisms responsible for, and the clinical features associated with, large artery atherosclerotic stroke or TIA.

Large-artery atherosclerosis

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

transhemispheric passage from a contralateral internal carotid stenosis via the circle of Willis.(Klijn *et al*, 1997)

Clinical features supportive of a diagnosis of stroke or TIA of large-artery atherosclerotic aetiology are ocular or cortical symptoms or signs in the territory of the internal carotid artery, previous TIA or stroke in the same vascular territory, and absence of symptoms, signs or radiological features in other vascular territories(Adams, Jr. *et al*, 1993) While patients with higher grades of carotid stenosis are at higher risk of stroke and TIA(Halliday *et al*, 2004;European Carotid Surgery Trialists' Collaborative Group, 1998) and may require carotid revascularisation, patients with lower grades of carotid stenosis may also experience stroke or TIA of large artery aetiology but do not benefit from carotid endarterectomy or endovascular treatment.(Halliday *et al*, 2004)

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

The degree of carotid stenosis on carotid angiography may be quantified via one of three methods: The Common Carotid method,(CAVATAS Investigators, 2001) the European Carotid Surgery Trial (ECST) method,(European Carotid Surgery Trialists' Collaborative Group, 1998) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) method.(Rothwell *et al*, 2003) With the Common Carotid method, the percent stenosis = 100 (C-A/C), where A = the diameter of the residual lumen at the point of maximal stenosis, and C = the width of the disease free common carotid artery below the bifurcation at a point where the walls are approximately parallel. With the ECST method, the percent stenosis = 100 (E-A/E), where A = the diameter of the residual lumen at the point of maximal stenosis, and E = the estimated normal diameter of the ICA at the point of maximal narrowing; therefore, one needs to 'guess' how wide the carotid bulb was

before it became narrowed by the atherosclerotic plaque. The NASCET method calculates the percent stenosis as 100 (N-A/N), where A = the diameter of the residual lumen at the point of maximal stenosis, and N = the diameter of the disease free distal ICA at a point where the walls are approximately parallel. The Common Carotid and ECST methods give rise to similar estimates of the degree of carotid stenosis.(CAVATAS Investigators, 2001) However, the NASCET method produces a lower estimate of the degree of carotid stenosis than the other two methods. Therefore, when interpreting the results of studies in patients with carotid stenosis, it should be remembered that 50 and 70% stenosis by the NASCET method are equivalent to 65% and 82% stenosis, respectively, by the ECST method.(Rothwell *et al*, 2003) To facilitate data interpretation and to guide best international clinical practice, it has been suggested that the NASCET method be adopted for grading ICA stenosis.(Rothwell *et al*, 2003)

1.1.4. Thrombus Formation in TIA and Ischaemic Stroke

In the following section, I will give a brief overview of thrombus formation under different conditions, and in particular, focusing on the interaction between platelets and the coagulation system.

Overview

There are two major physiological pathways which are thought to be important in mediating platelet thrombus formation: (a) the 'collagen pathway' which requires exposure of sub-endothelial collagen, and (b) the 'tissue factor pathway' which is independent of subendothelial collagen.(Furie & Furie, 2008) The exact contribution of the two pathways to platelet activation *in vivo* is unknown (Figure 1-1), and their relative contribution depends on the underlying disease process.(Furie & Furie, 2008) Furthermore, the pathways involved in platelet activation are also dependent on the levels of shear stress to which platelets are exposed *in vivo*.

(a) **The collagen pathway** may be activated when subendothelial collagen and tissue factor are exposed following traumatic injury to a blood vessel wall, thus promoting physiological haemostasis, or following rupture of an atherosclerotic plaque, thus promoting pathological thrombosis. In the resting state, platelets 'survey' the endothelium and roll on the endothelial surface without adhering to it.(Ruggeri, 2000) Following endothelial disruption, platelets adhere to exposed subendothelial collagen directly via the platelet glycoprotein (Gp) VI receptor, and via interaction of the platelet GpIb-V-IX receptor with von Willebrand factor bound to subendothelial collagen. These interactions also result in conversion of Factor XII to XIIa, initiating the process of thrombin formation, however platelet activation in this collagen-initiated pathway is initially independent of thrombin. Of interest, inhibition or deficiency of factor XII protects mice from ischemic brain injury, without causing a concurrent increase in rates

of intracerebral haemorrhage.(Kleinschnitz *et al*, 2006) The GpVI-collagen interaction predominates in settings of low shear stress (e.g. venous thrombosis, left atrial appendage thrombus), and conversely, the GpIb-V-IX / VWF / collagen interaction predominates under high shear stress conditions (e.g. following rupture of a stenosing carotid artery plaque). In addition to its role in mediating the adhesion of platelets to collagen, GpVI receptor binding also promotes further platelet activation and dense granule release.(Stoll *et al*, 2008) The platelet integrin $\alpha_{IIb}\beta_3$ (GpIIb/IIIa) plays a supportive, but not essential role in the interaction between platelets and collagen.

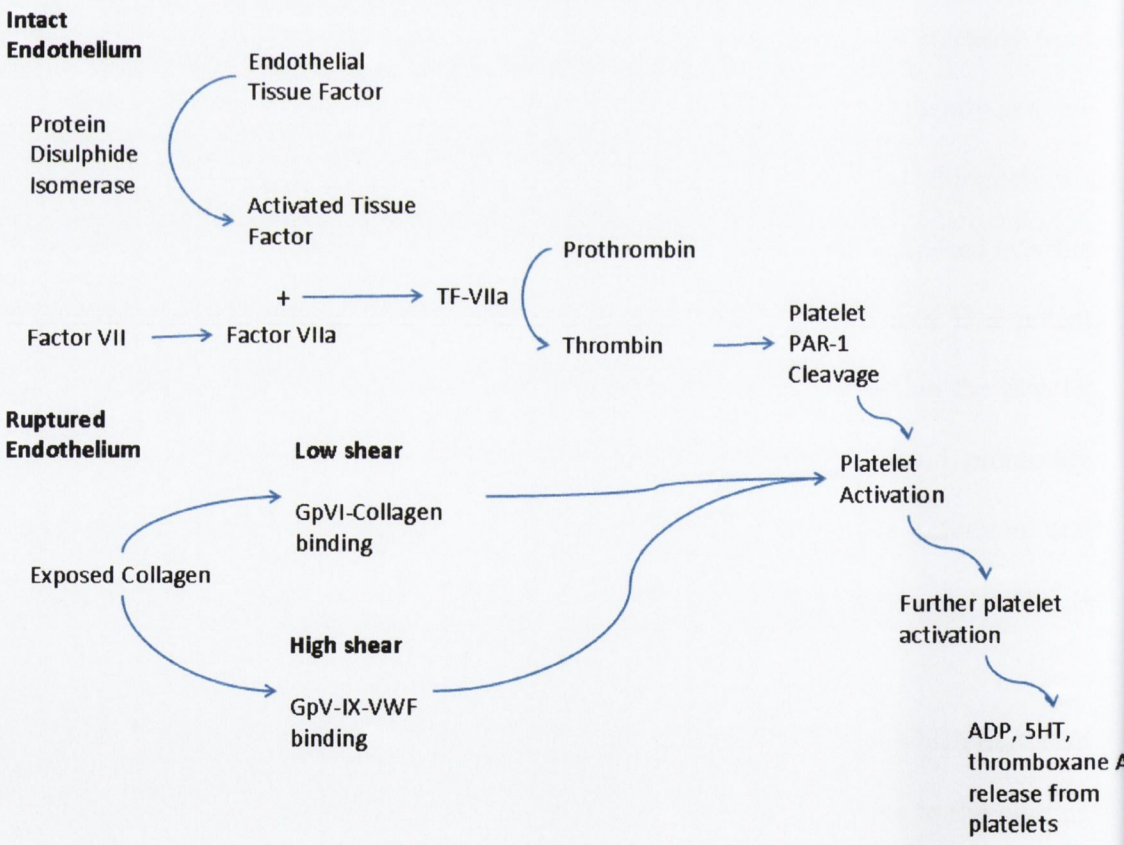
(b) **The tissue factor pathway** does not require disruption of the endothelium and is independent of von Willebrand factor and glycoprotein VI. Inactive tissue factor on the endothelial surface is activated by protein disulfide isomerise, which is released from activated endothelial cells and platelets. Activated tissue factor can subsequently activate factor X, which leads to the conversion of prothrombin to thrombin, and ultimately the conversion of fibrinogen to fibrin. It is unclear how, or if, platelets are recruited to a site of vessel injury when activated via the tissue factor pathway. Thrombin is a potent platelet agonist. Thrombin cleaves protease-activated receptor 1 (PAR1) on the platelet surface in humans,(Furie & Furie, 2008) thereby activating platelets and promoting platelet degranulation with the release of adenosine diphosphate (ADP), serotonin, and thromboxane A_2. Thrombin and these other agonists subsequently activate other platelets in a positive feedback loop.(Furie & Furie, 2008)

In addition to reacting to proteins generated by the coagulation cascade, which may lead to platelet-platelet aggregation as outlined above, platelets also contribute to the clotting cascade.(Monroe *et al*, 2002) These two events occur simultaneously, possibly initiated by the same agonists. Elevation in platelet cytosolic calcium during platelet activation leads to the exposure of procoagulant phosphatidylserine (PS) on the platelet surface.(van

der Meijden *et al*, 2005) The platelet surface, with exposed phosphatidylserine, serves as an assembly site for tenase and prothrombin complexes, thus promoting accelerated thrombin generation.(van der Meijden *et al*, 2005) Despite the fact that thrombin is a potent stimulator of platelets, and that during thrombus formation a saturating amount of thrombin should be generated to fully activate platelets, activation of the platelet $P2Y_{12}$ ADP receptor actually increases the time taken to achieve peak thrombin formation.(van der Meijden *et al*, 2005) This suggests that platelet activation by ADP may modulate the rate of thrombin formation, and this could theoretically, in turn, limit the extent of thrombin-induced platelet activation in settings with high levels of circulating ADP.

Figure-1-1 Platelet Activation Pathway

Two distinct pathways, which may act in parallel or separately to activate platelets. With intact endothelium, protein disulphide isomerise 'decrypts' circulating Tissue Factor which then activates Factor VII, and the activated Tissue Factor-Factor VIIa complex ultimately catalyses the conversion of Prothrombin to Thrombin. Thrombin then cleaves PAR-1 on the platelet surface to activate it, initiating platelet activation. In some disease states, Protein Disulphide Isomerise may not be required, as Tissue Factor may already be present in a decrypted form. **With a disrupted endothelium, for example due to trauma, or atherosclerotic plaque rupture, shear-dependant platelet surface receptors bind collagen (low shear) or VWF (high shear) leading to 'outside in' platelet activation.**



Platelet Thrombus Formation and Propagation

A developing platelet thrombus recruits circulating unstimulated platelets. Within the thrombus, platelet activation only occurs in a subset of recruited platelets.(Furie & Furie, 2008) Other platelets may remain loosely associated with the thrombus, do not undergo marked activation, and may ultimately disengage from the thrombus depending on the degree of shear stress and the strength of platelet integrin binding with relevant agonists. The platelet GpIIb/IIIa receptor (also called integrin $\alpha_{IIb}\beta_3$) mediates recruitment of platelets to the thrombus, as well as platelet–platelet interactions. Activation of GpIIb/IIIa, similar to tissue factor, requires protein disulfide isomerise.(Furie & Furie, 2008) Activation of platelets bound to the wall of the injured vessel causes a conformational transition in GpIIb/IIIa that increases it's affinity for fibrinogen and von Willebrand factor. At low shear rates, fibrinogen is the predominant GpIIb/IIIa ligand, whereas von Willebrand factor is the predominant ligand at higher shear rates.(Furie & Furie, 2008) However, neither von Willebrand factor nor fibrinogen are absolutely required for platelet adhesion or thrombus formation.(Ni *et al*, 2000) Platelets of mice deficient in von Willebrand factor and fibrinogen have been shown to accumulate fibronectin in their α-granules, suggesting that fibronectin could be the ligand supporting platelet aggregation in these mice.(Ni *et al*, 2000) During platelet activation, degranulation and release of the contents of platelet alpha and dense granules occurs, thus further promoting thrombus formation (see Chapter 1.2). Late signalling events also enhance platelet–platelet aggregation. Growth-arrest–specific gene 6, CD40 ligand, ephrin-Eph, and signalling lymphocyte activation molecule (SLAM) enhance platelet–platelet affinity by participating in the platelet-platelet synapse to create a protected environment within the clot that stabilizes the platelet thrombus.(Furie & Furie, 2008)

Delivery of tissue factor to the interior of the platelet thrombus is thought to be achieved via circulating platelet microparticles which express tissue factor on their surface. Activated platelets, express CD62P following platelet activation which binds to P-selectin glycoprotein ligand 1 (PSGL-1) on the surface of tissue factor-expressing microparticles. Recruitment of microparticles to the platelet thrombus increases the concentration of locally available membrane-bound tissue factor, which is then activated or "decrypted" by protein disulfide isomerise.(Stoll *et al*, 2008)

1.1.5. Summary

Ischaemic stroke is a heterogeneous disease involving thrombosis or thromboembolism in the majority of cases. A better understanding of the haemostatic/thrombotic mechanisms involved in TIA or ischaemic stroke in the setting of carotid stenosis should lead to improvements in secondary preventative therapy in these patients.

1.2. Platelets

1.2.1. Historical Background

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

In 1881, Professor Bizzozero of Turin repeated Zahn's experiment, this time using guinea-pigs or young rabbits, whose platelets are non-nucleated. He proposed the term 'Blut Plättchen' (blood platelet) to describe the third corpuscle in blood. He showed that the first step in thrombus formation *in vivo* was the adhesion of blood platelets to a damaged area of vessel wall, and described the formation of platelet 'plaques' (aggregates) and the subsequent plugging of small vascular punctures by the 'white platelet thrombus'.(Owen, 2001;Meade, 2005)

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

1.2.2. Platelet Structure

Resting anucleate platelets circulate as discoid cells that are 2 to 4 μm in diameter (Leeson, 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 1-2).(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, 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.(Monroe *et al*, 2002;Colman, 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*. 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) lines the channels of the surface-connected *open canalicular system* (OCS) (Shapiro, 1999) and participates in the transport of alpha and dense granules during platelet activation assisted by α-dystrobrevins.(Cerecedo *et al*, 2010) The OCS is an elaborate series of conduits that begin as indentations of the plasma membrane, course throughout the interior of the platelet and greatly increase its surface area.(Blockmans *et al*, 1995) The OCS facilitates the entry of plasma substances to the interior of the platelet, and the release of products from the interior of the cell.(Shapiro, 1999) It also serves as an extensive internal store of membrane that may be used in the process of platelet spreading and pseudopod formation after platelet adhesion.

(b) The sol-gel zone: This zone consists of a number of different components, lies beneath the peripheral zone, and forms the cytoskeletal framework of the platelet that comprises between 30 to 50% of the total platelet protein content.(Shapiro, 1999) A circumferential band of *microtubules*, composed of tubulin, lies beneath the submembrane skeleton of actin filaments, and is involved in maintaining the discoid shape of the unstimulated platelet.(Blockmans *et al*, 1995)

In addition to the integrated membrane actin filaments mentioned above, *cytoplasmic actin filaments* are also present within the platelet and they comprise the bulk of the platelet cytoskeleton.(Blockmans *et al*, 1995) The organisation of these actin filaments is maintained by their association with other proteins, such as actin-binding protein, tropomyosin and α-actinin.(Blockmans *et al*, 1995) During the process of platelet

activation, actin may bind to myosin,(Blockmans *et al*, 1995) and this contractile system mediates platelet shape change, pseudopod formation, internal contraction and release of granules contents.(Shapiro, 1999)

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

There are also four distinct populations of granules within the organelle zone: *α-granules*, *dense granules*, *lysosomes*, and *peroxisomes*.(Blockmans *et al*, 1995) *α-granules* are the most abundant granules.(Blair & Flaumenhaft, 2009) They contain membrane bound proteins, although most of these are already present on the resting platelet membrane, including integrins (e.g. αIIb, α6, β3), immunoglobulin family receptors (e.g. GPVI, Fc receptors, PECAM), leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), tetraspanins (e.g. CD9) and other receptors (CD36, Glut-3). Not all membrane-associated α-granule proteins, however, are present on the unstimulated platelet plasma membrane (e.g., the integral membrane proteins fibrocystin L, CD109, CD62P),(Blair & Flaumenhaft, 2009) but are expressed on platelet activation. α-granule VWF:Ag can contribute to, but is not necessary for, normal haemostasis and thrombus formation.(Blair & Flaumenhaft, 2009) Platelet α-granules also contain a number of coagulation factors and co-factors which participate in haemostasis. Factors V, XI, and XIII each localize in

α-granules and are secreted upon platelet activation. Factor V and XI are involved in activation of factor X, a reaction which is catalysed on the platelet surface. α-granules also store inhibitory proteases, such plasminogen activator inhibitor-1 (PAI-1) and α2-antiplasmin, which limit plasmin-mediated fibrinolysis, indicating a role of α-granules in perpetuating and stabilising the fibrin thrombus. α-granules store antithrombin, which cleaves activated clotting factors in both the intrinsic and extrinsic pathways, and C1-inhibitor, which degrades plasma kallikrein, factor XIa, and factor XIIa. Platelets secrete tissue factor pathway inhibitor (TFPI), protein S, and protease nexin-2 (amyloid β-A4 protein), which inhibits factors XIa and IXa. Although the definition of an α-granule is morphological, there are many variations in morphology that can be considered to represent an α-granule.(Blair & Flaumenhaft, 2009) It is possible that this heterogeneity in α-granule morphology means that there are different subtypes of α-granule, as yet not described, each of which may perform a predominant function or set of functions, depending on their structure or constituents.

Many of the proteins found in α-granules are present in plasma. This raises the question as to whether the α-granule counterparts of plasma proteins differ in structure or function. Plasma proteins, such as fibrinogen and immunoglobulin, are taken up into α-granules by either endocytosis or pinocytosis, and this is thought to continue throughout the life of the platelet.(Blair & Flaumenhaft, 2009) Also, while many important bioactive proteins are present, concentrated, and even modified in platelet α-granules, establishing the physiologic importance of a particular α-granular protein is challenging. Nonetheless, there is evidence that secreted α-granule proteins function in coagulation, inflammation, atherosclerosis, antimicrobial host defense, angiogenesis, wound repair, and malignancy.(Blair & Flaumenhaft, 2009)

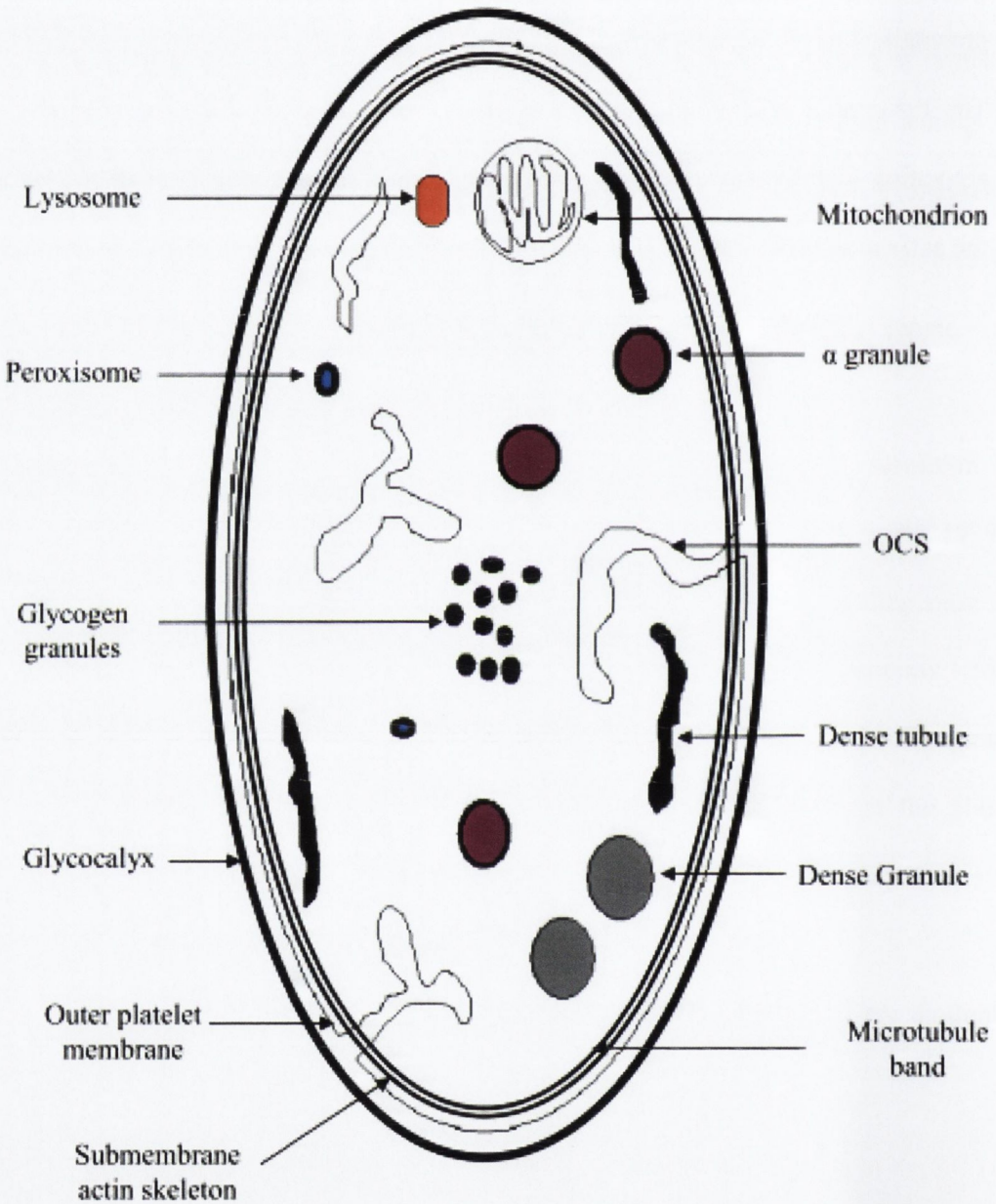
Dense granules contain CD62P, CD63,(Israels *et al*, 1992) ATP, ADP, serotonin and calcium, although a cytoplasmic pool of adenine nucleotides also exists.(Shapiro, 1999)

Lysosomes are small vesicles that contain β-glucuronidase, cathepsins, collagenase and elastase.(Ware, 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, 1995)

(d) The membrane zone: This includes the OCS and the dense tubular system.(Shapiro, 1999) The dense tubular system is a closed system of membrane bound tubules that is equivalent to the smooth endoplasmic reticulum in other cells. It is the site where calcium and protein disulfide isomerase is sequestered, and where the enzymes involved in prostaglandin synthesis are localised (Shapiro, 1999;Blockmans *et al*, 1995;van Nispen Tot Pannerden HE *et al*, 2009) Platelets may secrete thromboxane A_2 and platelet activating factor (in response to stronger stimuli) from the dense tubular system during the activation process.

Figure 1-2 Diagrammatic Representation of the Ultrastructure of a Platelet in Cross Section
(adapted from Figure 1 in Monograph by Shapiro, 1999)



1.2.3. Signalling mechanisms and second messengers in platelets

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

Guanine nucleotide binding regulatory (G) Proteins

Many platelet receptors are coupled to the second messenger generating enzymes, phospholipase C, phospholipase A_2, and adenylate cyclase via G proteins.(Blockmans *et al*, 1995) G proteins are composed of 3 subunits: 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 A_2.(Blockmans *et al*, 1995)

1.2.4. Second messenger generating enzymes and second messengers in platelets

The main second messengers involved in platelet activation/inhibition are calcium, inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG), TxA_2, PGs, cAMP and cGMP. The main enzymes involved in generating these second messengers will now be described.

Phospholipase C catalyses the breakdown of phosphatidylinositol 4,5-biphosphate (PIP_2) into IP_3 and DAG, and this enzyme is predominantly stimulated by agonists linked to G proteins.(Blockmans *et al*, 1995) IP_3 binds to specific receptors and induces the release of calcium from the dense tubular system, and promotes the influx of external calcium into the platelet.(Blockmans *et al*, 1995) ADP may induce calcium influx via receptor mechanisms that are independent of IP_3. Of note, all known platelet agonists increase intraplatelet calcium concentrations, and this will ultimately lead to phosphorylation of myosin and platelet shape change.

Calcium mobilisation leads to activation of phospholipase A_2, although this enzyme may also be activated directly by G protein mediated mechanisms.(Blockmans *et al*, 1995) Phospholipase A_2 liberates arachidonic acid from the dense tubular system and plasma membrane, with the subsequent formation of PGG_2, PGH_2, and the potent platelet agonist TxA_2. PGG_2, PGH_2, and TxA_2 can all induce platelet aggregation, but PGH_2, and TxA_2 are the main analogues involved in mediating platelet aggregation and secretion. PGH_2 can be converted to the inhibitory PGD_2, to the inactive or minimally inhibitory $PGF_2\alpha$, and to inhibitory or stimulatory PGE_2.

These prostaglandins may also 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, PGE1, and PGD2, to their respective G_s regulatory proteins. In contrast, thromboxane, ADP, epinephrine, and thrombin stimulate G_i regulatory proteins, with inhibition of adenylate cyclase. This leads to a resultant decrease in cAMP levels in platelets where cAMP was initially elevated.(Blockmans *et al*, 1995)

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

1.2.5. 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,(Andrews *et al*, 1997) Each receptor contains two GpIbα, two GpIbβ and two GpIX subunits, but only one GpV subunit.(Escolar & White, 2000) With the exception of patients with Bernard-Soulier syndrome, in whom this receptor may be deficient, there are approximately 25,000 copies of GpIb-IX (Andrews *et al*, 1997) per platelet, and approximately half as many copies of GpV.(Canobbio *et al*, 2004) The GpIbα (CD42b) subunit of the GpIb-IX-V complex is the predominant receptor for VWF on platelets, and this receptor mediates VWF-dependent platelet adhesion to the subendothelium (Gardiner *et al*, 2010) and platelet activation (Berndt *et al*, 2001) at high shear rates. GpIbα also possesses binding sites for thrombin, Mac-1, CD62P, high molecular weight kininogen and Factor XII.(Canobbio *et al*, 2004) The cytoplasmic tail of GpIbα contains a binding site for actin-binding proteins,(Andrews *et al*, 1997;Berndt *et al*, 2001) and the GpIbβ cytoplasmic tail contains a protein kinase A phosphorylation site that inhibits platelet actin polymerisation in response to agonist stimulation.(Andrews *et al*, 1997) In addition to these functions of the receptor subunits, GpIbα, GpIbβ and GpIX are all required for stable surface expression of the receptor complex.(Berndt *et al*, 2001) Until relatively recently, the exact physiological function of GpV was not known, but it appears that it is has a regulatory and inhibitory role in thrombin-dependent platelet activation when the

agonist binds to this receptor.(Berndt *et al*, 2001;Clemetson & Clemetson, 2001) Thrombin firstly cleaves GpV near the platelet membrane surface, releasing an extracellular soluble fragment, thus facilitating thrombin binding to GpIbα with the subsequent initiation of intraplatelet signalling responses and platelet activation.(Berndt *et al*, 2001) Overall, the signalling events induced by VWF (and perhaps other agonists) binding to GpIb-IX-V include opening of the transmembrane calcium channels, elevation of intraplatelet calcium levels, and activation of protein kinase C and tyrosine kinase(s).(Canobbio *et al*, 2004;Berndt *et al*, 2001) These signalling events ultimately result in 'inside-out' activation of the GpIIb/IIIa receptor and platelet aggregation.(Cosemans *et al*, 2008)

GpIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin) is the predominant platelet receptor, with up to 80,000 copies on each platelet surface.(Cosemans *et al*, 2008) Some GpIIb/IIIa receptors are also located in the OCS and in platelet α-granules,(Blockmans *et al*, 1995) but can be translocated to the platelet membrane surface upon platelet activation.(Du & Ginsberg, 1997) The GpIIb subunit consists of a heavy and a light chain linked by a disulphide bond, and this subunit is non-covalently bound to the GpIIIa subunit to form a heterodimer.(Calvete, 1995) The association between the GpIIb and GpIIIa subunits depends on a submicromolar concentration of extracellular calcium.(Du & Ginsberg, 1997;Abrams & Shattil, 1991;Moran N & FitzGerald GA, 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.(Du & Ginsberg, 1997) Thus, the receptor complex can bind several different ligands including fibrinogen, VWF, fibronectin, vitronectin and thrombospondin.(Moran N & FitzGerald GA, 1994) The surface binding ligand varies depending on the stimulus to activation e.g. fibrinogen is the predominant ligand in the

presence of thrombin, ADP or collagen, but VWF is the predominant ligand when aggregation is induced by high shear stress.(Litjens *et al*, 2000) In the resting platelet, GpIIb/IIIa is unable to bind fibrinogen or VWF in solution, but can adhere to these ligands if they are coated on a surface.(Bennett *et al*, 2009) However, platelet activation leads to a conformational change in this receptor that facilitates binding of soluble ligands, and this change is the final common pathway leading to platelet aggregation.(Shattil & Leavitt, 2001) When GpIIb/IIIa is activated and ligand binding occurs, this may trigger 'outside-in' signalling and induce profound changes in platelet shape and adhesiveness by interaction with the platelet cytoskeleton.(Shattil & Leavitt, 2001) These outside-in signals may also potentiate agonist-induced platelet secretion and procoagulant activity.(Shattil, 2009) However, the second messenger systems mediating the effects of ligand binding to GpIIb/IIIa are not fully understood.(Shattil & Leavitt, 2001) In addition, a variety of excitatory and inhibitory platelet receptors may exert their effect on platelets by 'inside-out' signalling that regulates the affinity and avidity of GpIIb/IIIa for ligands.(Clemetson & Clemetson, 2001) The involvement of the GpIIb/IIIa receptor in the platelet adhesion and aggregation process is described in more detail below.

GpIa/IIa ($\alpha_2\beta_1$ integrin) and GpVI are the two main *direct* receptors for collagen on platelets.(Cosemans *et al*, 2008) There are between 2000 and 4000 copies of GpIa/IIa,(Chen *et al*, 2002) and approximately 1250 GpVI receptors per platelet.(Chen *et al*, 2002) Under conditions of low shear stress, GpIa/IIa may bind to subendothelial collagen that is exposed during the process of vessel injury. GpVI also appears to be involved in the initial binding of platelets to collagen.(Arthur *et al*, 2011) Recently, downstream signalling has been described via TRAF4 and other redox relevant signalling proteins, and data suggest that GpVI exerts downstream effects on TRAF4/Nox2-dependent redox pathways.(Cosemans *et al*, 2008)

Under conditions of high shear stress, neither GpIa/IIa nor GpVI can mediate platelet adhesion to collagen. During platelet activation, $\alpha_2\beta_1$ converts to an active state.(Clemetson & Clemetson, 2001) Subsequently, the GpIb-VWF complex may bind to subendothelial collagen via a receptor for collagen on VWF. In this sense, the GpIb-IX-V complex acts as an *indirect* platelet receptor for collagen. The role of other potential platelet collagen receptors in the process of platelet adhesion e.g. GpIV (CD36) and CD31 is unclear.(Cosemans *et al*, 2008) Activated $\alpha_2\beta_1$ reinforces the GPVI-induced signaling to phospholipase Cγ2 activation, and Ca^{2+} release and secretion, in addition to integrin activation.(Clemetson & Clemetson, 2001) After the initial adhesion process, signalling events may facilitate more firm platelet adhesion, with further collagen binding to GpIa/IIa, activation of GpIIb/IIIa with subsequent fibrinogen binding, and perhaps binding of fibronectin to GpIc/IIa ($\alpha_5\beta_1$), and laminin to GpIc'/IIa ($\alpha_6\beta_1$).(Cosemans *et al*, 2008)

Procoagulant properties of integrins

Profound platelet stimulation, such as evoked by the GPVI interaction with collagen, or by the combination of thrombin and ADP, may cause a marked loss in platelet adhesion, loss of pseudopods and lamellipods, platelet disintegration, membrane bleb formation and microparticle formation.(Cosemans *et al*, 2008) These platelets characteristically show prolonged, high intracellular Ca2+ rises, and exposed phosphatidylserine on their surface, which stabilises tenase complexes, and accelerates thrombin formation.(Cosemans *et al*, 2008) Surface expression of GPIIb/IIIa is retained while fibrinogen and PAC-1 binding ability is lost in these platelets suggesting an additional conformational change in this integrin, which allows these non-adherent platelets to become separated from adherent platelets, allowing adequate access to plasma factor X.(Blockmans *et al*, 1995)

1.2.6. Excitatory 'non-glycoprotein' platelet receptors

The main 'non-glycoprotein' platelet receptor agonists that were studied directly or indirectly in this thesis were thromboxane A_2, 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 A_2 receptor(s)

Thromboxane A_2 (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. Arachidonic acid is metabolised to form thromboxane A_2 (TxA_2), which has a short half-life of less than 1 minute before being rapidly metabolised to form inactive thromboxane B_2. TxA_2 induces platelet shape change, aggregation, and secretion of granule contents via G protein-coupled platelet membrane receptors that are linked to phospholipase C breakdown of PIP_2, and elevation of intraplatelet free calcium.(Gachet, 2001;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;Hollopeter *et al*, 2001;Jin *et al*, 1998) The P2 receptors are divided into two main groups: (i) the G protein-coupled P2Y ADP receptors, and (ii) the ligand-gated, ion channel P2X ATP receptors which also bind ADP.

The P2Y ADP receptors are termed $P2Y_1$ (Gq- coupled) and $P2Y_{12}$ (Gi-coupled). They mediate different aspects of the response to this agonist.(Jennings, 2009) ADP must bind to both $P2Y_1$ and $P2Y_{12}$ receptors for normal ADP-induced platelet aggregation, but each receptor mediates its effects via different second messenger systems.(Jin *et al*, 1998) When ADP binds to $P2Y_1$, calcium is mobilised from intraplatelet stores, thus triggering platelet shape change and pseudopod formation,(Gachet, 2001) and the GpIIb/IIIa receptor is activated with resultant transient platelet aggregation.(Gachet, 2001) In contrast, ADP binding to $P2Y_{12}$ leads to inhibition of adenylate cyclase with a decrease in intraplatelet cyclic AMP (cAMP) levels, potentiation of the secretion of granule contents, and stabilisation of platelet aggregates through full activation of GpIIb/IIIa.(Kahner *et al*, 2006) Clopidogrel exerts its antiplatelet effects by selective and irreversible inhibition of the $P2Y_{12}$ ADP receptor.

The $P2X_1$ receptor is activated by binding of ATP. The effect of ATP is antagonised by binding of ADP.(Kahner *et al*, 2006) ATP-induced activation of the $P2X_1$ receptor results in influx of both divalent calcium and monovalent sodium at a ratio of 4:1.(Kahner *et al*, 2006) Activation of the receptor alone causes shape change, transient centralisation of granules, and formation of small microaggregates which may be insufficient alone to cause platelet activation, but important in amplifying the response to other agonists. $P2X_1$ receptor activation has been shown to be essential for enhanced platelet adhesion and thrombus formation under high shear rates.(Blockmans *et al*, 1995)

Epinephrine (Adrenaline) receptors

The term epinephrine will be used throughout this thesis to describe adrenaline, because this is the nomenclature used in the PFA-100® system. Platelets have stimulatory α_2 adrenoceptors and inhibitory β_2 adrenoceptors, and in most patients, the α_2 adrenoceptors

predominate.(Blockmans *et al*, 1995) There are approximately 200 to 300 copies of the α_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.(Helgason *et al*, 1993) 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.(Blockmans *et al*, 1995) Epinephrine may also sensitise platelets to the effects of other agonists.(Blockmans *et al*, 1995) However, because epinephrine only induces platelet aggregation in the presence of subphysiological calcium concentrations, and at epinephrine concentrations that are much greater than those found in the circulation, the importance of epinephrine-induced platelet aggregation *in vivo* is unclear.(Rivera *et al*, 2009)

Thrombin receptors

Thrombin is the most effective platelet agonist *ex vivo*, inducing shape change, secretion of the contents of platelet granules, synthesis and release of TxA_2, mobilisation of CD62P to the platelet surface, and activation of GpIIb/IIIa.(Rivera *et al*, 2009) It also induces the expression of procoagulant factors on platelets, which stimulates further thrombin generation, although this does not appear necessary for the development of a mature thrombus.(Furie & Furie, 2008;Coughlin, 2005)

Thrombin-induced platelet activation is mediated at least partially by the GpIb/IX/V complex, and mainly by protease activated receptor 1 (PAR1), but also by PAR4.(Coughlin, 2005) Protease activated receptors are activated by an intriguing mechanism. When thrombin binds to PAR1 on platelets, it cleaves part of the receptor and unmasks a new amino terminal domain, which in turn binds to the body of the

receptor itself and activates it.(Coughlin, 2005) It has been suggested that PAR1 and PAR4 mediate thrombin-induced platelet activation at low and high agonist concentrations, respectively, but that both receptors influence intraplatelet calcium signalling.(Rivera *et al*, 2009) Blockade of the thrombin-GpIbα interaction with antibodies impairs the cleavage of PAR1.(Rivera *et al*, 2009)

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

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

Platelet activating factor (PAF) receptor

PAF is formed in certain cells, including platelets, by the consecutive actions of phospholipase A_2 and acyltransferase on platelet membrane phospholipids.(Blockmans *et al*, 1995) PAF causes platelet shape change and aggregation, but because it is dependent on TxA_2 to mediate the secretion of granule contents, it is unlikely to play a major role in amplifying the response to other platelet agonists in humans. PAF is internalised during platelet activation, and this may be related to reorganisation of the plasma membrane.(Blockmans *et al*, 1995)

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 (V1) receptors that mediate platelet aggregation via phosphatidylinositol metabolism and an increase in the intraplatelet calcium concentration.(Blockmans *et al*, 1995)

1.2.7. Inhibitory 'non-glycoprotein' platelet receptors

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)

PGI_2 and PGE_1 receptor

Binding of PGI_2 and perhaps PGE_1 to this receptor also activates adenylate cyclase via a G protein-coupled mechanism, with an increase in cAMP production and inhibition of platelet function.(Blockmans *et al*, 1995)

PGD_2 receptor

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

PGE_2 receptor

It has been proposed that PGE_2 may inhibit platelet activation either by binding to its own receptor or the PGI_2 receptor (Blockmans *et al*, 1995). However, depending on the experimental conditions, PGE_2 may also potentiate platelet activation by activating protein kinase C or inhibiting cAMP formation.

1.3. The Symptomatic and Asymptomatic Carotid Atherosclerotic Plaque

In this section, I will briefly outline the literature regarding the morphology of symptomatic and asymptomatic carotid artery plaques in humans.

Atherosclerosis is a diffuse, multisystem, chronic inflammatory disorder involving the vascular, metabolic, and immune systems.(Ross, 1993) The atherosclerotic plaque at the carotid bifurcation is composed of a dense 'cap' made up of connective tissue embedded with smooth muscle cells overlying the centre of the plaque, which is composed of varying amounts of lipid and necrotic inflammatory debris (lipid rich / necrotic core or LR/NC).(Ross, 1993);(Golledge *et al*, 2000) The interaction between cells within the plaque including monocyte-derived macrophages, smooth muscle cells, and T lymphocytes, and connective tissue within the plaque appears to determine plaque development.(Ross, 1993);(Golledge *et al*, 2000) This interaction can lead to instability within the plaque, plaque rupture, and arterio-arterial embolisation or vessel occlusion causing ocular or hemispheric transient ischaemic attacks (TIAs) or ischaemic strokes.

However, it is still not entirely clear what causes a 'stable' asymptomatic carotid plaque to become 'unstable' with a subsequent high risk of causing ischaemic cerebrovascular symptoms. Comparisons of plaques from symptomatic and asymptomatic carotid stenosis patients have revealed certain characteristic features of 'unstable' carotid plaques. (Bassiouny *et al*, 1989);(Carr *et al*, 1996);(Sitzer *et al*, 1995) Previous studies, along with *in-vitro* work, suggest infiltration of inflammatory cells into the fibrous cap of the carotid plaque is a key initial step, with subsequent release of collagen-digesting enzymes and cytokines that promote smooth muscle cell apoptosis. This may weaken the surface of the plaque, thus promoting thrombosis, embolisation, and potentially arterial occlusion.(Golledge *et al*, 2000)

Symptomatic carotid plaques usually contain inflammatory cells, including macrophages (or foam cells), smooth muscle cells and T cells within the cap.(Jander *et al*, 1998);(Carr *et al*, 1996) Symptomatic plaques are also typically haemorrhagic and contain lipid material in a central necrotic core, which is usually closer to the cap in symptomatic than asymptomatic plaques.(Bassiouny *et al*, 1997) Symptomatic plaques also occasionally contain fresh thrombus which is usually within the vessel lumen, but may be present inside a fissured plaque.(Golledge *et al*, 2000)

Inflammation within the symptomatic plaque, can lead to increased plaque instability, with thinner fibrous caps at greatest risk of plaque rupture or ulceration.(Golledge *et al*, 2000);(Redgrave *et al*, 2006) The Oxford Plaque Study found that symptomatic carotid atherosclerotic plaques that have ruptured are more likely to have a minimum cap thickness <200 μm, whereas non-ruptured plaques tended to have a cap thickness >200 μm on histology.(Redgrave *et al*, 2008)

Most previous histological studies of carotid artery plaques excised from symptomatic and asymptomatic patients were usually too small or insufficiently detailed to reliably determine features of plaque instability, or to stratify analyses by the nature of the presenting symptoms, mainly due to the use of incomparable, non-reproducible methods.(Lovett *et al*, 2005) The largest-ever histological study of symptomatic carotid plaques from 526 consecutive patients who underwent endarterectomy, related accurate and detailed, reproducible histological assessments to the nature and timing of the presenting symptoms.(Redgrave *et al*, 2006) The researchers found that dense plaque inflammation, especially infiltration with macrophages, was the feature most strongly associated with cap rupture (odds ratio (OR) 3.39; $p < 0.001$), and was more likely to be seen in patients with recent (≤60 days) versus remote (>180 days) symptoms ($p = 0.008$). They also found strong negative associations with time since stroke onset for cap rupture

($p = 0.02$), overall plaque inflammation ($p = 0.003$), and "unstable plaque" appearances ($p = 0.001$). They concluded that the pathology of recently symptomatic carotid plaques was similar to that of symptomatic coronary plaques, with strong correlations between macrophage infiltration and plaque instability and recent symptoms. The finding that plaque inflammation and instability persisted over time after TIA, but decreased over time following stroke suggested that the nature of the underlying pathology may differ between these two symptomatic groups.(Redgrave *et al*, 2006)

Previous studies have identified several systemic and local factors that may influence the 'vulnerability' of the carotid plaque, or may be seen in patients with symptoms associated with carotid atherosclerotic stenosis.(Nighoghossian *et al*, 2005) Systemic factors include platelet count,(McCabe *et al*, 2005b) levels of platelet activation and / or levels of leucocyte activation as measured by lymphocyte-platelet, monocyte-platelet and neurophil-platelet complexes on fresh whole blood flow cytometry,(McCabe *et al*, 2005b) leucocyte and monocyte count,(Johnsen *et al*, 2005) fibrinogen levels,(Mauriello *et al*, 2000) or potentially C Reactive Protein (CRP) levels. Local factors include Intercellular Adhesion Molecule-1 (ICAM-1) or other related cytokines,(DeGraba *et al*, 1998) matrix metalloproteinases (MMPs), namely MMP-1, MMP-3 and potentially MMP-12,(Johnson *et al*, 1998);(Morgan *et al*, 2004) circulating transforming growth factor-β1,(Cipollone *et al*, 2004) tissue factor (TF) levels,(Jander *et al*, 2001) various cytokine polymorphisms,(Flex *et al*, 2004) plaque neovascularisation (Fleiner *et al*, 2004) and potentially shear stress from the local flow of blood (Traub & Berk, 1998) which is most turbulent at the vessel bifurcation.(Nighoghossian *et al*, 2005)

Asymptomatic carotid artery plaques, in contrast, usually contain caps which are less ulcerated or non-ulcerated, or unruptured with more fibrous material, but may occasionally contain similar amounts of intraplaque haemorrhage or luminal thrombus as

symptomatic plaques.(Golledge *et al*, 2000);(Carr *et al*, 1996);(Bassiouny *et al*, 1989);(Feeley *et al*, 1991) It has been demonstrated in studies using ultrasound analysis of carotid artery plaques that symptomatic plaques tend to be echolucent or hypoechoic and thus, presumably lipid-rich, whereas asymptomatic plaques more often exhibit echodense, hyperechoic appearances on B-mode ultrasound (Gronholdt, 1999);(Tegos *et al*, 2000);(Tegos *et al*, 2001b)

The selection of patients for carotid intervention has, in recent years, largely been based on the severity of luminal narrowing.(Golledge & Siew, 2008) However, available histological data are increasingly suggesting that other plaque features, including inflammation and fibrous cap thickness, may be very important in predicting future thromboembolic events. In future, the selection of acutely or remotely symptomatic patients, deemed high risk and most likely to benefit from timely surgical or endovascular treatment, or asymptomatic patients considered good candidates for intervention, may focus more on a combination of factors regarding the make-up of the carotid plaque itself and its propensity to cause distal embolisation.

There are emerging data to suggest that asymptomatic patients with microembolic signals on transcranial Doppler ultrasound imaging are at highest risk of developing subsequent stroke compared with those patients without microembolic signals on TCD.(Spence *et al*, 2005;Markus *et al*, 2010) Currently, the best predictor of whether a carotid stenosis patient is at high-risk of experiencing a subsequent cerebrovascular event is the occurrence of a recent TIA or stroke in the appropriate carotid territory, although many patients with recently symptomatic carotid stenosis do not have recurrent symptoms on medical therapy alone.

In future, a combined approach, including clinical assessment, risk factor profiling, multimodal neurovascular imaging with carotid ultrasound, high-resolution MR and / or

CT characterisation of the carotid plaque, (Chalela, 2009) molecular ultrasound, or MRI targeting of the thrombotic and inflammatory components of the plaque,(Nighoghossian *et al*, 2005), brain MR imaging, transcranial Doppler ultrasound monitoring for intracranial microemboli, and focused bio-markers may allow one to more accurately identify patients with vulnerable plaques.(Golledge & Siew, 2008). Such a comprehensive multimodal approach to risk-stratifying carotid stenosis patients was not studied in detail prior to the commencement of this thesis.

1.4 Review of Carotid Intervention Trials including endarterectomy and endovascular treatment studies

1.4.1 Asymptomatic Carotid Stenosis

Five to 10 per cent of people over 65 years of age have asymptomatic carotid stenosis (> 50%) of one extracranial carotid artery. (The European Carotid Surgery Trialists Collaborative Group, 1995) 1.6% of subjects over 50 years of age had > 80% asymptomatic carotid stenosis in one study.(Ricci *et al*, 1991) In the European Carotid Surgery Trial (ECST), asymptomatic moderate (^{ECST}30 - 69%) or severe (^{ECST}70 - 99%) carotid stenosis of the contralateral carotid artery was noted on digital subtraction angiography (DSA) 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 - 99% stenosis compared with those with 30 - 69% stenosis (5.7% versus 2.1%). (The European Carotid Surgery Trialists Collaborative Group, 1995) Because carotid endarterectomy has been shown to be effective in reducing the risk of subsequent stroke in patients with symptomatic severe (> 70%) extracranial carotid stenosis (see section 1.3),(Rerkasem & Rothwell, 2011) four 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) It was unclear from another study what proportion of patients were on antiplatelet therapy and on what particular agent, as it was left at the discretion of the managing clinician.(Halliday *et al*, 2004) The design of two of these studies did not allow for a direct meaningful comparison of the effects of carotid endarterectomy versus medical treatment alone. (Halliday *et al*, 2004;The CASANOVA Study Group, 1991). The results of these four trials will now be discussed with a brief discussion on two recent observational studies on patients with asymptomatic carotid stenosis. This will be followed by a summary of a meta-analysis of completed trials of treatment for asymptomatic carotid stenosis.

1.4.2 Review of the literature regarding treatment of asymptomatic 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 treatment in male patients with angiographically confirmed asymptomatic ^{NASCET}50 - 99% carotid artery stenosis.(Hobson *et al*, 1993) 211 patients were randomly assigned to undergo surgery and 233 to receive best medical treatment; both treatment groups received aspirin therapy (325 - 650 mg daily). The mean age of patients was 64.5 years, and mean duration of follow up was 2 years. Carotid endarterectomy reduced the absolute risk of ipsilateral TIA, transient monocular blindness or stroke by 12.6% versus 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 - 99% carotid stenosis were randomised to carotid endarterectomy ($n = 825$) or best medical treatment ($n = 834$) in 39 clinical centres in the USA and Canada.(Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995) The mean age of study subjects was 67 years, and 58% of patients had 70 - 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 were consistent with findings of a meta-analysis of five completed trials of carotid endarterectomy compared with medical treatment alone for asymptomatic 50 - 99% carotid stenosis. (Benavente *et al*, 1998) In comparison with best medical treatment, carotid endarterectomy significantly reduced the absolute risk of ipsilateral stroke or perioperative stroke or death by 2% over 3.1 years (0.65% per year).

The Asymptomatic Carotid Surgery Trial (ACST) was an international multicenter, randomised study which attempted to clarify the long-term effects of endarterectomy on overall stroke risk in patients with asymptomatic (no symptoms for at least 6 months) $\geq$ 60% carotid stenosis on colour Doppler ultrasound.(Halliday *et al*, 2004) All patients were treated otherwise as per their managing clinician and so no direct comparison between endarterectomy and best medical treatment was performed. 3120 asymptomatic patients were randomised equally between immediate endarterectomy (50% by 1 month, 88% by 1 year) and deferred endarterectomy until a more definite indication arose (4% per year) and were followed for up to 5 years (mean follow-up was 3.4 years). The risk of stroke or death within 30 days of endarterectomy was 3.1%. Combining perioperative and non-perioperative strokes in all patients undergoing endarterectomy, the 5-year risk for all strokes in the immediate versus deferred endarterectomy group was 6.4% versus 11.8% ($p < 0.0001$), indicating an average yearly reduction in stroke incidence of about 1.1% per year in the immediate versus deferred treatment group. As expected, the benefit was seen mainly for carotid territory ischaemic strokes (2.7% vs 9.5% respectively; $p < 0.0001$). Benefits were seen for both male and female subjects, mainly in subjects with > 70% stenosis on ultrasound and those < 75 years of age. Although there was a suggestion that the benefit of immediate or deferred carotid intervention in women may not be as high as in men, it should not completely exclude treatment in women as the study was not powered to test this hypothesis. Overall, this study showed that immediate

endarterectomy approximately halved the final 5-year 'all-stroke risk' from about 12% to 6%. It is important to note that these outcomes were for events in all vascular territories rather than just ipsilateral strokes, and so the overall benefit in reducing ipsilateral stroke only may have been even less.

A follow-on study, the Asymptomatic Carotid Surgery Trial-2 (ACST-2), has been designed to compare endarterectomy with carotid stenting in the prevention of stroke in patients with asymptomatic carotid stenosis. (Rudarakanchana *et al*, 2009) The investigators are aiming to study approximately 5000 patients with asymptomatic carotid stenosis to provide important evidence comparing the immediate and long-term safety and efficacy of carotid endarterectomy *versus* stenting in patients with asymptomatic carotid stenosis. Recruitment is ongoing, and it is to be determined whether recruitment targets will be met or not.

A small, single centre randomised clinical trial of patients with > 80% asymptomatic carotid stenosis investigated whether carotid endovascular treatment patients with asymptomatic stenosis were randomly allocated to undergo endovascular therapy or endarterectomy and followed up for 48 months.(Brooks *et al*, 2004) There were no major complications of cerebral ischaemia or death after either treatment modality. Peri-procedural complications included hypotension with or without bradycardia associated with endovascular treatment (n = 5), and cervical nerve injury (n = 3) or complications associated with general anaesthesia (n = 4) following endarterectomy. Although the investigators concluded that endovascular therapy and endarterectomy may be equally effective and safe in treating asymptomatic carotid stenosis, their numbers were far too small to make a conclusive statement.

From these data, the benefits of carotid endarterectomy in patients with asymptomatic $^{NASCET}50$ - 99% carotid stenosis are marginal, but in patients with $^{NASCET}70$ – 99%

stenosis, the benefits appeared clearer, particularly from the ACST results. However, the data from ACST indicate that up to 19 carotid endarterectomies would need to be performed in patients with 'asymptomatic' ≥ 60% carotid stenosis to prevent one stroke over a 5 year period (1/5.4 x 100). Further studies are required to identify patients with asymptomatic carotid stenosis, who are most likely to derive benefit from surgery, i.e. those at high risk of stroke despite best medical treatment alone, and with at least a 5-year life expectancy.(Chaturvedi *et al*, 2005).

Two recent, prospective, observational studies on the potential effects of modern, optimised medical treatment on the risk of stroke associated with asymptomatic carotid stenosis have been published. Marquardt *et al.* assessed the risk of TIA and stroke in 101 of 1153 consecutive patients who had presented with a stroke or TIA during the Oxford Vascular Study, and who were incidentally found to have ≥50% contralateral asymptomatic carotid stenosis.(Marquardt *et al*, 2010) All patients were treated with intensive 'contemporary' medical treatment. The average annual incidence of recurrent vascular events on optimal medical treatment was 0.34% for any ipsilateral ischaemic stroke, 0% for disabling ipsilateral stroke, and 1.78% for ipsilateral TIA. This important study highlighted the fact that the risk of stroke in the vascular territory supplied by a moderate or severe contralateral carotid stenosis on modern, intensive medical treatment was lower than that seen in patients randomised to 'best' medical treatment in the earlier trials of asymptomatic carotid stenosis. The authors concluded that this apparent reduction in risk was most likely attributed to improvements in medical treatment over time. However, it is of note that the combined annual risk of ipsilateral TIA or stroke distal to a ≥50% asymptomatic carotid stenosis was 2.12%. One cannot generalise these data to definitively guide patients who are incidentally noted to have an asymptomatic

moderate or severe carotid stenosis because it is possible that the risk may be even lower in patients who have never had symptoms at all.

Spence *et al.* assessed the effects of intensive medical therapy on the risk of subsequent vascular events and transcranial Doppler (TCD)-detected microemboli in patients with ≥60% asymptomatic stenosis.(Spence *et al*, 2010) 468 patients were recruited in 2 phases; in the 3 year period leading up to the end of 2002, and from 2003 – 2007 when improved medical therapy, including tighter control of plasma lipids with lipid-lowering therapy, had been instituted. All patients underwent at least 1 year of follow-up. They found that the risk of vascular events during follow-up (including stroke, MI, death or endarterectomy for subsequent symptomatic stenosis in the subsequent 2 years) was lower (5.2 vs. 17.6%; $p<0.001$), the prevalence of TCD microemboli was reduced (3.7% since 2003 vs.12.6% pre-2003; $p<0.001$), and the rate of carotid plaque progression was reduced ($p<0.001$) from 2003 onwards in association more intensive medical therapy. The group demonstrated that the incidence of any stroke in the cohort with MES was 10.3% in year 1, and 18.5% by year 2, but among patients without MES, this risk was 1.4% in year 1 and 1.8% by year 2. These data led the authors to suggest that <5% of patients with asymptomatic stenosis stand to benefit from revascularisation, and that all should receive intensive medical therapy and only be considered for revascularisation if microemboli are detected on TCD.

Data from these observational studies provide evidence for the increasing benefit seen with best medical therapy in asymptomatic carotid stenosis patients, and highlight that clinicians need to decide on a case-by-case basis which patients will derive most benefit from carotid revascularisation procedures in the future. The recently published Asymptomatic Carotid Emboli Study (ACES), indicated that the 2-year risk of ipsilateral stroke in patients with asymptomatic ≥70% carotid stenosis who have microembolic

signals (MES) detected on transcranial Doppler ultrasound is just over 7%, potentially aiding in the risk-stratification of patients with asymptomatic carotid stenosis who would most likely benefit from carotid intervention.(Markus *et al*, 2010)

Roffi *et al*, performed a recent metaanalysis of asymptomatic and symptomatic carotid stenosis trials comparing CEA to carotid stenting. (Roffi *et al*, 2009) The author discussed the lack of expertise and use of emboli protection devices in some randomised centres, as well as the issue of the use of older medical regimens in the initial trials, and the current use of lipid-lowering therapy, and statins in particular, possibly altering the magnitude of benefit of carotid intervention in some cases.(Roffi *et al*, 2009;Sillesen *et al*, 2008) Although there are guidelines supporting endarterectomy in carefully selected patients with asymptomatic ≥60% carotid stenosis under the age of 75, with a modest benefit evident if the perioperative risk of stroke or death is < 3% and the life expectancy of the patient is > 5 years,(Chaturvedi *et al*, 2005); (Biller *et al*, 1998) the dilemma facing clinicians will continue until further data are available regarding the TIA and stroke risk on optimal, modern medical treatment.

1.4.3 Review of the literature regarding the treatment of symptomatic carotid stenosis

Thirteen randomised controlled trials comparing the outcome after treatment with carotid endarterectomy versus non-surgical treatment of patients with symptomatic carotid stenosis have been published.(Rothwell, 2000); (Roffi *et al*, 2009); (Meier *et al*, 2010); (Brott *et al*, 2010) Two 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)

Ten published randomised clinical trials have compared the outcome of treatment with carotid endarterectomy with carotid endovascular treatment in patients with symptomatic carotid stenosis.(Naylor *et al*, 1998);(Carotid and Vertebral Artery Transluminal Angioplasty Study Group, 2001c);(Brooks *et al*, 2001); (Alberts M, 2001);(Yadav, 2004a);(Mas *et al*, 2006);(Ringleb *et al*, 2006);(Hoffmann A, 2008);(Ederle *et al*, 2010);(Brott *et al*, 2010) However, one of these studies, 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) Four of these studies were relatively small and randomised fewer than 300 patients in total and will not be described in any detail in this thesis.(Brooks *et al*, 2001); (Naylor *et al*, 1998); (Alberts M, 2001;Hoffmann A, 2008)

Regarding randomised controlled trials evaluating best medical management versus endarterectomy in symptomatic carotid stenosis, the final results of ECST and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) for patients with symptomatic severe ($^{\text{ECST or NASCET}}$70 to 100%) carotid stenosis will now be discussed. This will be followed by an overview of the results of a 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 a discussion of the data from the Stenting and Angioplasty with Protection in Patients at High Risk for Endarterectomy (SAPPHIRE) Trial, the Stent protected Percutaneous Angioplasty of the Carotid artery *vs.* Endarterectomy (SPACE) Trial, the Endarterectomy *vs.* Angioplasty in patients with Symptomatic Severe carotid Stenosis (EVA-3S) Trial, the International Carotid Stenting Study (ICSS), and the Carotid Revascularization Endarterectomy *vs.* Stenting Trial (CREST). The outcome after carotid intervention for patients with recently symptomatic 0 to 50% carotid stenosis will not be discussed further because endarterectomy has not been found to be beneficial in this patient population (The European Carotid Surgery Trialists Collaborative Group, 1995;North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991) and these patients were not studied in this thesis.

1.4.4 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 (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).(The European Carotid Surgery Trialists Collaborative Group, 1995) 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 > ^{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 3 years by 11.6% ($p = 0.001$) in patients with ^{ECST}80 to 100% carotid stenosis. 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%.(The European Carotid Surgery Trialists Collaborative Group, 1995)

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. 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)

Taking the results of ECST and NASCET together, carotid endarterectomy is beneficial in patients with recent symptoms associated with ^{ECST}70 to 99% or ^{NASCET}50 to 99% carotid stenosis,(Rothwell, 2000) although the benefit is greatest in patients with more than ^{ECST}80% or ^{NASCET}70% stenosis.

Rothwell et al published results of an analysis of the pooled data from ECST, NASCET, and the Veterans Affairs Trial (VA309) that compared outcomes after carotid endarterectomy (CEA) with best medical treatment in patients with recently symptomatic carotid stenosis.(Rothwell *et al*, 2003) These results confirmed the benefit of surgery in patients with recent symptoms in association with ≥ 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 [NASCET]70 to 99% stenosis without near-occlusion, 589 patients were randomised to undergo CEA and 506 to receive best medical treatment. CEA 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 Risk Reduction (ARR) 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 long-term secondary prevention: 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)

1.4.5. Endovascular compared with surgical treatment of symptomatic carotid stenosis

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.(Carotid and Vertebral Artery Transluminal Angioplasty Study Group, 2001b) 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 ^{ECST}86.4% (^{NASCET}77.3%) and ^{ECST}85.1% (^{NASCET}75.2%) in the endovascular and surgery groups, respectively.(Carotid and Vertebral Artery Transluminal Angioplasty Study Group, 2001a) 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, but these stents were not initially designed specifically for use in the carotid artery. 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 (CEA) 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 *et al*, 2005c) At one year, 32/173 (18.5%) endovascular patients compared with 9/174 (5.1%) surgery patients had severe carotid stenosis or occlusion ($p = 0.0001$). In follow-up paper in the same patient population, (Bonati *et al*, 2009) severe ≥70% carotid restenosis occurred three times more frequently following endovascular treatment than endarterectomy (estimated 5-year incidence 30.7% vs. 10.5%; adjusted hazard ratio [HR] = 3.17; $p<0.0001$). Ipsilateral non-perioperative stroke or TIA occurred more frequently in patients with ≥70% restenosis diagnosed in the first year after treatment than in patients who did not have ≥70% restenosis (5-year incidence 23%vs 11%; $p=0.04$).(Bonati *et al*, 2009) Ederle *et al*. found that although more patients in CAVATAS had strokes during follow-up in the endovascular compared to the CEA group, the incidence of ipsilateral non-perioperative stroke was low, and that there were no significant differences in the incidence of ipsilateral or any non-perioperative stroke between the two groups.(Ederle *et al*, 2009a)

A small, single-centre study, comparing the outcome after carotid endarterectomy with carotid angioplasty and stenting, looked at patients with recent TIA or stroke in the arterial territory supplied by a > 70% ICA stenosis within the preceding 3 months, who were randomly allocated to undergo CEA (n = 51) or carotid stenting (n = 53).(Brooks *et al*, 2001) It is important to note that the majority of patients in each treatment arm had TIA 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 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.

In the SAPPHIRE study, investigators at 29 international centres performed a randomised trial comparing carotid-artery stenting with the use of an emboli-protection device with carotid endarterectomy in 334 patients with coexisting conditions that potentially increased the risk of endarterectomy, including clinically significant cardiac or pulmonary disease, contralateral carotid occlusion or restenosis after endarterectomy.(Yadav *et al*, 2004b) Subjects had either ≥ 50% symptomatic or ≥ 80% asymptomatic stenosis, and the study was designed to assess whether endovascular treatment was 'non-inferior' to endarterectomy. 29.9% of patients in the stenting group and 27.7% of patients in the endarterectomy group had symptomatic stenoses. The primary end point was the cumulative incidence of any major cardiovascular event at 1

year (composite of stroke, death, or MI at 30 days, plus ipsilateral stroke or death between 31 days and 1 year).(Yadav *et al*, 2004b)

In the Intention to Treat (ITT) analysis, the composite primary end point occurred in 12.0% of the stenting and 20.1% of the CEA group (p=0.048). This difference was mainly as a result of the difference in the incidence of MI at 30 days (3% in the stenting group vs. 7.5% in the CEA group; p = 0.07). In the periprocedural period (up to 30 days), the cumulative incidence of stroke, MI, or death was 4.8% in the stenting group and 9.8% in the CEA group in the ITT analysis (p=0.09).(Yadav *et al*, 2004b) At one year, carotid revascularisation was repeated in fewer patients who had received stents than in those who had undergone endarterectomy (0.6 % vs. 4.3 %; intention-to-treat analysis: p = 0.04). The investigators concluded that carotid stenting in this patient population with the use of an emboli-protection device was not inferior to carotid endarterectomy. This was the first study to systematically use emboli-protection devices during stenting, and they were successfully employed in 95.6% of stenting cases.(Yadav *et al*, 2004b)

In a subsequent analysis of the 3 year results from SAPPHIRE, the major secondary end point i.e. the composite risk of stroke, MI or death within 30 days after the procedure, or ipsilateral stroke or death between 31 days and 3 years after the procedure occurred in 24.6% of patients in the stenting group and 26.9% of patients in the endarterectomy group (p = 0.71).(Gurm *et al*, 2008) There were a total of 15 strokes in each of the two groups, 11 ipsilateral (2 major, 9 minor) in the stenting group, and 9 ipsilateral (5 major, 4 minor) in the endarterectomy group. Overall, the investigators found no significant difference in long-term outcomes between patients who underwent carotid artery stenting with an emboli-protection device and those who underwent endarterectomy.

EVA-3S was a multicenter, randomised, non-inferiority trial comparing carotid stenting with endarterectomy in patients with ≥60% symptomatic carotid stenosis.(Mas *et al*,

2006) The use of emboli-protection devices was not mandatory according to the protocol. The primary end point was any stroke or death within 30 days of treatment. The trial was stopped prematurely after inclusion of 527 patients for reasons of "safety and futility". The 30-day incidence of any stroke or death was 9.6% after stenting (95% confidence interval [CI] 6.4-14.0) *vs.* 3.9% in the endarterectomy group (95% CI 2.0-7.2; relative risk of any stroke or death after stenting versus endarterectomy = 2.5; 95% CI 1.2-5.1). The 30-day incidence of disabling stroke or death was 3.4% after stenting (95% CI 1.7-6.7) *vs.* 1.5% after endarterectomy (95% CI 0.5-4.2; relative risk = 2.2; 95% CI 0.7-7.2). At 6 months, the incidence of any stroke or death was 11.7% after stenting and 6.1% after endarterectomy ($p = 0.02$).

91.9% of all patients who underwent stenting had a cerebral protection device used. The relative risk of stroke or death from stenting versus endarterectomy did not differ significantly before systematic use of a cerebral protection device was recommended in the trial (RR = 2.0; 95% CI 0.8-5.0) compared with after (RR = 3.4; 95% CI 1.1-10.0; $p=0.50$). There were more major local complications after stenting (3.1% versus 1.2%) and more systemic complications after endarterectomy, mainly pulmonary (3.1% versus 1.9%), but neither differences were significant. Cranial-nerve injury was more common after endarterectomy than after stenting (7.7% versus 1.1%; $p < 0.001$), and the use of antiplatelet agents was more frequent in the stenting than the endarterectomy group, both pre- and post-procedurally (52.5% *vs.* 49.0%; $p=0.43$). Overall, the investigators found that the risk of stroke and death at 1 and 6 months was lower with endarterectomy than with stenting ($p\leq0.01$). The authors reported that the trial involved centres with varying degrees of experience in carotid stenting, including investigators treating subjects under supervision. However, they did not find any significant differences in outcome related to

the number of stenting procedures performed in individual centres or to the experience of the interventionalists.(Mas *et al*, 2006)

A four-year follow-up analysis found that the cumulative probability of periprocedural stroke or death, and non-procedural ipsilateral stroke after 4 years of follow-up was higher with stenting than with endarterectomy (11.1% versus 6.2%; p = 0.03), with these differences largely accounted for by the higher periprocedural risk within 30 days of stenting compared with endarterectomy.(Mas *et al*, 2008) After the periprocedural period (>31 days post procedure), the 4-year risk of ipsilateral stroke was relatively low (1.26% in the stenting group and 1.97% in the CEA group; HR = 0.75; 0.17–3.37). The authors concluded that these data suggested that carotid stenting may be as effective as endarterectomy in the medium-term prevention of ipsilateral stroke, but the safety of carotid stenting in the periprocedural period must be improved to reduce the risk of complications in the initial peri-procedural period.

The SPACE Study was another non-inferiority, randomised trial conducted in 35 centres in Germany, Austria and Switzerland which investigated whether carotid stenting was non-inferior to endarterectomy.(Ringleb *et al*, 2004;Ringleb *et al*, 2006) 1200 patients with symptomatic carotid stenosis were randomly assigned within 180 days of TIA or moderate stroke (modified Rankin scale score $\leq$ 3) to undergo carotid artery stenting (n=605) or endarterectomy (n=595); emboli-protection devices were used in 27% of stenting cases. The primary endpoint was ipsilateral ischaemic stroke or death from time of randomisation to 30 days after the procedure. The null hypothesis was that the difference between the event rates in the stenting group and the CEA group would be $\geq$2·5% (the 'non-inferiority margin').(Ringleb *et al*, 2006) This study was terminated early after interim analysis showed that a much larger sample size would be needed to prove equivalence, and funding was not available to support further recruitment. The

incidence of ipsilateral ischaemic stroke or death from randomisation to 30 days after the procedure was 6.84% in the stenting group and 6.34% with carotid endarterectomy (one-sided p value for non-inferiority = 0.09, indicating a 9% chance of concluding that carotid stenting was 'not inferior' to endarterectomy). Based on the pre-planned power calculations, this study failed to prove non-inferiority of stenting compared with endarterectomy for the 30-day complication rate. The authors concluded that these data did not justify the routine use of carotid stenting rather than carotid endarterectomy for the treatment of carotid artery stenoses.(Ringleb *et al*, 2006) An editorial subsequently commented that SPACE did not provide evidence to support stenting over CEA or vice versa.(Naylor, 2006)

2-year follow up data from SPACE showed that in both the intention-to-treat and per-protocol analyses, estimates of ipsilateral ischaemic stroke incidence up to 2 years after the procedure, and any periprocedural stroke or death did not differ between the carotid stenting and endarterectomy groups (intention to treat analysis: 9.5% versus 8.8%; p = 0.62; per protocol analysis: 9.4% versus 7.8%; p = 0.31).(Eckstein *et al*, 2008) However, recurrent ≥70% stenosis based on ultrasound criteria alone was significantly more frequent in the stenting than in the endarterectomy group (10.7% versus 4.6% in the ITT analysis (p=0.0009).(Eckstein *et al*, 2008) The authors acknowledged that the degree of in-stent stenosis may have been slightly overestimated by conventional ultrasound criteria. However, these data raise concerns about the long-term durability of modern carotid stenting techniques, especially if carotid restenosis predisposes to a higher risk of recurrent TIA or stroke during long-term follow up.(Bonati *et al*, 2009) A 2009 Cochrane systematic review of 10 trials involving 3178 patients commented that the data were difficult to interpret due to trial heterogeneity.(Ederle *et al*, 2009b) The authors concluded that the results do not support a change in clinical practice away from

recommending CEA as the treatment of choice for suitable carotid artery stenosis, but overall supported continued recruitment into large ongoing clinical trials.(Ederle *et al*, 2009b)

An interim safety analysis of data from the International Carotid Stenting Study (ICSS) has been recently published. ICSS was a multicentre, randomised controlled trial with blinded adjudication of outcomes in patients with recently symptomatic (<12 months before randomisation) moderate or severe carotid artery stenosis (Ederle *et al*, 2010). The primary outcome measure is the 3-year incidence of fatal or disabling stroke in any territory, but these data have not yet been analysed. The main outcome measure for the interim safety analysis was the 120-day rate of stroke, death, or peri-procedural myocardial infarction. The trial enrolled 1713 patients (stenting group, n = 855; endarterectomy group, n = 858). Between randomisation and 120 days, there were 34 (4.0%) disabling strokes or deaths in the stenting group versus 27 (3.2%) events in the endarterectomy group (Hazard Ratio [HR] = 1.28, 95% CI 0.77-2.11; p=0.34). The incidence of stroke, death, or peri-procedural MI was 8.5% in the stenting group versus 5.2% in the endarterectomy group (p = 0.006). The incidence of any stroke or death was higher in the stenting group than in the endarterectomy group (8.5% *vs*. 4.7%; p=0.001). Three peri-procedural MIs were recorded in the stenting group, all of which were fatal, compared with four, all non-fatal, in the endarterectomy group. There was one (0.1%) cranial nerve palsy in the stenting group compared with 45 (5%) in the endarterectomy group (p<0·0001).(Ederle *et al*, 2010) Completion of follow-up is required to establish the long-term efficacy of carotid artery stenting compared with endarterectomy in ICSS.

An imaging substudy of ICSS compared the incidence of new ischaemic brain lesions on MRI between the two study groups.(Bonati *et al*, 2010b) Among 231 patients with data available for analysis, 50% of patients in the stenting group and 17% of patients in the

endarterectomy group had at least one new DWI lesion detected on scans performed at a median of 1 day after treatment ($p<0.0001$). At 1 month, there were changes on fluid-attenuated inversion recovery (FLAIR) sequences in 33% of patients in the stenting group *vs.* 8% of patients in the endarterectomy group ($p=0.0003$). This indicates that new ischaemic lesions on DWI were approximately three times more frequent after carotid stenting than endarterectomy.(Bonati *et al*, 2010b) In patients treated with cerebral protection devices (CPDs), 73% in the stenting group and 17% in the CEA group had at least one new DWI lesion on post-treatment scans (adjusted OR=12.20), whereas in patients treated without CPDs, 34% in the stenting group and 16% in the CEA group had new DWI lesions (adjusted OR=2.70; $p=0.019$). These data indicated that emboli-protection devices did not appear to be effective in preventing cerebral ischaemia during stenting.(Bonati *et al*, 2010b)

The Carotid Revascularization Endarterectomy vs. Stenting (CREST) trial randomly assigned patients with symptomatic or asymptomatic carotid stenosis to undergo either carotid artery stenting (n=1262) or carotid endarterectomy (n=1240).(Brott *et al*, 2010) The primary composite end-point included peri-procedural stroke, MI, or death from any cause, or any ipsilateral stroke within 4 years after randomisation. Secondary outcome measures included any peri-procedural stroke or death or post-procedural ipsilateral stroke or MI. 47.1% of subjects in the stenting group and 47.3% in the endarterectomy group were asymptomatic, and a similar proportion in each treatment arm had contralateral occlusion. Most patients in each treatment group were on antiplatelet therapy before (97.7% in the stenting group and 92.1% in the CEA group) and after the procedure (99.0% in the stenting group and 91.1% in the CEA group).(Brott *et al*, 2010)

At least 48 hours before stenting, patients received aspirin 325mg twice daily and clopidogrel 75mg twice daily. When stenting was scheduled for within 48 hours after

randomisation, 650mg aspirin and 450mg clopidogrel were given ≥4 hours before the procedure. After stenting, patients received one or two 325mg doses of aspirin daily for 30 days, and either clopidogrel 75 mg daily or ticlopidine 250 mg twice daily for 4 weeks. Antiplatelet therapy was continued for at least 4 weeks in all post-stenting patients. In the CEA group, at least 48 hours before the procedure, patients received 325mg aspirin daily and continued that dose for ≥ 1 year. Alternatives to this regimen were ticlopidine 250mg twice daily, clopidogrel 75mg daily, aspirin 81mg daily, or aspirin and extended-release dipyridamole twice daily. The incidence of peri-procedural stroke was higher (4.1% vs. 2.3%; p=0.01), whereas the incidence of peri-procedural MI was lower (1.1% vs. 2.3%; p=0.03) in the stenting than in the CEA group. After this initial 30-day period, the incidence of ipsilateral stroke in the stenting and CEA groups was similar during follow up over a median duration of 2.5 years (2.0% versus 2.4%, p = 0.85). There was no significant difference in the 'estimated' 4-year incidence of the primary composite endpoint between the stenting and CEA groups (7.2% versus 6.8%, respectively; p = 0.51). The 4-year incidence of stroke or death was 6.4% in the entire stenting group and 4.7% in the entire CEA group (p = 0.03). The 4-year incidence of stroke or death was 8.0% in the symptomatic stenting group *vs.* 6.4% in the symptomatic CEA group (p = 0.14), and 4.5% in the asymptomatic stenting group *vs.* 2.7% in the asymptomatic CEA group (p = 0.07).(Brott *et al*, 2010) A subsequent analysis of data by the CREST investigators revealed a slightly higher incidence of the periprocedural primary composite endpoint in women undergoing carotid stenting versus CEA (8·9% *vs.* 6·7% in women (HR = 1·35, 95% CI 0·82–2·23), with no significant difference seen in men.(Howard *et al*, 2011) The results of CREST are broadly in line with results from other large, modern randomised trials assessing carotid artery stenting versus endarterectomy for the treatment of carotid stenosis (Davis & Donnan, 2010).

There are recent data suggesting that the use of stenting for symptomatic carotid stenosis should be avoided in patients ≥70 years of age, with the estimated 120-day risk of stroke or death with carotid stenting twice that with endarterectomy (12.0% vs 5.9%; p=0·0053) in pooled analyses of data from EVA-3S, SPACE and ICSS.(Bonati *et al*, 2010a);(Bonati & Fraedrich, 2011)

A recent meta-analysis of 11 randomized trials up to 2009 (excluding CREST) showed that CEA was associated with reduced risk of periprocedural stroke or death compared with carotid stenting (p=0.025).(Meier *et al*, 2010) Until longer term clinical and restenosis data are available from EVA-3S, SPACE, ICSS and CREST, and until one sees whether carotid restenosis is more common in all modern stenting studies than after endarterectomy, carotid surgery remains the preferred treatment for most patients with symptomatic carotid stenosis.(Ederle *et al*, 2010);(Ederle *et al*, 2009b) However, based on clinical experience rather than data from large clinical trials, it is still reasonable to consider carotid endovascular treatment as an empirical treatment of first choice in selected patients with e.g. symptomatic carotid restenosis after prior endarterectomy, radiotherapy-induced carotid stenosis, and in patients with carotid lesions that are not accessible for surgery.(Ringleb *et al*, 2010;Marquardt & Barnett, 2011) Optimal timing of acute or 'hyperacute' intervention in cases of recently symptomatic carotid stenosis will also need to be critically appraised in future large trials, along with the impact of operator experience.(Marquardt & Barnett, 2011;Naylor & Ricco, 2011)

2. Platelet activation, function and reactivity in atherosclerotic carotid artery stenosis: A systematic review of the literature

2.1. Introduction

Approximately 25-30% of all TIAs or ischaemic strokes may be attributed to thromboembolism from, or less commonly from haemodynamic ischaemia distal to a moderate or severe (50 – 99%) atherosclerotic carotid stenosis or occlusion.(Petty *et al*, 1999;Golledge *et al*, 2000;Ringelstein *et al*, 1988)

Prior studies have indicated that the two-year risk of stroke in the arterial territory supplied by a ≥ 60% internal carotid artery (ICA) stenosis is approximately 4% in 'asymptomatic' medically-treated patients in whom the stenosis is incidentally picked up. (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995);(The European Carotid Surgery Trialists Collaborative Group, 1995) More recent data have suggested that the 2-year risk of stroke may have fallen to approximately 0.68% in patients with asymptomatic ≥ 50% ICA stenosis receiving modern secondary preventive treatment.(Marquardt *et al*, 2010) However, additional information from transcranial Doppler ultrasound (TCD) monitoring may aid risk stratification and treatment decisions regarding carotid intervention in patients with asymptomatic carotid stenosis.(Markus *et al*, 2010), (Spence *et al*, 2010) The recently published Asymptomatic Carotid Emboli Study (ACES) indicated that the 2-year risk of ipsilateral stroke in patients with asymptomatic ≥70% carotid stenosis who have microembolic signals (MES) detected on transcranial Doppler ultrasound was 3.6%.(Markus *et al*, 2010) In contrast, the two-year risk of ipsilateral stroke may be as high as 26% in medically-treated patients with 'symptomatic' severe (≥ 70%) carotid stenosis who have had a recent TIA or stroke.

(North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991) The recent 'Clopidogrel plus aspirin versus aspirin alone for reducing embolisation in patients with acute symptomatic cerebral or carotid artery stenosis (CLAIR) study revealed that therapy with clopidogrel and aspirin is more effective than aspirin alone in reducing MES in patients with predominantly intracranial symptomatic stenosis.(Wong *et al*, 2010) Recent studies have illustrated the potential role of MES detection and carotid plaque imaging in risk-stratifying asymptomatic carotid stenosis patients to identify those who may derive most benefit from best medical rather than surgical therapy.(Madani *et al*, 2011;Spence *et al*, 2005;Topakian *et al*, 2011) However, the potential usefulness of adding specific platelet activation and /or reactivity data to these investigations to guide treatment decisions in patients with carotid stenosis is uncertain.

The mechanisms responsible for this disparity in stroke risk between asymptomatic and symptomatic severe carotid stenosis patients are not fully understood, but may relate to differences in the morphology of the atherosclerotic plaque, differences in endothelial activation, or differences in the thrombogenicity of the circulating blood.(McCabe *et al*, 2005b) Histological studies indicate that the carotid plaque is composed of a dense 'cap' composed of connective tissue embedded with smooth muscle cells, with varying amounts of central plaque lipid and necrotic inflammatory debris.(Ross, 1993) Compared with asymptomatic carotid artery plaques, symptomatic carotid plaques are reported to have thinner, less fibrous caps, infiltrated with foamy macrophages and T cells that are more prone to rupture or ulceration,(Golledge *et al*, 2000;Redgrave *et al*, 2006) and to contain a central, lipid-rich necrotic core which may be closer to the cap.(Bassiouny *et al*, 1997) Several studies have suggested that the prevalence of intraplaque haemorrhage, or thrombus adherent to the plaque surface is similar in symptomatic and asymptomatic carotid stenosis patients,(Golledge *et al*, 2000;Bassiouny *et al*, 1989;Feeley *et al*,

1991;Carr *et al*, 1996) although one study found that fibrous cap thinning, plaque rupture, infiltration of the cap with foam cells, and intraplaque fibrin was more common in symptomatic than asymptomatic patients.(Carr *et al*, 1996)

Inflammation and instability in recently symptomatic carotid plaques is strongly associated with macrophage infiltration, with plaque instability and rupture more frequently seen with a critical plaque cap thickness of < 200 μm.(Redgrave *et al*, 2006),(Redgrave *et al*, 2008) Non-invasive carotid ultrasound studies have also suggested that symptomatic plaques tend to be more echolucent or hypoechoic, assumed to be secondary to a larger lipid-rich core, whereas asymptomatic plaques are more often echodense with smaller lipid cores on B-mode ultrasound.(Gronholdt, 1999;Tegos *et al*, 2000;Tegos *et al*, 2001b)

Although activation of the endothelium and coagulation system may contribute to pathogenesis of symptoms in patients with carotid stenosis,(With Noto *et al*, 2008) (With Noto *et al*, 2006) platelets also play a pivotal role in arterial thrombosis and thromboembolism. Exposure to high shear stress, as might occur in response to turbulent flow of blood through a stenosed artery, may activate platelets,(Merten *et al*, 2000) promote platelet hyperreactivity, and potentially increase the risk of subsequent thrombosis and thromboembolism from a stenosing carotid plaque. Platelets may also play a role in the progression of atherosclerosis,(Pitsilos *et al*, 2003) and lipid-rich carotid atheromatous plaques may stimulate monocyte-platelet complex formation and platelet–dependant thrombus formation *in vitro*.(Penz *et al*, 2005) Increased expression of the endothelial adhesion molecule, P-selectin (CD62P),(Schumacher *et al*, 2001) and of Platelet Factor 4 (PF4)- a chemokine released by activated platelets,(Pitsilos *et al*, 2003) in symptomatic versus asymptomatic carotid plaques, suggests that persistent platelet activation may contribute to the evolution of atherosclerotic lesions.

To investigate the potential pathogenic role of platelet activation and function in patients with carotid stenosis, and the potential for accessible platelet biomarkers to improve risk stratification of carotid stenosis patients, we performed a systematic review of the literature to critically assess available data on *ex vivo* platelet activation and platelet function/reactivity in blood in this patient population. The main aims of this review were to consolidate available data, and to stimulate further research in this important area of translational platelet science, vascular neurology and stroke medicine.

2.2. Methods

A systematic review of the literature was performed by searching Pubmed, Medline, Ovid, Embase and Web of Science / Web of Knowledge for human studies on platelet activation or platelet function in symptomatic or asymptomatic carotid stenosis published in the English language between 1975 – August 2011. The following search terms were used: 'carotid stenosis' and 'platelet activation', 'platelet function', 'platelet reactivity', 'platelet aggregation', 'flow cytometry', We excluded case reports, case series and review articles. One author (JAK) personally read all abstracts and articles returned, hand-searched the reference lists of published articles and relevant PhD theses in our department, and identified all papers suitable for inclusion in this review. After searching through all databases, 1,467 articles and one relevant PhD thesis were retrieved. The articles were categorised *a priori* according to whether they compared data from patients with symptomatic or asymptomatic carotid stenosis versus controls or other TIA or stroke subtypes, from patients with symptomatic versus asymptomatic carotid stenosis, or from symptomatic or asymptomatic carotid stenosis patients undergoing intervention. After critical review of all manuscripts, 213 articles relating to assessment of platelet activation and / or function in patients with cerebrovascular disease were identified, and 26 were deemed suitable for inclusion in this systematic review. We subsequently categorised the evidence within these subject groups according to the method of assessment of platelet activation and function *ex vivo*.

2.3. Symptomatic Carotid Artery Stenosis versus Controls

2.3.1. Platelet activation

Urinary (Uyama *et al*, 1992) and soluble plasma markers of platelet activation (Frijns *et al*, 1997;Hashimoto *et al*, 2003;McCabe *et al*, 2004b;Balla *et al*, 2006;Blake *et al*, 2003;Shah *et al*, 1985;Jurk *et al*, 2010) have been studied in patients with symptomatic carotid artery disease. Another study assessed urinary markers of platelet activation in patients with carotid stenosis undergoing endovascular intervention, but symptomatic status or degree of stenosis was not mentioned.(Cavalca V *et al*, 2010)

Urinary markers

Urinary 11-dehydrothromboxane B_2 levels were increased in patients with chronic cerebral infarction with "distinct" carotid atherosclerotic lesions (n = 6; degree of stenosis not reported) compared with controls (n = 44), or compared with patients with chronic cerebral infarction without distinct carotid atherosclerotic lesions (n = 6; $p < 0.01$).(Uyama *et al*, 1992) Another recent study found elevated creatinine-corrected urinary levels of 11-dehydrothromboxane B2 in subjects with carotid stenosis (n = 15) versus healthy controls with no stenosis (n = 20; absolute values not given; $p = 0.01$).(Cavalca V *et al*, 2010) These data do not inform us of the potential usefulness of urinary markers of platelet activation in patients with specific degrees of carotid stenosis.

Soluble markers (Table 2.1a)

Two studies found increased soluble P-selectin levels in patients with recently (≥ 7 days) symptomatic ≥ 30% carotid stenosis versus controls without cardiovascular disease (n = 34 for each group, $p < 0.0001$),(Frijns *et al*, 1997) and in symptomatic patients with ≥

50% carotid stenosis compared with those with < 50% carotid stenosis (n = 27 *vs.* 274; p < 0.05).(Hashimoto *et al*, 2003) Although these data suggested that sP-selectin levels reflect enhanced platelet activation in recently symptomatic patients compared with controls, a further single centre study did not reveal elevated sP-selectin levels in either the early or late phase after TIA or stroke onset associated with ≥ 70% carotid stenosis compared with controls (n = 19 early phase patients, and 16 late phase patients *vs.* 27 controls, P ≥ 0.09).(McCabe *et al*, 2004a) It is important to note that sP-selectin is generated by platelets and endothelial cells, so is not a specific marker of platelet activation.(McCabe *et al*, 2004a)

Balla *et al*. assessed soluble serum CD40 and soluble CD40 ligand (CD40L) levels in 60 patients with carotid artery disease (16 ICA occlusions; 44 with > 30% ICA stenosis) and 30 age- and sex-matched controls without carotid plaques.(Balla *et al*, 2006) Soluble CD40 levels did not differ between patients and controls (p = 0.34), but CD40L levels were higher in patients than controls (6.9 vs. 4.5 ng/mL, p = 0.038).

Another imaging-based study demonstrated elevated soluble CD40L levels in 49 patients with ≥ 30% CCA or ICA stenosis who had increased intraplaque lipid on high-resolution MRI compared with those who did not have these plaque characteristics (p < 0.02).(Blake *et al*, 2003) Thirteen of these patients were 'symptomatic', but the proportion who were 'recently symptomatic' was unclear.

An earlier study demonstrated increased β-thromboglobulin (p < 0.001) and platelet factor-4 levels (p < 0.05) in 'thromboembolic TIA or stroke' patients versus controls; 10 patients (77%) had 'internal carotid artery stenosis or occlusion', but stenosis severity was not specified, and levels in the subgroup of patients with carotid stenosis were not specified.(Shah *et al*, 1985)

A recent case-control study by Jurk *et al.* of 25 patients with acute (< 4 days) symptomatic >70% carotid stenosis, 48 patients with asymptomatic >50% carotid stenosis, and 30 age-matched healthy controls, found that soluble P-selectin ($P < 0.05$) and CD40L levels were higher in symptomatic patients than controls (2.99 *vs* 1.16 ng/ml; $p < 0.05$).(Jurk *et al*, 2010)

Surface markers (Table 2.1b)

More recent studies have employed the sensitive and specific technique of flow cytometry to assess platelet activation (CD62P, CD63 and CD40L expression, and leucocyte-platelet complexes) in platelet rich plasma (PRP) (Zeller *et al*, 1999;Yip *et al*, 2006) or whole blood (Cha *et al*, 2003;Liu *et al*, 2009;McCabe *et al*, 2004a;Jurk *et al*, 2010) in symptomatic carotid stenosis patients versus controls.

Zeller *et al* found elevated platelet surface CD62P ($p < 0.001$) and CD63 ($p = 0.003$) in 47 patients within 36 hours of symptom onset in association with symptomatic >50% carotid artery stenosis/carotid occlusion ($n = 34$; exact proportion with moderate or severe carotid stenosis alone not specified), and vertebral or basilar artery stenosis ($n = 13$) versus controls.(Zeller *et al*, 1999) Yip *et al* found elevated CD62P in patients at least 2 months after TIA or stroke associated with ≥ 70% symptomatic ICA stenosis compared with 'at-risk controls' and 'healthy volunteers' ($p < 0.0001$).(Yip *et al*, 2006) Another whole blood flow cytometry study of 25 patients with acute (< 24 hours) 'atherosclerotic ischaemic stroke' (proportion with > 50% carotid stenosis not specified) found elevated platelet surface CD62P, CD63 and CD40L ($p < 0.01$) levels compared with healthy controls (N=24).(Cha *et al*, 2003) Subgroup data from a further longitudinal, case-control whole blood flow cytometry study revealed that CD62P levels were not increased within 4 weeks, but were significantly higher in patients with severe symptomatic carotid

stenosis ≥3 months after TIA or stroke onset than in controls (P = 0.01).(McCabe *et al*, 2004a) However, the authors did not find higher levels of other platelet activation markers (CD63, PAC1, or leucocyte-platelet complexes) in patients than controls, although this study was not powered to look at individual TIA or stroke subtypes.(McCabe *et al*, 2004a) Jurk *et al*. found that CD62P ($p < 0.05$), CD63 ($p < 0.05$), circulating monocyte-platelet (43.3% *vs*. 17.4%, $p < 0.01$) and neutrophil-platelet complexes (22.4% *vs*. 10.3%, $p < 0.05$) were elevated in recently symptomatic severe carotid stenosis patients versus controls.(Jurk *et al*, 2010)

2.3.2. Platelet function (Table 2.1c)

One pilot substudy assessed *ex vivo* inhibition of platelet function with aspirin monotherapy on a platelet function analyser, called the PFA-100, with both collagen-ADP (C-ADP) and collagen-epinephrine (C-EPI) cartridges in 11 patients in the early phase (≤ 4 weeks) and 9 in the late phase ≥ 3 months) after TIA or stroke secondary to ≥ 70% carotid stenosis; data were compared with those from 23 healthy controls who were not on aspirin.(McCabe *et al*, 2005a) Prior data from healthy controls have indicated that C-EPI closure times are increased in 83-100% of healthy controls on aspirin.(Kundu *et al*, 1994;Harrison *et al*, 1999;Mammen *et al*, 1995;Mammen *et al*, 1998) Median collagen-epinephrine (C-EPI) closure times were not significantly prolonged in the early phase ($p = 0.3$), but were prolonged in the late phase after symptom onset compared with controls ($p < 0.001$). Seven of 11 (64%) patients in the early phase, and 1/9 (11.1%) in the late phase had high 'on-treatment platelet reactivity' / 'aspirin non-responsiveness' on the PFA-100.(McCabe *et al*, 2005a) However, six of these nine patients in the late phase had undergone carotid endarterectomy or stenting before repeat testing, potentially

explaining in part why the vast majority of these patients subsequently became more responsive to aspirin *ex vivo*.

2.4. Asymptomatic Carotid Artery Stenosis versus Controls

2.4.1. Platelet activation

Three studies compared soluble markers,(Novo *et al*, 2005;Balla *et al*, 2006;Enomoto *et al*, 2010) one compared surface markers,(Cha *et al*, 2003) and 2 compared both soluble and surface markers of platelet activation (Stolz *et al*, 2002;Jurk *et al*, 2010) between asymptomatic carotid stenosis patients and healthy controls.

Soluble markers (Table 2.2)

Elevated soluble CD40L levels ($p = 0.038$), but not soluble CD40 levels ($p = 0.34$) were identified in 60 patients with > 30% carotid stenosis or occlusion who had not had a TIA or stroke within the preceding 6 months compared with 30 healthy controls. (Balla *et al*, 2006) Elevated soluble CD40L levels were reported in patients with < 50% asymptomatic carotid stenosis compared with healthy controls ($p < 0.0001$).(Novo *et al*, 2005) Stolz *et al* also found elevated soluble P-selectin levels in a pilot study of patients with asymptomatic >70% carotid stenosis compared with controls ($p < 0.01$).(Stolz *et al*, 2002) Another pilot study revealed elevated soluble CD40L ($p < 0.01$) and 11-dehydro-TXB2 ($p < 0.05$) levels in PRP in 10 patients with >50% asymptomatic carotid stenosis versus 8 patients without carotid stenosis. (Enomoto *et al*, 2010) A further study did not reveal any difference in soluble P-selectin or soluble CD40L levels between asymptomatic carotid stenosis patients and controls.(Jurk *et al*, 2010)

Surface markers (Table 2.3)

In keeping with their pilot sP-selectin data outlined above, Stolz *et al* found elevated platelet surface CD62P expression in 19 patients with asymptomatic > 70% carotid

stenosis ($p < 0.001$) and 11 patients with asymptomatic < 70% carotid stenosis (mean stenosis severity 26 ± 20%; $p < 0.01$) versus healthy controls (n = 13).(Stolz *et al*, 2002) CD62P expression was also higher in patients with > 70% than those with < 70% asymptomatic carotid stenosis ($p < 0.001$). A whole blood flow cytometry study reported that patients with asymptomatic > 50% carotid stenosis (n = 20) had higher CD62P and CD63 expression ($p < 0.01$), but not CD40L expression than healthy controls (n = 24).(Cha *et al*, 2003) However, Jurk *et al*. did not find any differences in the expression of surface CD62P and CD63 between asymptomatic carotid stenosis patients and controls.(Jurk *et al*, 2010)

2.5. Symptomatic versus Asymptomatic Carotid Stenosis

One study compared soluble markers (Jurk *et al*, 2010) and three studies directly compared platelet surface activation marker expression with whole blood flow cytometry in symptomatic versus asymptomatic carotid stenosis patients. (Cha *et al*, 2003;McCabe *et al*, 2005b;Jurk *et al*, 2010)

2.5.1. Platelet activation

Soluble markers (Table 2.4)

Jurk *et al*. found elevated soluble P-selectin levels ($p < 0.05$, absolute values not given), but not CD40L levels (2.99 *vs* 1.49 ng/ml; $p > 0.05$) in symptomatic severe versus asymptomatic >50% carotid stenosis patients. (Jurk *et al*, 2010)

Surface Markers (Table 2.5)

Platelet surface CD40L expression was found to be higher in patients with 'acute large artery atherosclerotic ischaemic stroke' than in patients with asymptomatic carotid stenosis ($p < 0.01$), but there were no differences in CD62P or CD63 expression between the groups.(Cha *et al*, 2003) However, the authors did not specify whether the symptomatic ischaemic cerebrovascular event occurred in the territory of a carotid, vertebral or intracranial stenosis.

Another pilot, longitudinal study identified an increased platelet count in both the early and late phases after TIA or stroke in patients with ≥ 70% symptomatic carotid stenosis compared with those with asymptomatic severe carotid stenosis ($p \leq 0.05$); 8 of 16 patients had undergone successful carotid endarterectomy or stenting by the late stage of follow up.(McCabe *et al*, 2005b) Circulating neutrophil-platelet, monocyte-platelet and

lymphocyte-platelet complexes were higher in the early ($p \leq 0.046$), but not late phase after symptoms in symptomatic than asymptomatic patients.(McCabe *et al*, 2005b) However, there were no differences in CD62P, CD63 or PAC-1 expression at any time point between the groups. Jurk *et al*. did find higher CD62P and CD63 expression in symptomatic severe compared with asymptomatic > 50% carotid stenosis patients ($p < 0.05$; absolute values not given).(Jurk *et al*, 2010)

2.5.2. Platelet Function

There are no data directly comparing platelet function in whole blood in patients with symptomatic versus asymptomatic carotid stenosis.

2.6. Symptomatic and Asymptomatic Carotid Artery Stenosis Patients undergoing Carotid or Pharmacological Intervention

Four studies assessed unstimulated platelet activation status,(Robless *et al*, 2002)-(Vogten *et al*, 2008) and five assessed inducible platelet activation/reactivity with flow cytometry or platelet function testing in symptomatic carotid stenosis patients undergoing carotid endarterectomy (CEA).(Robless *et al*, 2002;Hayes *et al*, 2003;Assadian *et al*, 2008;Webster *et al*, 2004;Payne *et al*, 2004) Three studies assessed platelet aggregometry in carotid stenosis patients around the time of carotid intervention.(Payne *et al*, 2004;Vogten *et al*, 2008);(Szapary *et al*, 2009) Two recent studies assessed platelet aggregometry in symptomatic carotid stenosis patients after alteration of antiplatelet therapy.(King *et al*, 2011;Markus *et al*, 2005)

Unstimulated platelet activation (Table 2.6a)

Compared with levels at the time of initial skin incision and 1 and 24 hours post-operatively, platelet surface CD62P expression in whole blood temporarily increased after carotid dissection ($p < 0.01$) and immediately after cross-clamp removal ($p < 0.05$) in 40 carotid stenosis patients who underwent CEA on aspirin monotherapy; 90% were symptomatic, but the severity of stenosis was not specified.(Robless *et al*, 2002) Platelet surface PAC-1 expression also temporarily increased after carotid dissection ($p < 0.05$) compared with levels following initial incision, and 1 and 24 hours post-operatively.

A further longitudinal study in patients with ≥ 70% symptomatic carotid stenosis found no differences in platelet surface PAC-1 binding or circulating monocyte-platelet complexes in the immediate pre-carotid stenting phase compared with three separate phases up to 6 days after carotid stenting.(Liu *et al*, 2009) A small peri-operative study in

symptomatic "high-grade" carotid stenosis patients undergoing CEA found increased CD41 ($p = 0.002$) and CD62P expression ($p < 0.001$) during surgery, but no changes in leucocyte-platelet complex formation in the peri-operative phase.(Assadian *et al*, 2008)

In a randomised controlled trial, Payne *et al*. assessed 100 carotid stenosis patients (mean degree of stenosis 80%; 84% symptomatic) immediately before carotid endarterectomy.(Payne *et al*, 2004) All patients were stabilised on 150mg of aspirin daily for 4 weeks before intervention, and were randomised to receive 75mg of clopidogrel or placebo the day before surgery. Whole blood flow cytometry was performed on all patients prior to surgery, before and after addition of clopidogrel or placebo. There was no difference in platelet fibrinogen binding between patients randomised to aspirin and clopidogrel versus aspirin and placebo.

Vogten *et al*. assessed 27 patients who were scheduled to undergo carotid endarterectomy on either aspirin monotherapy ($N = 18$) or aspirin and clopidogrel combination therapy ($N = 9$; stenosis severity and proportion of symptomatic patients not specified).(Vogten *et al*, 2008) Platelet surface CD62P expression was assessed at seven timepoints pre-operatively, intra-operatively and post-operatively; there were no differences in CD62P expression between patients on the two different antiplatelet regimens at any peri-operative timepoint.

Inducible and unstimulated platelet activation and platelet function

Assadian *et al* assessed 'inducible platelet activation' (ADP- or TRAP-6-induced platelet surface CD62P and CD41 expression), as well as unstimulated leucocyte-platelet complex (LPC) formation and the numbers of reticulated platelets with flow cytometry, along with platelet reactivity on the PFA-100 in 20 'high-grade' symptomatic ICA stenosis patients undergoing eversion CEA ≥ 3 months after symptom onset.(Assadian *et*

al, 2008) Patients received either intraoperative unfractionated heparin (UFH) or enoxaparin. ADP-induced CD62P ($p = 0.03$) and CD41 expression ($p < 0.001$), and TRAP-6-induced CD41 expression ($p = 0.002$) increased during surgery (after administration of UFH or enoxaparin, and after the anastomosis was sutured and declamped) compared with pre-operatively and 30 minutes post-operatively. However, there were no differences between the subgroups treated with UFH and enoxaparin. There were no differences in LPC formation between the immediate pre- and post-op phases in the enoxaparin and UFH groups combined. However, LPC formation was increased in the post-op compared with the pre-op phase in the UFH subgroup, and this increase was higher in the UFH subgroup than in the enoxaparin subgroup ($p = 0.034$). There was a slight decrease in platelet count ($p = 0.036$), and an increase in reticulated platelets ($p = 0.001$) during carotid endarterectomy between cross-clamping and reestablishment of blood flow through the artery. There were no differences in platelet reactivity on the PFA-100 between the immediate pre-operative phase and up to 30 minutes after completion of the surgery.(Assadian *et al*, 2008)

Hayes *et al* assessed ADP- and thrombin-induced platelet fibrinogen binding with whole blood flow cytomtery, and ADP-induced platelet aggregometry in PRP in 120 patients with carotid stenosis (severity not specified) one day prior to CEA; 90% of patients were symptomatic.(Hayes *et al*, 2003) Platelet fibrinogen binding was higher in patients with >25 versus <25 emboli detected on 3 hours of post-op transcranial Doppler Ultrasound monitoring (TCD) of the ipsilateral middle cerebral artery ($p < 0.0001$).The authors also found that ADP-induced platelet aggregation was greater in patients with >25 emboli than in those with <25 emboli detected on TCD following carotid endarterectomy ($p = 0.0012$).(Hayes *et al*, 2003)

Robless *et al* found elevated spontaneous ($p < 0.001$) and ADP-induced platelet aggregation ($p < 0.01$) in whole blood in patients at carotid clamping, and after clamp release ($p < 0.01$) versus initial incision; spontaneous platelet aggregation remained elevated up to one hour after clamp release compared with levels at the time of initial incision. ($p < 0.05$).(Robless *et al*, 2002)

A cross-sectional *ex-vivo* platelet aggregometry study in 41 patients undergoing CEA for carotid stenosis (symptomatic status and degree of stenosis not clearly specified) found that administration of a heparin bolus immediately before CEA caused abrupt and significant elevations in the degree of arachidonic acid-induced platelet aggregation in PRP ($p < 0.0001$) versus pre-operative values before heparin administration. The authors postulated that this potentially explained why some patients are at increased risk of acute vascular events during carotid endarterectomy despite apparently appropriate pre-op aspirin therapy.(Webster *et al*, 2004)

In the randomised trial alluded to above, the addition of clopidogrel to aspirin the day before CEA decreased ADP-induced platelet-fibrinogen binding on whole blood flow cytometry compared with treatment with aspirin alone (66.76% vs 75.52%; $p = 0.03$.(Payne *et al*, 2004)

Szapary *et al*. assessed 18 patients with ≥70% carotid stenosis (10 with previous TIA or stroke, 8 asymptomatic) before carotid endovascular treatment, and found elevated ADP-induced platelet aggregation 5 days following intervention compared with values measured immediately after the procedure ($p < 0.0001$).(Szapary *et al*, 2009) However, no absolute values were provided by the authors, and the number of patients in this study was very small.

Platelet function after altering antithrombotic therapy in symptomatic and asymptomatic carotid stenosis patients

King *et al.* assessed platelet aggregation in PRP in patients with recently (< 1 month) symptomatic ≥ 50% carotid stenosis who were initially on treatment with aspirin monotherapy. Unmatched data from 30 patients subsequently randomised to receive clopidogrel (300 mg loading dose, followed by 75mg daily) were compared with data from 30 patients randomised to receive 200 mg of dipyridamole MR bd in addition to aspirin. ADP- and collagen-induced platelet aggregation were similar in both groups at baseline ($P \leq 0.83$). ADP-induced aggregation was lower in the aspirin + clopidogrel than in the aspirin + dipyridamole group (86.5 vs 101.7%; $P < 0.001$), but there was no difference in collagen-induced aggregation between the groups 48 hours after commencing the relevant combination treatment. (40.6 vs 48.4%; $P = 0.06$) Longitudinal, matched data in each subgroup before and after changing treatment were not presented.

Payne *et al.* also performed platelet aggregometry in PRP in response to arachidonic acid in 100 carotid stenosis patients immediately before CEA, as outlined above.(Payne *et al*, 2004) The addition of clopidogrel to aspirin the day before CEA did not significantly influence arachidonic acid-induced platelet reactivity compared with treatment with aspirin alone (3.8 vs 4.3; $p = 0.51$).

Vogten *et al.* found that baseline arachidonic acid-induced platelet aggregation was higher in their carotid stenosis patients, described above, who were on aspirin monotherapy than in those on aspirin and clopidogrel combination therapy (14.5% *vs.* 10.3%; $p < 0.05$).(Vogten *et al*, 2008) Five minutes after administration of heparin and 3 minutes before carotid artery clamping, arachidonic acid-induced platelet aggregation increased to 19.7% in the aspirin group, and 22.5% in the aspirin and clopidogrel group

versus baseline pre-operative arachidonic acid-induced platelet aggregation (10.7%; $p < 0.01$ and 7.5%; $p < 0.05$, respectively). In the 24-hour period following surgery, platelet aggregation gradually returned to pre-operative values in both groups. ADP-induced platelet aggregation did not change significantly in response to surgery or heparin administration.

The Clopidogrel and Aspirin for Reduction of Emboli in Symptomatic Carotid Stenosis (CARESS) Study, was a randomised, double-blind study in patients with recently (<3 months) symptomatic ≥50% carotid stenosis.(Markus *et al*, 2005) All patients had transcranial Doppler ultrasound monitoring, and if cerebral microembolic signals were detected, they were randomised to receive either aspirin monotherapy or aspirin and clopidogrel combination therapy for 7 days. Collagen-induced platelet aggregation was performed in PRP at baseline and at day 7 following randomisation. The 'mean maximum intensity of platelet aggregation' was 106.7% of baseline at day 7 in the aspirin monotherapy group (n – 40), and 70.9% of baseline in the aspirin and clopidogrel group (N = 31); implying that the addition of clopidogrel resulted in a significant reduction in collagen-induced platelet aggregation over aspirin alone.

2.7. Discussion

This review illustrates that relatively few studies have assessed platelet activation or platelet function / reactivity in patients with symptomatic or asymptomatic carotid artery stenosis. The existing data on indirect urinary markers or soluble plasma markers of platelet activation do not provide convincing, reproducible evidence of excessive platelet activation that is of potential clinical relevance to the modern-day management of patients with well-categorised asymptomatic and symptomatic carotid stenosis, in whom stenosis severity markedly influences treatment decisions.

Most, but not all, small-medium case-control studies included in this review have indicated that patients with mild, moderate or severe symptomatic carotid stenosis exhibit increased platelet activation compared with healthy controls. However, only 3 studies (McCabe *et al*, 2004b;McCabe *et al*, 2005a;McCabe *et al*, 2005b) assessed the same symptomatic patients in both the early and late phases after TIA or stroke onset or carotid intervention so it is unclear whether these findings are reflective of an acute phase response, whether increased platelet activation predisposes to TIA or stroke in patients with carotid stenosis, or whether there is a combination of both factors at play. Furthermore, many of these studies did not clearly define the exact proportion of symptomatic patients with ≥50% and ≥70% carotid stenosis in whom carotid intervention should be considered ,(Uyama *et al*, 1992;Frijns *et al*, 1997;McCabe *et al*, 2004a;Blake *et al*, 2003;Shah *et al*, 1985;Zeller *et al*, 1999;Cha *et al*, 2003;Assadian *et al*, 2008;Hayes *et al*, 2003;Webster *et al*, 2004) or the interval between TIA or stroke onset and study inclusion.(Uyama *et al*, 1992;Hashimoto *et al*, 2003;Blake *et al*, 2003;Shah *et al*, 1985;Liu *et al*, 2009;Assadian *et al*, 2007;Robless *et al*, 2002;Hayes *et al*, 2003;Webster *et al*, 2004) Allowing for these limitations, the majority of case-control studies included

patients with symptomatic carotid stenosis already on anti-thrombotic therapy at the time of recruitment, thus indicating that platelets were excessively activated in these patients **despite** treatment with the secondary preventative regimen in question.

Within the symptomatic carotid stenosis subgroup, very preliminary data from one case-control study suggest that it is possible that surgical removal or endovascular treatment of a severely stenosing atherosclerotic plaque may reduce platelet activation and hyper-reactivity *ex vivo*, and decrease the likelihood of *ex vivo* aspirin non-responsiveness/high on-treatment platelet reactivity in the laboratory at least 3 months after intervention.(McCabe *et al*, 2005a) However, these findings might also reflect resolution of the acute phase response following cerebral or ocular ischaemia or infarction.

There is some evidence of enhanced spontaneous and inducible platelet activation, and platelet hyper-reactivity as measured by platelet aggregometry in PRP and whole blood in the perioperative phase around the time of carotid endarterectomy, and also in "higher than lower risk" carotid stenosis patients with more active embolisation on TCD. These data provide some insight into the potential platelet-mediated mechanisms that may predispose to TIA or stroke around the time of carotid endarterectomy. However, these findings need to be assessed in larger patient cohorts, preferably in longitudinal studies, where patients in the acute phase following TIA or stroke are followed up to the late phase after intervention to assess whether changes in platelet reactivity persist over a more prolonged period.

Some case-control studies have revealed evidence of increased platelet activation in patients with asymptomatic carotid stenosis compared with healthy controls, and that the degree of stenosis may influence the level of platelet activation. These data highlight the potential role played by 'clinically silent' carotid atherosclerotic plaques in contributing to platelet activation, although it is also possible that excessive platelet activation may

contribute to plaque formation in this patient population in the first instance. Follow up studies in asymptomatic carotid stenosis patients undergoing carotid endarterectomy are required to determine whether removal of the plaque may reduce platelet activation in these patients. However, due to the small sample size, heterogenous severity of the degree of asymptomatic carotid stenosis between studies, and differences in vascular risks between patients and control subjects, additional work is required to re-investigate platelet activation status in asymptomatic carotid stenosis patients on modern preventative regimens.

To our knowledge, there are only three studies comparing platelet activation in symptomatic versus asymptomatic moderate or severe carotid stenosis.(Cha *et al*, 2003;McCabe *et al*, 2005b;Jurk *et al*, 2010) These pilot studies provide some evidence that platelet production is increased in the early and late phases after symptom onset,(McCabe *et al*, 2005b) and that platelets are excessively activated in recently symptomatic compared with asymptomatic moderate or severe carotid stenosis and controls.(Cha *et al*, 2003;McCabe *et al*, 2005b;Jurk *et al*, 2010) Measurement of circulating leucocyte-platelet complexes was found to be a more sensitive method of detecting increased platelet activation than measurement of platelet surface marker expression in one study,(McCabe *et al*, 2005b) and may partly explain the higher risk of recurrent vascular events in symptomatic than asymptomatic carotid stenosis patients over time. However, as per other subgroup data outlined above, adequately powered longitudinal studies simultaneously assessing platelet surface markers and leucocyte-platelet complexes are required to readdress this issue.

There are very limited longitudinal data on the impact of altering antiplatelet therapy on platelet activation and function in patients with asymptomatic or symptomatic moderate

or severe carotid stenosis. The differences in methodology employed between studies do not allow one to make any definitive conclusions re this issue at present.

The data outlined in this systematic review highlight the fact that although this aspect of translational platelet science in cerebrovascular disease is relatively under-investigated, excessive platelet activation and / or platelet hyper-reactivity is likely to play an important role in the pathogenesis of first or subsequent TIA or stroke in carotid stenosis patients. However, larger studies in asymptomatic carotid stenosis, and further studies comparing platelet activation and function in symptomatic versus asymptomatic carotid stenosis are warranted to improve our understanding of the mechanisms responsible for TIA or stroke, and to improve risk-stratification in this patient population. If longitudinal studies in patients with asymptomatic carotid stenosis confirm that the subgroup of patients with the highest levels of platelet activation are at highest risk of subsequent cerebrovascular events, these patients could be targeted with more aggressive primary prevention regimens, with or without surgery. Furthermore, if one could identify symptomatic patients with excessive platelet activation or platelet hyper-reactivity, and prove that these patients are at highest risk of recurrent stroke or TIA than those with less marked activation or hyper-reactivity, these patients could be treated with more aggressive secondary preventative medical treatment and undergo hyperacute surgical or endovascular intervention, as deemed appropriate. Longitudinal follow up studies will also be needed to determine whether a subgroup of symptomatic carotid stenosis patients, who have less marked platelet activation, might be treated successfully with a comprehensive medical secondary prevention regimen and avoid undergoing surgical intervention altogether, with the attendant health and economic benefits.

Table 2.1a: Symptomatic Carotid Stenosis versus Controls – Soluble markers of platelet activation (↑ = increased; ↔ = not significantly different). Where data are available, absolute values are reported as mean±SD or mean±2SD. B-Throm = Beta-Thromboglobulin, PF-4 = Platelet Factor-4

Author	Year	Study Design	Patient population	Control population	sP-Selectin	sCD40	sCD40L	B-Thromb	PF-4	Antiplatelet Agents	Sample Type
Frijns [32]	1997	Case-control	≥30% carotid stenosis/ occlusion (n=34)	No cardiovascular, malignant, inflammatory or autoimmune diseases (n=34)	↑ (200±73 vs. 135±41 ng/ml; p<0.0001)					Aspirin, Dipyridamole, Ticlopidine	Plasma
Hashimoto [33]	2003	Case-case	≥50% carotid stenosis (n=27)	<50% carotid stenosis (n=274)	↑ (53.2 ±27.5 vs 38.9±20.5 ng/ml; p<0.05)					Aspirin, Ticlopidine	Serum
McCabe [40]	2004	Longitudinal, Case-control	Early phase post TIA / stroke (including ≥70% ICA stenosis/ occlusion)(total n=79; n=19 atherothrombotic)	No history cerebrovascular disease or carotid stenosis on ultrasound (n=27)	↔(data not shown; p≥0.09)					Aspirin, Dipyridamole, Clopidogrel	PPP
Balla [35]	2006	Case-control	>30% carotid stenosis/ occlusion (n=60)	Healthy subjects with no carotid plaques (n=30)		↔ (85 ± 56.9 *vs.* 79.3 ± 18.7 pg/mL; p=0.34)	↑ (6.9 ± 5 *vs.* 4.5 ± 3.0 ng/mL; p<0.038)			Aspirin, Clopidogrel Ticlopidine	Serum
Blake [36]	2003	Case-case	≥30% ICA/CCA stenosis (n=46)	N/A			↑ (2.54 *vs.* 1.58 ng/ml; p<0.02) in patients with intraplaque lipid on high-resolution MRI			Not specified	Plasma
Shah [37]	1985	Case-control	Prior TIA / stroke (n=58)	Young, healthy individuals (n=20) and elderly subjects age matched with study subjects (n=15)				↑ in thromboembolic (50.2±4.1 ng/ml), cardioembolic (56.3±5.2 ng/ml), uncertain aetiology (56.0±4.8 ng/ml) TIA / stroke *vs.* controls (31.1 ±2.5 ng/ml; p<0.001)	↑ in thrombo-embolic TIA / stroke (14.9±3.5 ng/ml) *vs.* controls (6.5±1.4 ng/ml; p<0.05)	Aspirin	PPP
Jurk [38]	2010	Case-control	Symptomatic >70% (n=25)	Age-matched healthy individuals without cardiovascular disease (n=30)	↑ (values not stated; p<0.05)		↑ (2.99 ± 1.08 *vs.* 1.16 ± 0.55ng/ml , p<0.05)			Aspirin, Ticlopidine	Whole Blood

Table 2.1b: Symptomatic carotid stenosis versus controls – Surface markers of platelet activation (↑ = increased; ↔ = not significantly different with P > 0.05)). Where data are available, absolute values are reported as mean ± SD or mean ± 2SD. NPC = Neutrophil-Platelet Complexes; MPC = Monocyte-Platelet Complexes; LPC = Lymphocyte–Platelet Complexes

Author	Year	Study Design	Patient population	Control population	CD62P	CD63	CD40L	PAC-1	NPC	MPC	LPC	Antiplatelet agent	Sample Type
Zeller [41]	1999	Case-control	Acute Symptomatic Carotid occlusion or >50%carotid, vertebral or basilar stenosis (n=47)	Volunteers with no history of cerebrovascular disease (n=72)	↑ (9.4 ± 5.8 *vs.* 4.9 ± 2.8%; p<0.001)	↑ (15.7 ± 8.9 *vs.* 10.8 ± 4.5%; p<0.001)						Aspirin	PRP
Yip[42]	2006	Longitudinal, Observational, Case-control	≥70% Symptomatic ICA stenosis (n=35)	subjects with angina pectoris undergoing cardiac catheterization as at-risk controls (n=20); age- and sex-matched healthy volunteers (n=20)	↑ (2.93 ± 1.09 % *vs.* 1.74 ± 0.54 % in at-risk controls, and 1.40 ± 0.64 % in healthy controls ; p<0.0001)							Aspirin, Clopidogrel	PRP
Cha[43]	2003	Longitudinal, Observational, Case-control	Atherothrombotic ischaemic stroke (n=25)	Healthy subjects (n=24)	↑ (p<0.01)	↑ (p<0.01)	↑ (p<0.01)					Aspirin	Whole Blood
McCabe [40]	2004	Longitudinal, Observational, Case-control	Early and late phase post TIA / stroke (≥70% ICA stenosis/ occlusion)(n=19)	No history cerebrovascular disease or carotid stenosis on ultrasound (n=27)	↑ in late phase (p=0.01)	↔		↔	↔	↔	↔	Aspirin, Clopidogrel, Dipyridamole	Whole Blood
Jurk [38]	2010	Case-control	Symptomatic >70% (n=25)	Age-matched healthy individuals without cardiovascular disease (n=30)	↑ (p<0.05)	↑ (p<0.05)			↑ (22.42 ± 3.2% *vs.* 10.3 ± 2.7% (p<0.05)	↑ 43.3 ± 3.4% *vs.* 17.4 ± 10.8 % (p<0.01)		Aspirin, Ticlopidine	Whole Blood

Table 2.1c: Symptomatic carotid stenosis versus controls – CVD = cerebrovascular disease; CT = closure time; Markers of platelet function and reactivity (↑ = increased; ↔ = not significantly different)

Author	Year	Study Design	Patient population	Control population	PFA-100 C-EPI CT	PFA-100 C-ADP CT	Platelet Aggregometry	Aspirin Non-responders	Antiplatelet Agent	Sample Type
McCabe [45]	2005	Longitudinal, Observational, Case-control	Stroke/ TIA in territory of ≥70% carotid stenosis/ occlusion(n=11)	Healthy subjects with no cerebrovascular disease (n=23)	↔ early phase (p=0.3), ↑ late phase (p<0.001)	↔	No late phase patients defined as aspirin on PFA-100 were non-responders on aggregometry	(7/11) 64% early phase, 11.1% late phase on PFA-100 C-EPI cartridge	Aspirin	Whole blood for PFA-100; PRP for aggregometry

Table 2.2: Asymptomatic carotid stenosis versus Controls – Soluble and Surface markers of platelet activation (↑ = increased; ↔ = not significantly different). Where data are available, absolute values are reported as mean ± SD or mean ± 2SD or median (range).

Author	Year	Study Design	Patient Population	Control population	Sample Type	Platelet Marker						Antiplatelet Agent
						Soluble	P-Selectin	CD63	sCD40	CD40L	11-dehydro-TxB2	
Balla [35]	2006	Case-control	>30% carotid stenosis/ occlusion (n=60)	Healthy subjects with no carotid plaques (n=30)	Serum				↔ (85 ± 56.9 *vs.* 79.3 ± 18.7 pg/mL; p=0.34)	↑ (6.9 ± 5 vs. 4.5 ± 3.0 ng/mL p=0.038)		Aspirin, Clopidogrel Ticlopidine
Novo [50]	2005	Case-control	<50% Carotid stenosis (n=42)	Subjects without carotid stenosis (n=21)	Plasma					↑ 6.2 (1.4–15.8) *vs.* 1.4 (0.5–4.5) ng/ml; p<0.0001)	-	Aspirin
Stolz [52]	2002	Case-control	>70% (n=19) and <70% (n=11) Carotid Stenosis	Healthy subjects (n=13)	Serum	-	↑ (452.7 ± 169.7 *vs.* 256.7 ± 74.8 ng/ml in >70% carotid stenosis *vs.* controls; p<0.01)					Not reported
Enomoto [51]	2010	Case-control	>50% Carotid Stenosis (n=10)	Age-matched non-atherosclerotic Controls (n=8)	PRP					↑ (p<0.01)	↑ (p<0.05)	Aspirin
Jurk [38]	2010	Case-control	Asymptomatic >50% (n=48)	Age-matched healthy individuals without cardiovascular disease (n=30)	Whole blood		↔ (p > 0.05)			↔ (1.49 ± 0.82 *vs.* 1.16 ± 0.55; p > 0.05)		Aspirin, Ticlopidine
						Surface	**CD62P**	**CD63**	**CD40**	**CD40L**		
Stolz[52]	2002	Case-control	>70% (n=19) and <70% (n=11) Carotid Stenosis	Healthy subjects (n=13)	Whole blood		↑ 35.7 ± 9.9% in >70% stenosis (p<0.001); ↑ 21.2 ± 12.6% in <70% stenosis (p<0.01) versus controls 7.5 ± 2.2%					Not reported
Cha[43]	2003	Longitudinal, Observational, Case-control	>50% Carotid Stenosis (n=20)	Healthy subjects (n=24)	Whole blood		↑ (p<0.01)	↑ (p<0.01)		↔		Aspirin
Jurk [38]	2010	Case-control	Asymptomatic >50% (n=48)	Age-matched healthy individuals without cardiovascular disease (n=30)	Whole blood		↔ (p>0.05)	↔ (p>0.05)				Aspirin, Ticlopidine

Table 2.3: Symptomatic versus asymptomatic carotid stenosis – Soluble and Surface markers of platelet activation; NPC = Neutrophil-Platelet Complexes; MPC = Monocyte-Platelet Complexes; LPC = Lymphocyte–Platelet Complexes (↑ = increased; ↔ = not significantly different).

Author	Year	Study Design	Patient Population	Platelet Marker: Soluble								Sample Type	Antiplatelet Agent
				P-Selectin	CD63	CD40L	11-dehydro-TXB2						
Jurk [38]	2010	Case-control	Symptomatic >70% (n=25) *vs.* asymptomatic >50% carotid stenosis (n=48)	↑ ($p<0.05$)		↔ (2.99 *vs.* 1.49 ng/ml; $p>0.05$)						Plasma	Aspirin, Ticlopidine
Author	**Year**	**Study Design**	**Patient Population**	**Platelet Marker: Surface**									
				CD62P	CD63	CD40L	PAC-1	NPC	MPC	LPC	Platelet Count	Sample Type	Antiplatelet Agent
Cha [43]	2003	Longitudinal, Observational, Case-control	'Atherothrombotic stroke' (n=25) *vs.* Asymptomatic carotid stenosis (n=20)	↔ ($p > 0.05$)	↔ ($p>0.05$)	↑ ($p<0.01$)						Whole Blood	Aspirin
McCabe [14]	2005	Longitudinal, Observational, Case-control	Acute symptomatic (n=19) *vs.* asymptomatic (n=16) ≥70% carotid stenosis	↔ (1,3% *vs.* 1.7%; $p\geq0.09$)	↔ (8.8% *vs.* 7.5%; $p\geq0.2$)		↔ (2.9% *vs.* 2.6%; $p\geq0.3$)	↑ in acute phase: (2.7% *vs.* 2.2%; $p=0.004$)	↑ in acute phase (5.2% vs. 4.4%; $p=0.046$)	↑ in acute phase (2.6% *vs.* 2.1%; $p=0.02$)	↑ in acute symptomatic ($275x10^9$/l *vs.* $223x10^9$/l $p=0.02$ in EDTA; $231x10^9$/l *vs.* $193x10^9$/l; $p=0.04$ in citrate)	Whole Blood	Aspirin, Clopidogrel, Dipyridamole
Jurk [38]	2010	Case-control	Symptomatic >70% (n=25) vs asymptomatic (n=48) >50% carotid stenosis	↑ ($p<0.05$)	↑ ($p<0.05$)							Whole Blood	Aspirin, Ticlopidine

Table 2.4a: Assessment of surface markers of platelet activation in symptomatic carotid stenosis patients undergoing intervention; LPC = Leucocyte–Platelet Complexes (↑ = increased; ↓ = decreased; ↔ = not significantly different).

Author	Year	Study Design	Patient Population	CD62P	CD41	PAC-1	LPC	Reticulated Platelets	Platelet Count	Fibrinogen platelet binding	Antiplatelet Agent	Sample Type
Robless [53]	2002	Longitudinal, observational	Carotid stenosis on aspirin undergoing CEA (90% symptomatic; n=40)	↑ after carotid dissection ($p<0.01$) and cross-clamp removal - ($p<0.05$)		↑ after carotid dissection ($p<0.05$)					Aspirin, Dipyridamole	Whole Blood
Liu [44]	2009	Longitudinal, observational	≥70% symptomatic carotid stenosis undergoing carotid stenting (n=40)			↔ ($P > 0.05$ at 30 mins, 18 hours and 6 days post-op *vs.* baseline		↔ ($P > 0.05$ at 30 mins, 18 hours and 6 days post-op *vs.* baseline			Aspirin, Clopidogrel	Whole Blood
Assadian [54]	2008	Longitudinal, observational	Symptomatic carotid stenosis undergoing CEA (n=20)	↑ serially during operation ($p<0.001$)	↑ serially during operation ($p=0.002$)		↑ post surgery in unfractionated heparin group ($p\leq0.034$)	↑ between cross-clamping and reestablishment of blood flow ($p=0.001$)	↓ during CEA ($p=0.036$)		None	Whole Blood
Payne [55]	2004	Randomised controlled trial	Carotid stenosis pre CEA (84% Symptomatic; mean stenosis 80%; n=100)							↔ after addition of clopidogrel to aspirin	Aspirin, clopidogrel	Whole blood
Vogten [56]	2008	Longitudinal, observational	Carotid stenosis pre CEA on Aspirin *vs.* ASA + clopidogrel combination (Proportion symptomatic unclear; n=27)	↔ at 7 timepoints pre-operatively, intra-operatively and post-operatively							Aspirin, clopidogrel	Whole blood

Table 2.4b: Assessment of platelet reactivity in symptomatic carotid stenosis patients undergoing intervention. (↑ = increased; ↓ = decreased; ↔ = unchanged)

Author	Year	Study Design	Patient Population	PFA-100 C-EPI CT	Platelet Aggregometry	Flow Cytometry Reactivity (inducible activation)	Antiplatelet Agent	Sample Type
Hayes [57]	2003	Longitudinal, observational	Carotid Stenosis undergoing CEA (n=120; 10% asymptomatic)		↑ ADP-induced aggregometry with >25 *vs.* <25 emboli (p<0.0012)	↑ platelet response to ADP with >25 *vs.* <25 emboli (p<0.0001)	Aspirin	Whole blood (flow cytometry); PRP (aggregometry)
Robless [53]	2002	Longitudinal, observational	Carotid stenosis on aspirin undergoing CEA (90% symptomatic; n=40)		↑ spontaneous (21 to 42% ; p<0.001) and ADP-induced aggregation (56 to 80%; p<0.01)		Aspirin, Dipyridamole	Whole blood
Webster [58]	2004	Longitudinal, observational	Carotid stenosis undergoing CEA (? proportion symptomatic; n=41)		↑ Arachidonic acid-induced aggregation post heparin (p≤0.006)		Aspirin	PRP
Assadian [54]	2008	Longitudinal, observational	Symptomatic carotid stenosis undergoing CEA (n=20)	↔	N/A	↑ ADP-induced CD62P (p=0.03) and CD41 P<0.001), and TRAP-6 induced CD41 expression (p=0.002) during surgery	None	Whole blood
Payne [55]	2004	Randomised controlled trial	Carotid stenosis before CEA (84% symptomatic; mean stenosis 80%; n=100)		↔ (addition of clopidogrel to aspirin did not influence arachidonic acid induced platelet aggregation	↓ (ADP-induced platelet-fibrinogen binding with clopidogrel & aspirin *vs.* aspirin alone (66.76% vs 75.52%; p = 0.03)	Aspirin, clopidogrel	Whole blood
King [59]	2011	Longitudinal, observational	Symptomatic carotid stenosis starting aspirin/clopidogrel (n=30) or aspirin/dipyridamole (n=30)		↓ ADP-induced aggregation in aspirin-clopidogrel *vs.* aspirin-dipyridamole group (86.5 ± 28.8 *vs.* 101.7 ± 13.0; p<0.001)	-	Aspirin, Clopidogrel, Dipyridamole	PRP
Szapary [61]	2009	Longitudinal, observational	≥70% carotid stenosis (10 symptomatic, 8 asymptomatic; n=18)		↑ ADP-induced platelet aggregation 5 days post-intervention *vs.* immediately post-procedure (p < 0.0001)		Aspirin, Clopidogrel	PRP
Vogten [56]	2008	Longitudinal, observational	Carotid stenosis pre CEA on ASA vs. ASA/clopidogrel combination (proportion symptomatic unclear; n=27)		↑ baseline arachidonic acid induced platelet aggregation in aspirin vs. aspirin + clopidogrel group (14.5% vs 10.3%; p < 0.05). 5 minutes after administration of		Aspirin, clopidogrel	PRP

					heparin, platelet aggregation 19.7% in aspirin group ($p < 0.01$) and 22.5% in aspirin and clopidogrel group ($p < 0.05$)			
Markus [60]	2005	Randomised, double blind study	Recently (<3 months) symptomatic ≥50% carotid stenosis on aspirin (n= 40) *vs.* aspirin and clopidogrel (n = 31)		↑ mean maximum intensity of collagen-induced platelet aggregation at day 7 to 106.7% of baseline in aspirin monotherapy group, and ↓ to 70.9% of baseline in aspirin & clopidogrel group		Aspirin, Clopidogrel	PRP

PRP = platelet rich plasma; TCD = transcranial Doppler ultrasound

3. General Methods

3.1. Study Subjects

The general inclusion and exclusion criteria for subjects involved in each study described in this thesis are outlined below. These inclusion and exclusion criteria apply to patients recruited to the studies discussed in Chapters 4, 5, 6, 7, 8, 9 and 10. Chapter 11 contains a different patient population which will be outlined in that Chapter.

3.1.1. Ethical approval

The study was fully approved by the St. James Hospital/Adelaide and Meath Hospital Research Ethics Committee (Project/REC Reference: 2007/03/01). Written informed consent (or written assent from a relative or next of kin, where appropriate) was obtained in all cases following detailed discussions regarding the nature of the study and procedures and after all study subject were given detailed study information sheets.

3.1.2. Inclusion criteria

Patients with asymptomatic moderate or severe carotid artery stenosis

Consecutive eligible patients older than 18 years of age, were included in the 'asymptomatic carotid stenosis' group if incidentally noted to have moderate (50 - 69%) or severe (≥ 70%) carotid stenosis identified on colour Doppler ultrasound (CDUS), e.g. during vascular work-up in a patient with a carotid bruit or coronary artery disease. Asymptomatic patients either had no history of TIA or stroke, or had not had any TIA or stroke within the preceding three years.

Patients with symptomatic moderate or severe carotid artery stenosis

Consecutive eligible patients older than 18 years of age, were included in the 'symptomatic carotid stenosis' group if they recently had a TIA or ischaemic stroke in the vascular territory supplied by a moderate or severe carotid artery stenosis or carotid occlusion within the preceding 4 weeks (early phase), and symptoms were attributed to the stenosed carotid artery of interest. Patients with carotid occlusion were only included if no other cause for stroke or TIA was identified. These patients were prospectively reassessed at least three months after symptom onset or after surgical or endovascular intervention (late phase). If antiplatelet therapy was altered by their treating physician in the early phase after presentation, patients were invited to undergo repeat blood testing approximately 14 days later if they had not undergone carotid intervention by that stage. Details regarding antiplatelet regimens, dose and duration of therapy were recorded.

Controls

Control subjects of similar age and gender to the patient population and who had no prior history of cerebrovascular disease were recruited. These inclusion and exclusion criteria apply to control subjects recruited to the studies discussed in Chapter 5. Chapter 11 contains a different control subject population which will be outlined in that Chapter.

3.1.3. Exclusion Criteria

Patients with asymptomatic or symptomatic carotid stenosis with:

- Myocardial infarction, PE or DVT within the preceding 3 months
- Platelet count < 120 x 10^9/L or > 450 x 10^9/L
- Urea >10mmol/l or GFR < 30ml/min
- Major surgery within the preceding 3 months
- Systemic haemorrhage within the preceding three months (Haemoglobin drop of >1g/dl in one day, or requiring transfusion)
- Ongoing unstable symptomatic coronary or peripheral vascular disease
- Prior history of primary intracerebral haemorrhage
- Known bleeding/ clotting diathesis, including known platelet disorders
- Active vasculitis or other inflammatory conditions
- Active neoplasia
- Current active infection (Clinical signs of infection, white cell count > 11 x 10^9/L)
- NSAID intake within the previous 14 days

Patients were subsequently excluded from the symptomatic group if there was evidence of a potential cardioembolic source of embolism detected within 3 months of study recruitment.

Control Subjects

The exclusion criteria for control subjects were the same as those for patients, as outlined above, with the exception that subjects were also excluded from the control group if they had a history of stroke or TIA in the past, carotid stenosis or if they were on antiplatelet therapy.

3.1.4. Recruitment Source

Patients were recruited from the One-Stop Rapid Access Stroke Prevention Clinic, vascular surgery and general neurology clinics and from the inpatient population of the Neurology and Age-Related Health Care and Stroke service, as well as the vascular surgery service, at AMNCH and St James's Hospital. Both University Teaching Hospitals accept direct referrals from primary care and emergency department physicians, and hospital consultants in the region. Control subjects were recruited from amongst the staff at AMNCH and from the local population. Spouses of patients and control subjects were also recruited.

3.1.5. Baseline Clinical Assessment

All study subjects and controls were clinically assessed and underwent detailed neurovascular assessment by one of three examiners (Justin A Kinsella, W Oliver Tobin, Dominick JH McCabe) using a standardised protocol prior to recruitment, to confirm that asymptomatic patients met inclusion criteria, and to confirm a diagnosis of large artery atherosclerotic TIA or stroke in the symptomatic cohort. Information regarding vascular risk factors, including hypertension, prior TIA or stroke, ischaemic heart disease, atrial fibrillation, valvular heart disease, diabetes mellitus, hyperlipidaemia, peripheral vascular disease, migraine, family history of stroke, medication intake (including anti-thrombotic therapy), smoking status, alcohol intake, and the method of detection of carotid stenosis was collected prospectively. The degree of disability following ischaemic stroke was quantified by using the modified Rankin scale. A score of 0 on this scale indicates full recovery with no residual symptoms or disability, and a score of 5 indicates severe disability.(Farrell *et al*, 1991) Colour Doppler ultrasound of carotid and vertebral arteries was performed to rule out moderate or severe carotid or vertebral stenosis. TIA or stroke

work up was performed in all patients by their primary physician according to ESO recommendations (European Stroke Initiative Recommendations for Stroke Management., 2008), along with their research assessment by one of three examiners (JAK, WOT, DJHM) using a standardised protocol. The diagnosis of asymptomatic or symptomatic carotid stenosis was confirmed in all cases by Dr Kinsella and/or Dr McCabe, and patients were not included if Dr Kinsella or Dr McCabe had any doubt about the working diagnosis.

CT and/or MRI brain was performed in all symptomatic patients, and magnetic resonance angiography (MRA) or CT angiography (CTA) was performed where deemed appropriate by the treating physician to establish concordance between CDUS and another non-invasive imaging modality. Chest radiograph, electrocardiograph (ECG), 24-hour ECG (Holter) recording and transthoracic or transoesophageal echocardiography were obtained in all symptomatic patients.

Medication adherence in inpatients was confirmed by checking the inpatient drug chart. All late stage symptomatic carotid stenosis patients were phoned before their appointment to stress the importance of medication adherence in the week prior to assessment. Adherence in this group was assessed by history taking alone. Patients in whom there was any concern about non-adherence to their antithrombotic regimen were invited back for reassessment 14 days later. However, one must stress that the effects of antiplatelet therapy on circulating reticulated platelets was not a primary aim of this component of our study, as it was addressed by another colleague during his PhD studies in our lab (Dr W Oliver Tobin).

3.2. Sample collection and separation

3.2.1. Sample collection

All subjects were rested for at least 20 to 30 minutes before venepuncture to standardise conditions and to minimise platelet activation *in vivo*. A tourniquet was applied to the arm and careful venepuncture was performed in all cases. Blood was collected from a free-flowing vein using a sterile 21G Butterfly® needle (Venisystems™, Abbott, Ireland) and a Vacutainer® system with a luer adaptor (Becton Dickinson Vacutainer® Systems, U.K.) as previously described.(McCabe *et al*, 2004a)

For all studies, the tourniquet was released during collection of the first 4 ml of blood which was drawn into two 2 ml sterile Vacutainer® tubes containing K_2 EDTA. The first 2ml EDTA sample was used for measurement of a full blood count (FBC), including measurement of the mean platelet volume (MPV) and platelet distribution width (PDW), and the second 2ml EDTA tube containing whole blood was stored at –70°C for future planned genetic studies (see later). Full blood count (FBC) was measured in EDTA Vacutainer® tubes between 2 and 4 hours after venepuncture on a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd.).

Eight further 3 ml samples were collected into sterile Vacutainer® tubes containing 0.105 M (3.2%) buffered sodium citrate. All samples were gently inverted five to eight times to ensure thorough mixing of the anticoagulant with the blood sample. The first 3 ml citrate-anticoagulated blood sample was used for whole blood flow cytometric analysis on a Beckman Coulter XL MCL flow cytometer and for measurement of platelet function in whole blood using the platelet function analyser (PFA-100®, Dade-Behring, Germany; see below). The next four citrate-anticoagulated samples were used to prepare platelet poor plasma (PPP). The remaining sample was used for measurement of the platelet count, MPV and PDW in citrate-anticoagulated whole blood. Platelet count, MPV and

PDW were assessed in both EDTA and citrate-anticoagulated samples to quantify these parameters in an anticoagulant used in most clinical laboratories for routine FBC measurements (EDTA), and also to assess these parameters in the anticoagulant used for platelet flow cytometric and platelet function studies (citrate). We intentionally did not correct for the 1:10 dilution of the sodium citrate anticoagulant when reporting our results because we wanted the platelet data from the FBC in citrate-anticoagulated whole blood to match the platelet parameters during our platelet activation and functional assays.

Three 6ml K_2 EDTA tubes were also taken: the first two 6 ml K_2 EDTA tubes were used to prepare PPP for assessment of antiplatelet drug levels in a collaborative high performance liquid chromatography (HPLC) study, and the last K_2 EDTA-containing tube was used for ABO blood grouping.

Results of routine haematological (FBC), coagulation (PT/APTT), biochemical (renal and liver profiles) and blood glucose testing were collected prospectively.

3.2.2. Sample separation

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

Platelet poor plasma (PPP)

PPP was prepared from three 3.2% (0.105 M) buffered sodium citrate anticoagulated blood samples within one hour of venepuncture. The samples were centrifuged at 2250g for 15 minutes at room temperature. The upper two thirds of each sample was carefully aspirated using a plastic Pasteur pipette and pipetted into a 12 x 75 mm polypropylene sample tube. The sample was centrifuged again at 2250g for 15 min. Double-spun PPP was then recovered from the upper two thirds of this sample, aliquoted into three

polypropylene tubes (Sarstedt®, Germany) and immediately frozen at –70°C. The remaining PPP was removed from the lower third of the centrifuge tube and also stored in a polypropylene tube at -70°C; this sample was not considered to be double-spun.

3.3. Flow Cytometry

3.3.1. General principle

The flow cytometer used in this study was a Coulter® EPICS® XL-MCL (Beckman Coulter United Kingdom Ltd.), and the information below applies to the operation of this model.

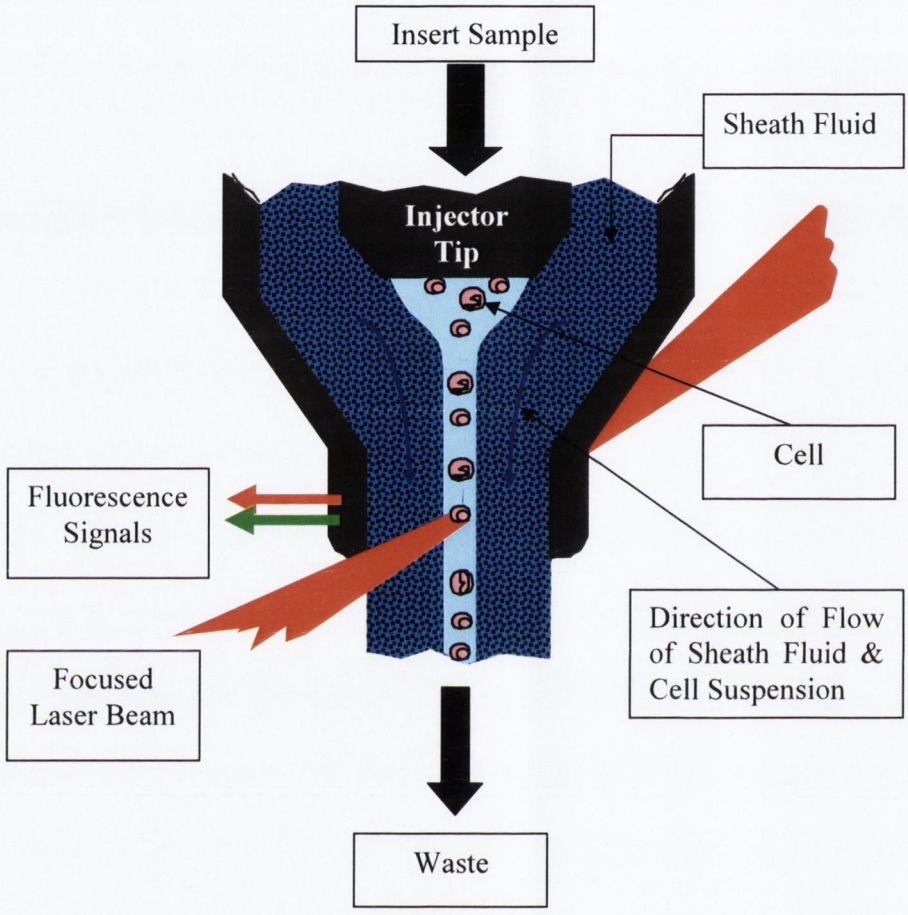
Flow cytometry is a method used for sensing cells or particles as they flow in a liquid stream through a laser beam.(Macey, 1994) The signals that are ultimately produced by the flow cytometer provide information about the cell. The process begins with the insertion of a cell suspension into the flow cell, through which sheath fluid (ISOTON II®, Beckman Coulter, U.K.) also flows. The sheath fluid is filtered with a 0.2 μm filter. It contains bacteriostatic and fungistatic agents and is transparent and non-fluorescent in response to 488 nm laser light. The sheath fluid exerts a constant pressure on the cell suspension and, using a low flow rate of 10μl/min, the cells are aligned in single file by a process known as 'hydrodynamic focusing'.

A 488 nm argon ion laser is used in the Coulter® EPICS® XL-MCL (see Figure 3.1). When a cell passes through the laser beam, light is scattered in different directions. The degree of light scatter in the forward direction (forward scatter (FS)) is proportional to the size of the cell, with larger cells producing more FS. Light from the laser beam also enters the cell and is reflected and refracted by the internal structures and granules within the cell. This produces side scatter (SS) light, and the degree of SS is proportional to the granularity of the cell. The cells may also be labelled with fluorochrome-linked antibodies, or stained with fluorescent dyes. Fluorochromes or fluorescent dyes absorb laser light energy and emit fluorescence at different colour wavelengths.

The FS sensor is positioned behind the sample stream, whereas the SS and other fluorescence sensors are positioned at 90° to the laser beam and sample stream. A series

of dichroic mirrors (beam splitters) direct the different components of the transmitted light to appropriate sensors, with filters removing unwanted wavelengths of light. The sensors are called photomultiplier tubes (PMTs). They serve as detectors and amplifiers that convert the transmitted light to a voltage pulse that rises and falls depending on the amount of light entering the PMT. Smaller cells or particles generate smaller voltage pulses, whereas larger cells yield larger pulses. The process of logarithmic amplification may amplify the pulses. This makes smaller pulses much larger, but amplifies larger pulses to a lesser degree, thus accentuating differences between two small pulses.(Macey, 1994)

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

Table 3-1 Fluorochromes / Fluorescent Dye Used In Flow Cytometry
***RPE-Cy5 is a combination of two fluorochromes [R-phycoerythrin [RPE] and Cyanine 5 [Cy5]) that are covalently coupled to one another. The argon ion laser excites the RPE at 488 nm, and the emitted light energy excites the Cy5 closely bound to the RPE molecule. The Cy5 then fluoresces at 670 nm and the FL3 detector detects this fluorescence**

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

The fluorochromes and fluorescent dye (Thiazole Orange [TO]) used for whole blood flow cytometric analysis in this thesis are listed in Table 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, 1996) All blood samples will exhibit some degree of non-specific fluorescence because of (i) autofluorescence of the sample and (ii) non-specific staining of the cell with a given monoclonal antibody or fluorochrome.(Schmitz *et al*, 1998)

To account for this non-specific fluorescence when monoclonal antibodies were used, matched 'isotype control' monoclonal antibodies that were conjugated to the same fluorochrome, but that did not recognise target antigens on platelets were employed.(Schmitz *et al*, 1998) To quantify the degree of non-specific fluorescence in the TO assay, a matched control sample that was incubated in the absence of the fluorescent dye was used (see below). For each assay, the 'antibody positive population' was determined by using an analysis marker placed to the right of a histogram of 'fluorescence versus intensity' from a matched control sample.(Michelson, 1996) Expressing results as percent positive platelets is simpler and, unlike MFI, is independent of variation in signal amplification caused by changes in PMT voltage or gain over time, because the isotype control signal changes in proportion with the test sample.(Michelson, 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, 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.

3.3.2. 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 on a daily basis. These 10 μm fluorescent beads are excited by the 488 nm argon ion laser and emit within the wavelength range of 525 nm to 700 nm. 5,000 events were collected for each sample tested at a rate of < 200 events per second, and a half peak coefficient of variation of ≤2.0 on the relevant histograms was deemed acceptable.

3.3.3. Platelet surface markers

GpIb (CD42b) - Principle

Using flow cytometry, platelets can be distinguished from red and white blood cells by their characteristic pattern of forward and side scatter, which is dependent on their size and granularity respectively. The GpIbα (CD42b) subunit of the GpIb-IX-V complex is the predominant receptor for von Willebrand Factor (VWF:Ag) on platelets.(Ruggeri, 1997;Clemetson, 1997;Escolar & White, 2000) With the exception of patients with Bernard-Soulier syndrome, in whom this receptor may be deficient, if one stains platelets with an 'activation-independent' platelet-specific antibody directed against GpIb and conjugated to PE, the flow cytometer threshold can be set to detect only PE-positive particles i.e. GpIb-positive platelets and platelet-derived microparticles.(Abrams & Shattil, 1991) The method used in our laboratory for identifying GpIb-positive platelets was adapted from the whole blood flow cytometric protocol described by Shattil *et al.*(Shattil *et al*, 1987) GpIb expression can also be used as a marker of platelet activation because there is some degree of GpIb-IX-V receptor redistribution from the platelet surface membrane to the membranes of the surface connected open canalicular system upon platelet activation.(Abrams & Shattil, 1991;Schmitz *et al*, 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, the reduction in GpIb binding was not quantified in this thesis.

Reagents

- Anti-IgG_1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration : 6.25 ug/ml)
- Anti-CD42b-PE mouse monoclonal antibody (IgG1) (Immunotech, Beckman Coulter, Marseille, France. Concentration : 1.56 ug/ml)
- HEPES buffered saline (HBS) - made up with NaCl 0.145mol/l, KCl 5 mmol/l, MgSO4 1 mmol/l and HEPES 10mmol/l, dissolved in distilled water and pH adjusted to 7.4.(Chronos *et al*, 1994) The solution was filtered with a 0.2 μm filter prior to storage at 4°C, but allowed to reach room temperature before use.
- Fixative: 0.2% formalin saline solution – made up of 0.25 ml of 40% formaldehyde solution diluted 1:200 with 50 ml of 0.9% NaCl. The fixative was freshly prepared each day and filtered with a 0.2 μm filter to remove debris.

Methods

Because the concentration of the anti-IgG_1-PE isotype control antibody was 6.25 μg/ml, and the concentration of the anti-CD42b-PE monoclonal antibody was 1.56 μg/ml, 5 μl of the anti-IgG_1-PE control antibody was diluted 1:4 with 15 μl of HBS before use in the assay. 5 μl of citrate anticoagulated whole blood was aliquoted into the control and test

polypropylene tube within 45 minutes of venepuncture. 5 µl of diluted anti-IgG_1-PE antibody was added to the isotype control sample and 5 µl of anti-CD42b-PE antibody to the test sample. 40 µl of HBS was then added to each sample, the samples were gently mixed, covered and incubated at room temperature for 20 minutes. The samples were then fixed with 1 ml of fixative and flow cytometric analysis began within 45 minutes of fixation.

A protocol was designed in which only cells in the control sample with a particular forward and side scatter profile consistent with platelets were analysed. A gate was manually positioned around the platelet cloud on the 'log FS' versus 'log SS' histogram, and the gating settings were saved and used for subsequent platelet activation studies on the same sample. 10,000 platelet events were analysed in all assays, using a low flow rate, unless otherwise stated.

The non-specific fluorescence of the anti-IgG_1-PE isotype control monoclonal antibody was measured using a histogram that plotted the platelet count versus log fluorescence detected in FL2 (Figure 3-2). The 'positive analysis function' on the flow cytometer was set at 0.5% for this and all other assays performed as part of the panel set-up i.e. the percentage of 'antibody-positive' platelets in the test sample was determined by measuring those platelets with a fluorescence intensity exceeding that of 99.5% of the control sample. Therefore, 0.5% of the matched control sample histogram was included in the calculation of percent positive cells in the test sample to avoid excluding any weakly positive cells in the analysis. To confirm that the cells within the gate in the test sample were platelets, the % GpIb binding in the test sample was then measured in FL2; the majority of cells within the gate were considered to be platelets if the % GpIb binding was $\geq$ 95%. If the % GpIb binding was < 95%, the process was repeated until $\geq$ 95% GpIb positivity was obtained for the test sample (Figure 3-3).

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.

Figure 3-2 Scatterplot and Histogram from a GpIb Control Sample

Figure 3-2-I: Scatterplot of log FS versus log SS from a GpIb control sample identifies platelets within region B by their characteristic light scatter profiles; gate 'A' is manually positioned around the platelet cloud.
Figure 3-2-II: Histogram of cell count (Count) versus log fluorescence detected in FL2 from an anti-IgG1-PE isotype control sample to calculate the degree of nonspecific background fluorescence of the control sample. The positive analysis function includes 0.5% of the control sample in region E. The entire gated region is represented by the blue line in the histogram.

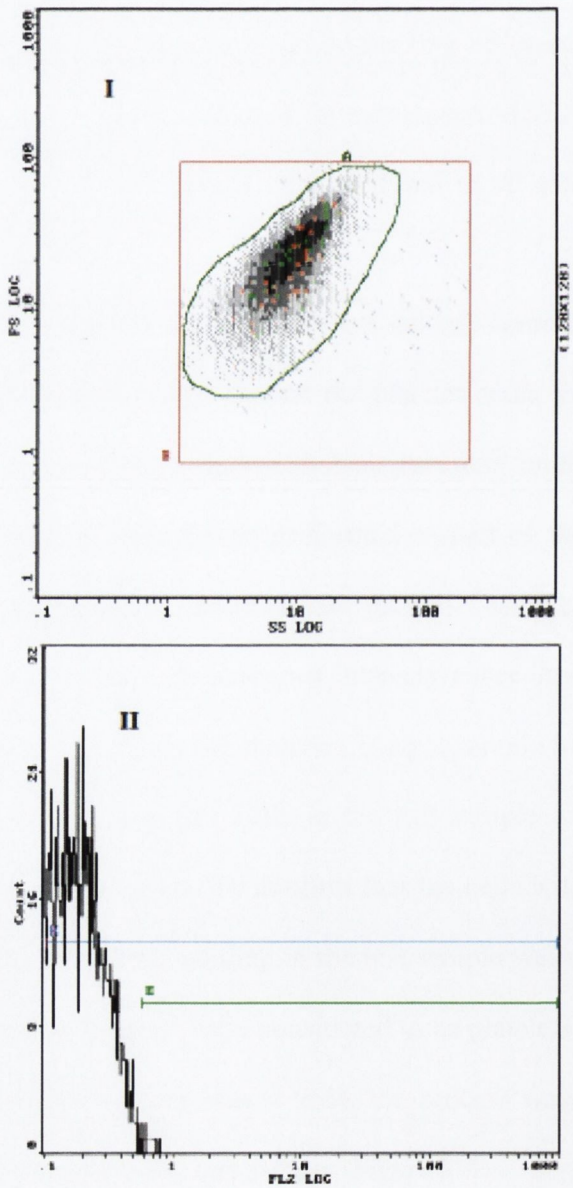
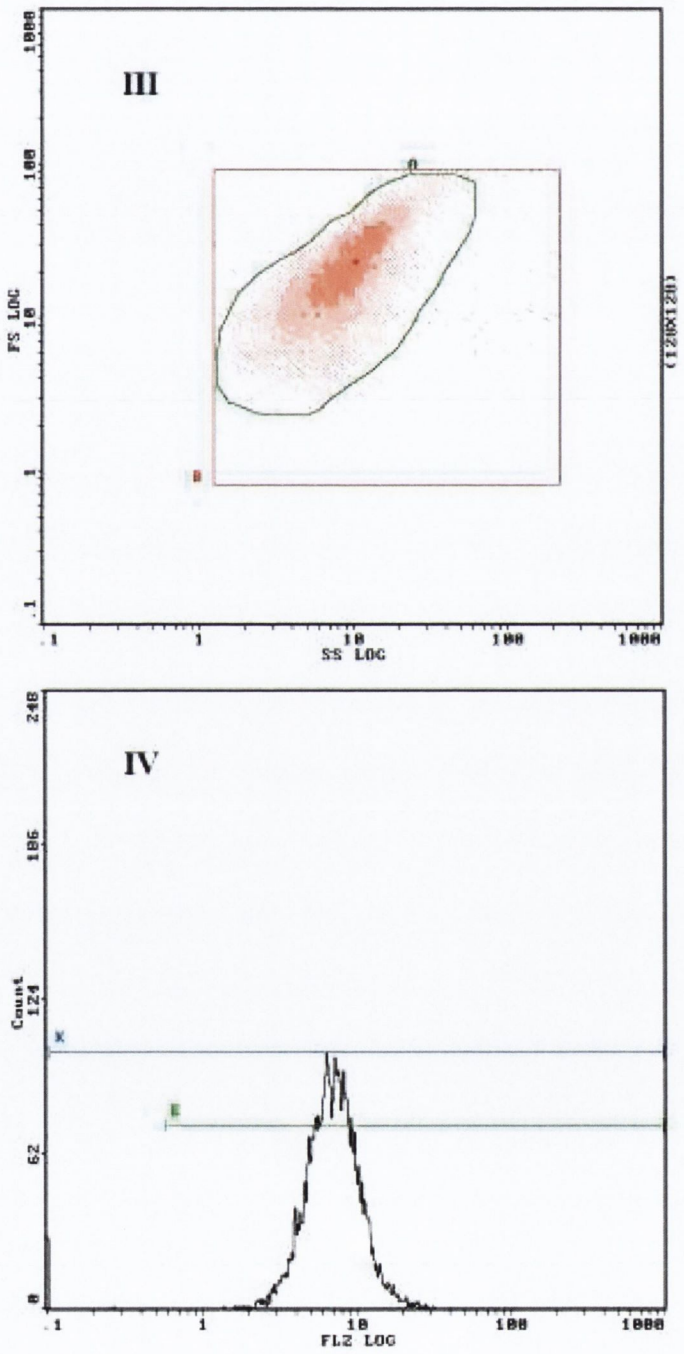


Figure 3-3 Scatterplot and Histogram from a GpIb Test Sample

Figure 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 3-2-I.**

Figure 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. The entire gated region is represented by the blue line in the histogram.**



CD62p and CD63 - Principle

'Activation-dependent' monoclonal antibodies bind minimally, or not at all, to unstimulated platelets, but bind specifically and saturably to activated platelets. (Abrams & Shattil, 1991) CD62P is only expressed on the platelet surface membrane after alpha (α)-or dense-granule secretion, and the CD63 antigen is expressed on the platelet surface after its release from lysosomes or dense granules.(Grau *et al*, 1998;Israels *et al*, 1992) By adding 'activation-dependent' monoclonal antibodies that are conjugated to a fluorochrome and specific for CD62P or CD63, whole blood flow cytometry can be used to quantify the sample fluorescence, and hence, the expression of these activation markers on the platelet surface.

CD62P

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

Reagents

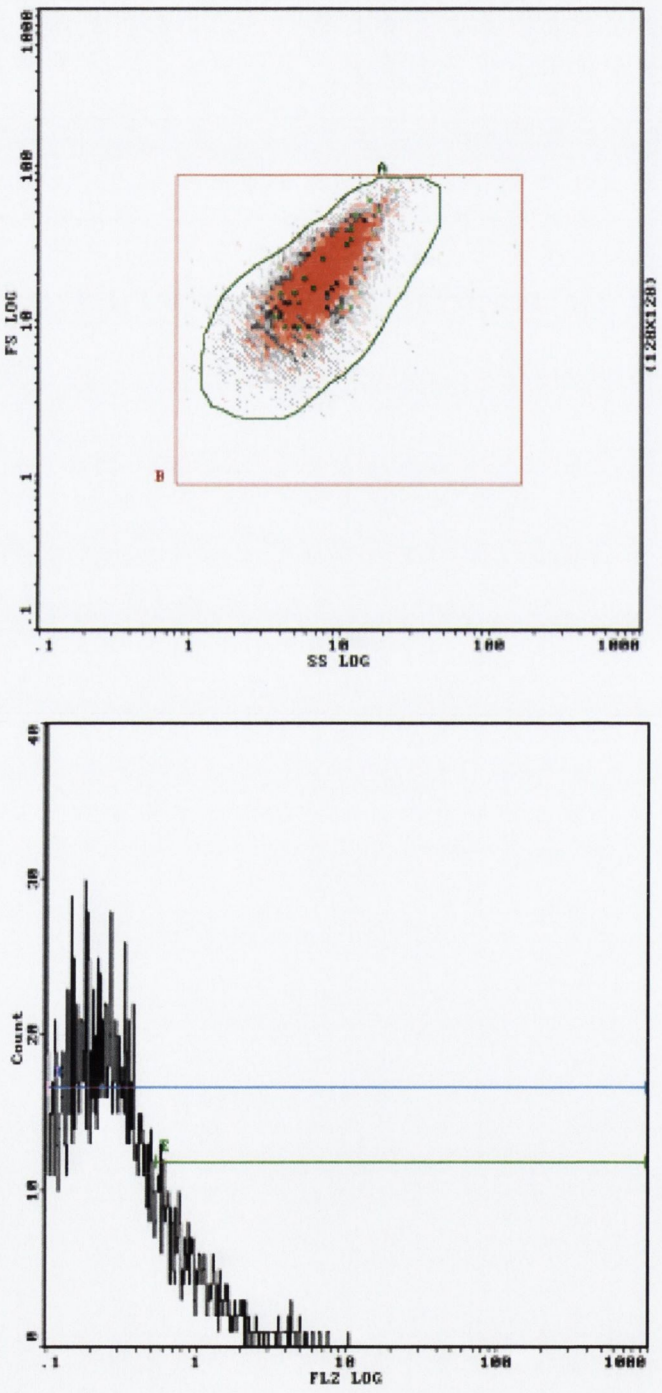
- Anti-IgG_1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25ug/ml)
- Anti-CD62P-PE mouse monoclonal antibody (IgG_1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25ug/ml)
- HBS
- Fixative: 0.2% formalin saline solution

Methods

The initial methodology used for the CD62P assay was identical to that used for the GpIb assay with the exception that 5 µl of anti-IgG_1-PE antibody was added to the isotype control sample and 5 µl of anti-CD62P-PE antibody to the test sample. The platelet cloud was identified on the log SS versus log FS histogram using the gating settings from the GpIb assay. The non-specific fluorescence of the control sample was calculated, and the % CD62P positivity was then measured in FL2 (Figure 3-4).

Figure 3-4 Scatterplot and Histogram from a CD62P Test Sample

Figure 3-4 -I: Scatterplot of log FS versus log SS from a CD62P test sample. Figure 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. The entire gated region is represented by the blue line in the histogram.



CD63

Reagents

- Anti-IgG_1-FITC isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 50 ug/ml)
- Anti-CD63-FITC mouse monoclonal antibody (IgG_1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 50 ug/ml)
- HBS
- Fixative: 0.2% formalin saline solution

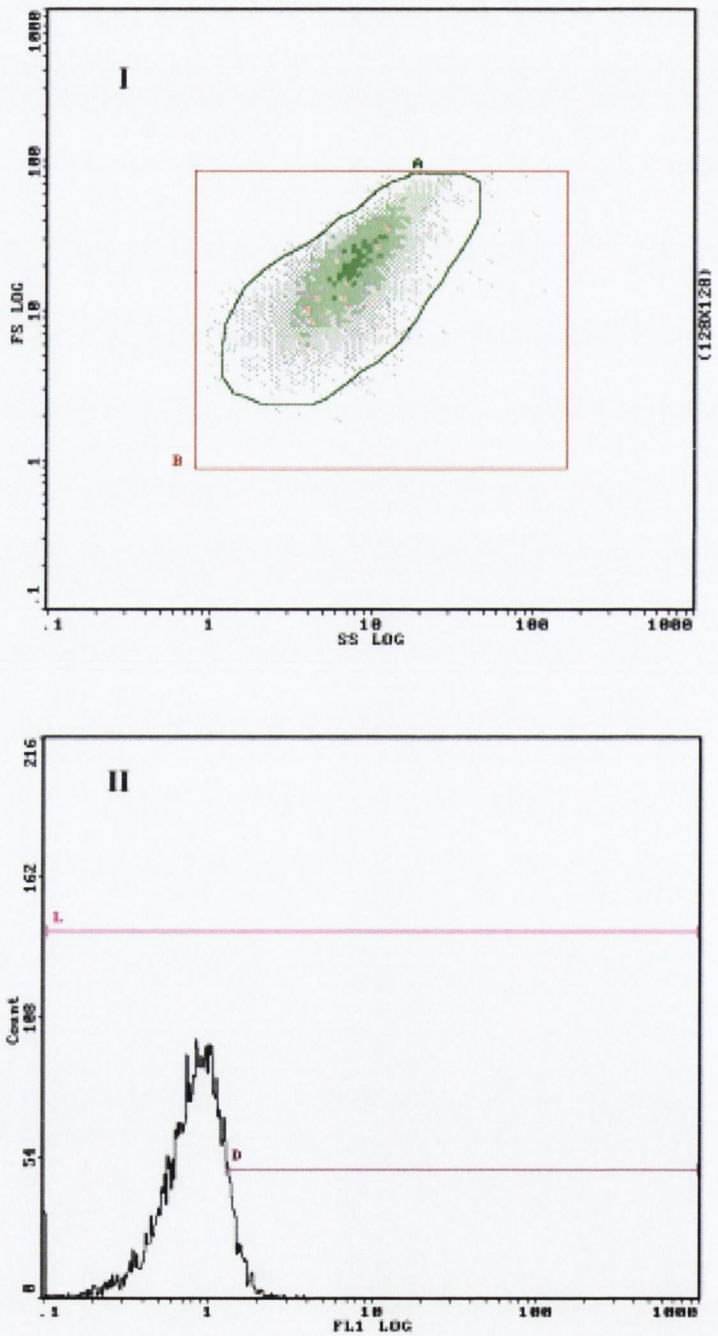
Methods

The initial methodology used for the CD63 assay was also identical to that used for the GpIb assay with the exception that 5 µl of anti-IgG_1-FITC antibody was added to the isotype control sample and 5 µl of anti-CD63-FITC antibody to the test sample. The platelet cloud was identified on the log SS *vs.* log FS histogram using the gating settings from the GpIb assay. The non-specific fluorescence of the control sample was calculated, and the % CD63 positivity was then measured in FL1 (Figure 3-5).

Figure 3-5 Scatterplot and Histogram from a CD63 Test Sample

Figure 3-5 -I: Scatterplot of log FS versus log SS from a CD63 test sample.

Figure 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. The entire gated region is represented by the pink line in the histogram.



3.3.4. Leucocyte-platelet Complexes (LPCs)

Principle

CD62P expressed on the platelet surface membrane mediates the adhesion of platelets to leucocytes, including neutrophils, monocytes and lymphocytes.(de Bruijne-Admiraal *et al*, 1992) The predominant receptor for CD62P on leucocytes is P-selectin glycoprotein ligand-1 (PSGL-1),(Furie *et al*, 2001) and the percentage of leucocytes that are bound to platelets can be measured by flow cytometry.(Li *et al*, 1997;Furman *et al*, 1998) Because CD62P is rapidly shed from the surface of circulating degranulated platelets,(Michelson, 1996) elevated CD62P expression may not be found in patients with platelet activation unless the blood sample is drawn immediately distal to the site of platelet activation, the sample is taken within 5 minutes of the activating stimulus, or there is an ongoing stimulus to platelet activation.(Michelson, 1996) 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, 2006a;Michelson *et al*, 2000;Michelson *et al*, 2001) For these reasons, flow cytometry was used to measure the percentage of leucocyte-platelet aggregates in addition to measurement of the other surface markers of platelet activation.

Reagents

- Anti-IgG_1-PE isotype control mouse monoclonal antibody (Immunotech, Beckman Coulter, Marseille, France. Concentration: 6.25 ug/ml)
- Anti-CD42b-PE mouse monoclonal antibody (IgG_1) (Immunotech, Beckman Coulter, Marseille, France. Concentration: 1.56 ug/ml)
- Anti-CD45-RPE-Cy5 (Dako, Glostrup, Denmark. Concentration: 200 ug/ml)
- HBS
- 10 x Hanks Balanced Saline Solution (HBSS) without calcium, magnesium or phenol red (Gibco BRL, Life Technologies, Paisley, UK)
- Distilled H_2O
- 10% formaldehyde solution – 0.25 ml of 40% formaldehyde solution diluted 1:4 with 0.75ml of distilled H_2O
- Diluent fixative – made up of 0.5 ml of 10% formaldehyde solution, 0.6 ml of 10 x HBSS and 0.9 ml of distilled H_2O.

Method

Because the concentration of the anti-IgG_1-PE isotype control antibody was 6.25 µg/ml, and the concentration of the anti-CD42b-PE monoclonal antibody was 1.56 µg/ml, 5 µl of the anti-IgG_1-PE control antibody was diluted 1:4 with 15 µl of HBS before use in the assay.

The method used was based on one that had been established by Joseph *et al.(Joseph et al, 2001)* Initially, 5 µl of diluted anti-IgG_1-PE control antibody was aliquoted into the isotype control sample tube, and 5 µl of anti-CD42b-PE antibody into the test sample tube. 5 µl of anti-CD45-RPE-Cy5 antibody (a pan-leucocyte marker), followed by 65 µl of HBS were then added to both tubes, the samples were gently mixed, and covered until use. Within five minutes of venepuncture, 25 µl of whole blood was aliquoted into both

the control and test tubes, and the samples were incubated at room temperature for 10 minutes. Then, the samples were fixed with 84 µl of diluent fixative, and after a further 10 minute incubation period, 840 µl of distilled water was added to each tube in order to induce erythrocyte lysis. The samples were analysed on the flow cytometer within three hours of venepuncture. A low flow rate was used to minimise the possibility of detecting dual events i.e. the simultaneous passage of a single leucocyte and a single, unattached platelet through the flow chamber. A protocol was set up in which only CD45-positive events (leucocytes) were detected i.e. a scatterplot of log fluorescence of anti-CD45-RPE-Cy5-positive cells was plotted against side scatter, and a listmode gate employed to exclude red cell debris from the analysis. A scatterplot of SS versus FS was then drawn to further analyse events in the gated region and to identify three distinct subpopulations of leucocytes (i.e. neutrophils, monocytes, and lymphocytes); these were separated by manually drawing a gate around each of the leucocyte subpopulations (Figure 3-6). 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 (Figure 3-7). The assay was stopped after 1,000 monocyte events were detected.

Figure 3-6 Scatterplot from an LPC Test Sample

Figure 3-6 -I: Only anti-CD45-RPE-Cy5-positive cells (leucocytes) are identified in region 'B' with RPE-Cy5 fluorescence detected in FL4.
Figure 3-6 -II: Three distinct leucocyte subpopulations can be identified by their light scatter profiles, and gates drawn around each subpopulation: C = neutrophils, D = monocytes, E = lymphocytes.

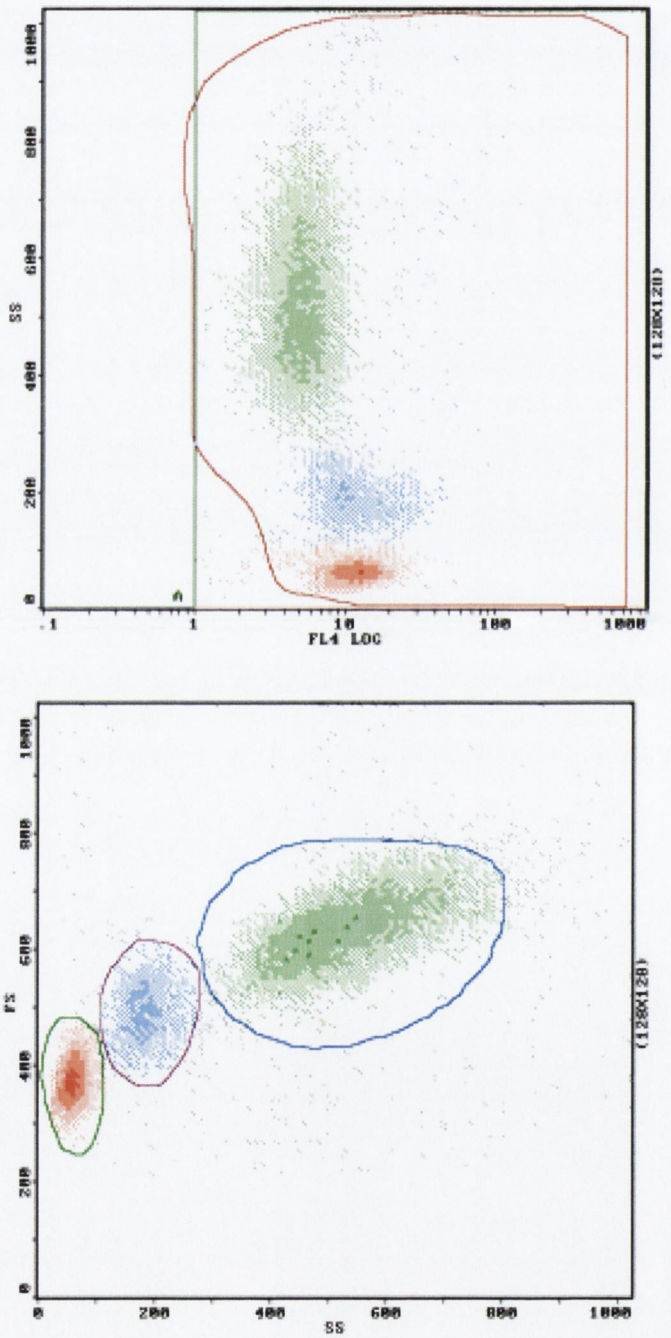
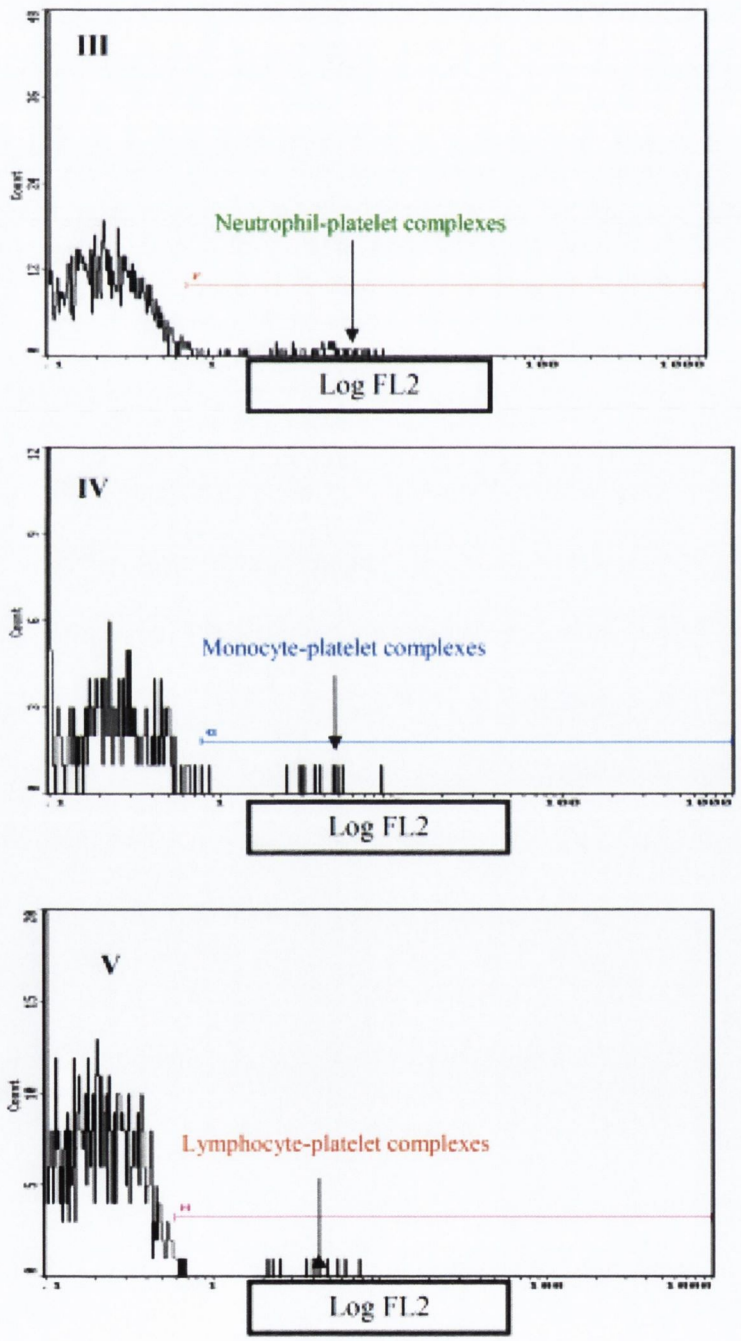


Figure 3-7 Histogram from an LPC Test Sample

Figure 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.



3.3.5. Reticulated platelets

Principle

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

Thiazole orange (TO) is a fluorescent dye originally synthesised for erythrocyte reticulocyte analysis.(Michelson, 1996) It readily permeates live cell membranes without the need for a permeabilisation step, and fluoresces at 533 nm on binding to nucleic acids, especially RNA more than DNA.(Ault *et al*, 1992;Michelson, 1996) Therefore, TO can be used to label reticulated platelets within the circulation. Whole blood flow cytometric methods have been developed to identify reticulated platelets using TO, but there is no 'gold standard' reference method available and no standardised control against which the results obtained can be compared.(Harrison *et al*, 1997;Robinson *et al*, 2000b) It has been shown that high concentrations of TO can non-specifically label dense granules, whereas low concentrations of the dye do not result in non-specific

labelling.(Robinson *et al*, 2000a;Robinson *et al*, 1998;Robinson *et al*, 2000b) A low concentration of TO was therefore used in this assay.

Reagents

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

Methods

Experiments in our laboratory have shown that the uptake of thiazole orange by platelets is stable and reproducible if sample processing begins within 30 to 60 minutes after venepuncture. Subsequent experiments that were performed in the laboratory also showed that the percentage of reticulated platelets remains stable if the sample is stored in the fridge at 4°C, and processing begins between 1 and 6 hours after venepuncture. However, analyses were performed on non-refrigerated samples in this thesis. 1 ml of Isoton® II alone was aliquoted into the control tube, and a 1:10 dilution of Retic-COUNT™ was performed by adding 900 µl of Isoton® II to 100 µl of Retic-COUNT™ in the test sample tube.(Robinson *et al*, 2000a) 5 µl of citrate anticoagulated whole blood was then added to the control and test tubes, respectively, between 30 and 60 minutes after venepuncture. The samples were covered, incubated for exactly 30 minutes and then centrifuged at 1200 g for 2.5 minutes. The supernatant was discarded to prevent further incubation of the test sample with Retic-COUNT™, and the remaining pellet was resuspended in 1 ml of Isoton® II before being analysed on the flow cytometer within an hour of resuspension.

The platelet cloud in the control sample tube was identified on a scatterplot of log FS versus log SS that had been saved on the flow cytometer (Figure 3-8). The non-specific fluorescence of the control sample was calculated, and the % of TO-positive (reticulated) platelets in the test sample was then measured in FL1 (Figure 3-9).

Figure 3-8 Scatterplot of Log FS vs. SS from TO Control and Test Sample.

Figure 3-8 -II: Scatterplot of log FS vs. log SS from a TO control sample identifies platelets within region B by their characteristic light scatter profiles; gate 'A' is manually positioned around the platelet cloud.

Figure 3-8 -III: Scatterplot of log FS vs. log SS from a TO test sample shows that the TO-positive platelets (blue cells) are amongst the largest and most granular in the platelet cloud.

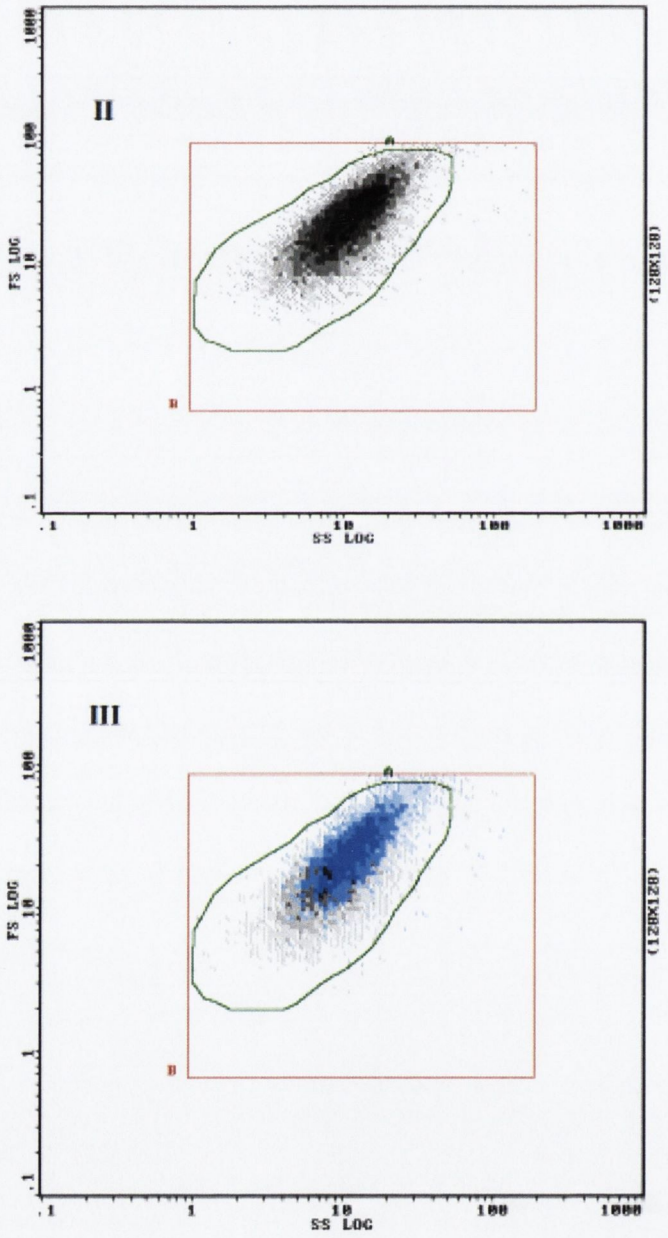
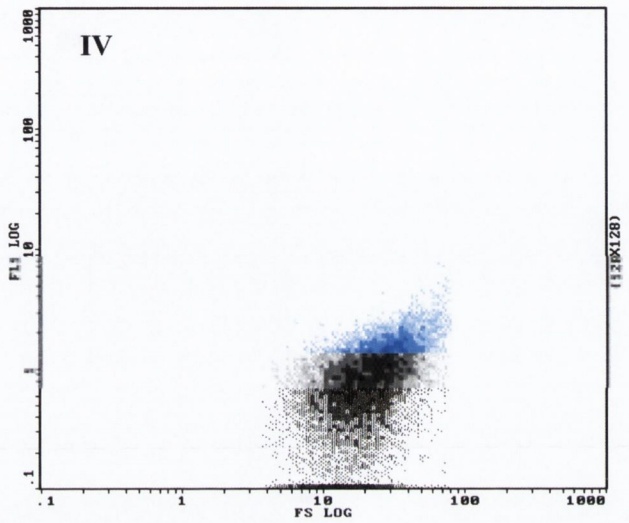
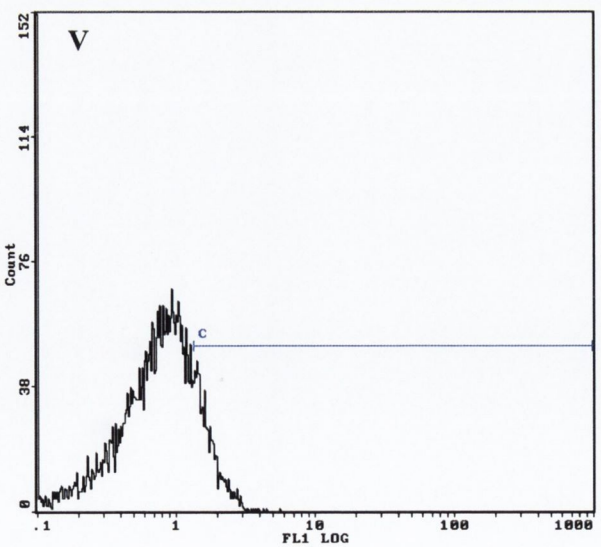


Figure 3-9 Scatterplot of Log FS vs. SS from TO Control and Test Sample

Figure 3-9 -IV: Scatterplot of log fluorescence in FL1 versus log FS from a TO test sample confirms that the TO-positive platelets (blue cells) are amongst the largest of the platelet cloud.

Figure 3-9 -V: Histogram of cell count versus log fluorescence detected in FL1 from the same sample; flow cytometric analysis showed that 16.8% of platelets in the region of interest in this patient sample were TO-positive.





3.4. Platelet Function Analyser - PFA-100®

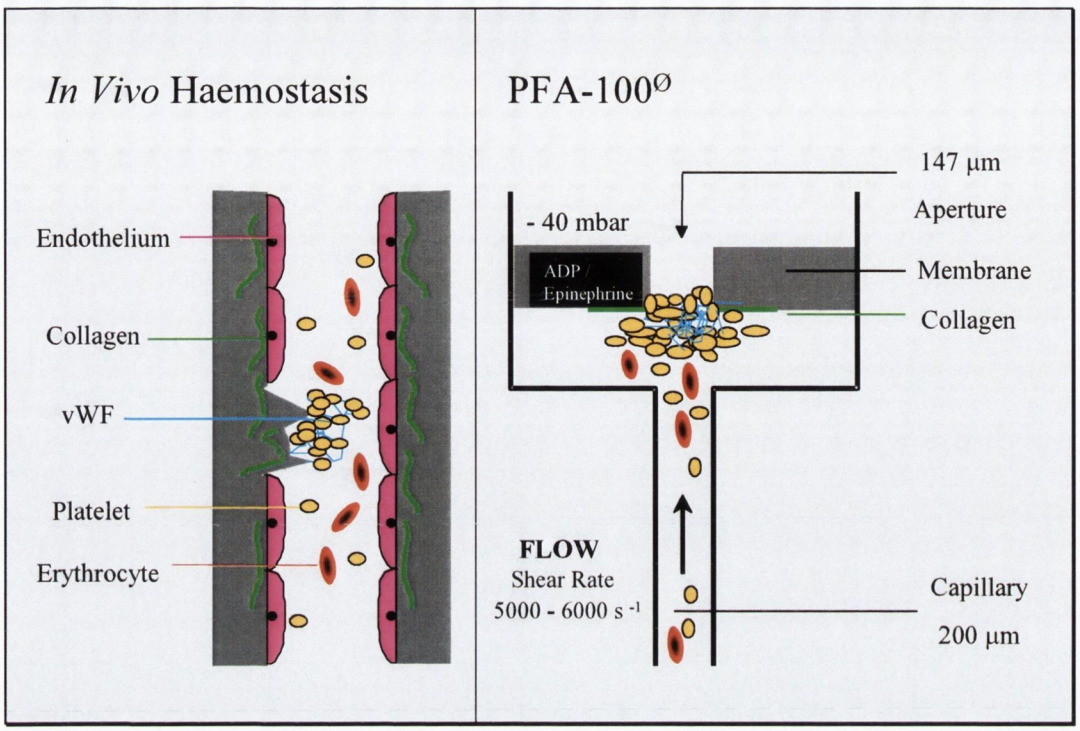
3.4.1. Background

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 in patients with and without carotid stenosis. 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.(Kerenyi *et al*, 1999) Platelet aggregometry has been considered to be the 'gold standard' test of platelet function over the past five decades, and is based on the principle that platelets aggregate in response to exogenous agonists added to the system.(Harrison, 2000;BORN & CROSS, 1963) However, because aggregometry is often performed using platelet rich plasma (PRP), platelet function is not usually studied in the physiological milieu of whole blood. The test also requires a considerable amount of sample preparation, and it is labour intensive and reasonably expensive.

3.4.2. General Principle

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

Figure 3-10 Diagram of the in vivo haemostatic process and the PFA-100®



Legend for Figure3-10:

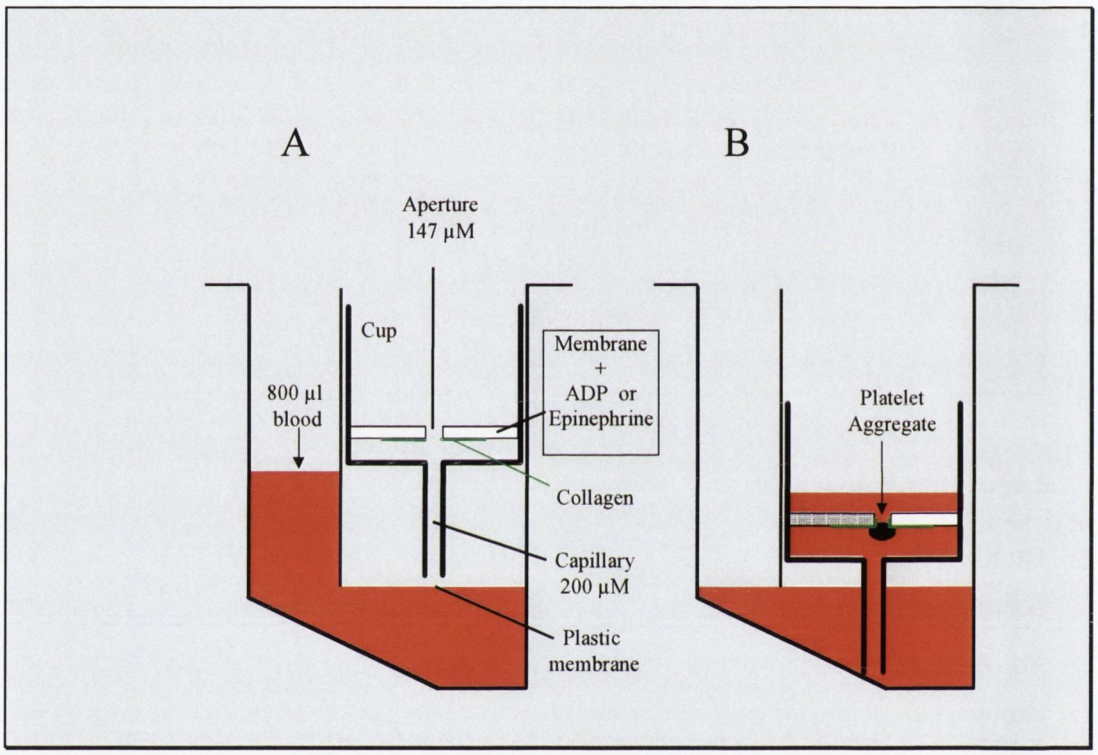
In Vivo Haemostasis, If there is a defect in the endothelial lining of an artery, as may occur if there is rupture of a stenosing atherosclerotic plaque, blood flow at and distal to the stenosis will become turbulent, thus increasing the shear stress that platelets are exposed to. This will in turn,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

PFA-100®, Diagrammatic representation of a PFA-100® cup-capillary system within a test cartridge showing a platelet plug occluding the central aperture of the biologically active membrane (see text) (Figure redrawn from slide kindly donated by Dade-Behring, Germany). The carousel rotates and places the cartridges under the vacuum chuck inside the instrument, and heats the samples to 37°C prior to analysis. The blood sample is aspirated at a moderately high shear rate (5000 to 6000 s^{-1}) through a 200 µm capillary to a nitrocellulose membrane with a central 147 µm aperture (**Figure 3-11**).

Figure 3-11 PFA-100® Cup-capillary system

A: The 800 µl blood sample is loaded into the sample loading port at the start of the test and is separated from the cup-capillary system by a plastic membrane.

B: During the test, the capillary tube is forced down through the membrane into the sample reservoir, and blood is aspirated through the capillary until a platelet plug forms at the aperture (see text) (redrawn from slides kindly donated by Dade-Behring, Germany)



The shear rate that the blood sample is exposed to is equivalent to that seen in a moderately stenosed artery.(Kroll *et al*, 1996) The membrane is coated with collagen (2 µg) in combination with either ADP (50 µg in the C-ADP cartridge) or epinephrine bitartrate (10 µg in the C-EPI cartridge). At the beginning of the test, a predetermined volume of saline trigger solution is dispensed onto the membrane to solubilise the ADP or epinephrine. The combination of high shear stress and biochemical stimulation activates the platelets, they adhere to the membrane and aggregate to one another, thus forming a platelet plug that ultimately occludes the aperture (Figures 3-10 & 3-11).

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)

3.4.3. Quality control

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

3.4.4. 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 $\leq$ 1 minute, and subsequently at 10 and 60 minutes after venepuncture. They reported that the median C-EPI closure time was significantly prolonged at $\leq$ 1 minute after venepuncture, but significantly shortened relative to this baseline level at 10 and 60 minutes after venepuncture using 0.106 M buffered sodium citrate. In contrast, the median C-EPI closure time was persistently prolonged throughout this time period using 0.129 M buffered sodium citrate. The authors suggested that PFA-100® testing for aspirin-induced platelet dysfunction should only be performed using 0.129 M buffered sodium citrate. However, the results obtained from this small subgroup were not consistent with those from a larger group of 80 patients who had discontinued aspirin therapy within the preceding 24 hours, and in whom C-EPI closure times were measured between 10 and 20 minutes after venepuncture. In the larger study, the median C-EPI closure time was prolonged in comparison with normal controls when 0.106 M buffered sodium citrate was used as an anticoagulant, despite the interval to sample analysis of 10 to 20 minutes.

Although these studies were not directly comparable because of the different blood collection systems and concentrations of sodium citrate used, the disparity in results did raise some uncertainty about the effects of the time interval between venepuncture and sample processing on the results obtained. For this reason, all tests in this thesis were performed between 120 and 150 minutes after venepuncture to standardise the time interval between venepuncture and PFA-100® analysis. In addition, because closure times tend to be longer with 0.129 M compared with those seen with 0.105 M or 0.106 M buffered sodium citrate blood collection systems, only 0.105 M (3.2%) buffered sodium citrate Vacutainer® tubes were used in our laboratory.(von Pape *et al*, 2000;Heilmann *et al*, 1997;Mammen *et al*, 1995)

3.5. Routine Haematology Investigations

3.5.1. Full Blood Count

For each study subject, 4 ml of blood was drawn into two 2 ml sterile Vacutainer® tubes containing freeze-dried K_2 EDTA, and a further 3 ml sample was collected into a sterile Vacutainer® tube containing 0.105 M (3.2%) buffered sodium citrate, as described previously. A full blood count was performed in all subjects using a Sysmex XE-2100 haematology analyser (Sysmex U.K. Ltd., Milton Keynes, U.K.). Furthermore, because EDTA causes more platelet swelling over time compared with citrate, and because the sodium citrate solution in the Vacutainer® tube dilutes the sample and reduces the platelet count more than EDTA,(Bath, 1993) measurements of the total platelet count and MPV were performed using both anticoagulants. In addition, because the MPV increases and the platelet count decreases over time with both anticoagulants,(Bath, 1993) it was planned that the FBC measurements would be performed between 2 and 4 hours after venepuncture to standardise the effect of the delay between venepuncture and sample analysis on the results obtained.

3.5.2. Coagulation Assays

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

3.6. Endothelial Activation

3.6.1. General principle

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

In this thesis, I assessed von Willebrand factor antigen (VWF:Ag) and von Willebrand factor propeptide (VWF:Ag II) levels as markers of endothelial activation in patients with asymptomatic carotid stenosis and in patients with early symptomatic carotid stenosis who were followed up to the late phase to assess for differences in levels between the patient groups and to assess for changes in these parameters over time. The nature and functions of these proteins has been discussed in Chapter 7 'Assessment of endothelial activation and the role of von Willebrand Factor (VWF:Ag) and von Willebrand Factor propeptide (VWF:Ag II) in symptomatic and asymptomatic moderate and severe carotid artery stenosis'.

3.6.2. Patient Groups

(a) Patients with asymptomatic ≥50% carotid stenosis were compared with patients with early and late phase ≥50% symptomatic carotid stenosis.

(b) *Nested longitudinal study:* Patients with early symptomatic carotid stenosis were followed up to the late phase after symptom onset or intervention. Symptomatic carotid stenosis patients who were enrolled in the platelet activation and platelet function studies, outlined in Section 0 [Flow Cytometry] and Section 0 [Platelet Function Analyser – PFA-100®], were assessed at each time point: i.e. within 4 weeks of symptom onset (early

phase), and again at > 3 months (late phase) following symptom onset or carotid intervention.

3.6.3. Laboratory Methods

Platelet Poor Plasma (PPP)

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

Enzyme Linked Immunosorbant Assay (ELISA)

Reagents

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

Methods

The concentration of VWF:Ag and VWF:Ag II in each PPP sample was quantified, as previously described.(O'Donnell *et al*, 2005;Preston *et al*, 2009) Double spun PPP that had been collected and prepared as outlined in section 3.2 was warmed to 37°C. Polyclonal Rabbit Anti-Human VWF antibody (DAKO) was used as coating antibody, and polyclonal rabbit Anti-Human VWF/HRP antibody (DAKO) as a detection antibody for the VWF:Ag ELISA. M193902 CLB-Pro 35 coating antibody (Plesmanlaan 125) and M103904HRP CLB-Pro 14.3 detection antibody (Plesmanlaan 125) were used for VWF:Ag II quantification. The ELISA data were quantified by spectrophotometry at 490nm, using a VERSA Max Tuneable Microplate Reader. VWF:Ag and VWF:Ag II levels were recorded as μg/mL.

3.7. Coagulation System Potential

3.7.1. General principle

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

3.7.2. Patient Groups

We performed this study in the subject groups as described in Section 3.6 [Endothelial Activation] above.

3.7.3. Laboratory Methods

Platelet Poor Plasma (PPP)

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

Thrombin Generation Assay:

Reagents

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

Methods

The method is briefly described here, but described in more detail in Chapter 8**Error! Reference source not found.** [Assessment of Coagulation System Potential in Asymptomatic and Symptomatic moderate and severe Carotid Artery Stenosis]. Coagulation system potential was measured, as previously described, in a 96-well polystyrene plate.(Faber *et al*, 2003) Fluo-Substrate was added to the warmed Fluo-Buffer shortly before the experiment to prepare FluCa, and warmed in a water bath at 37°C prior to the experiment.

20 uL of 5pM PPP-Reagent was added to 80 uL of platelet poor plasma in triplicate. Thrombin Calibrator was suspended in 1 ml of deionized water. 20 uL of Thrombin Calibrator was added to 80 uL of platelet poor plasma from the same patient in a fourth well to act as an internal control. The plate was then pre-heated to 37°C for 5 min in the Fluroskan Ascent® microplate fluorometer. The device automatically dispensed 20 µl of

premixed warmed FluCa into each of the wells, so that the final reaction mixture contained 5 pM tissue factor and 4 μM phospholipids.

The Fluroskan Ascent® microplate fluorometer automatically measured the fluorescent signal that corresponded to thrombin generation every 20 seconds. Corrected thrombin generation curves were calculated by the Thrombinoscope™ software, after accounting for the fluorogenic signal from the internal control.

3.8. Transcranial Doppler Ultrasound Detection of Cerebral Microembolic Signals

Details of the methodology employed for cerebral microembolic signal detection by transcranial Doppler ultrasound are described in detail in Chapter 9.

3.9. Carotid Plaque Characterisation on Ultrasound

Details of the methodology employed for carotid plaque morphology characterisation on colour Doppler ultrasound are described in Chapter 10.

3.10. Platelet 'On-Treatment' Reactivity Assessment using the VerifyNow and PFA-100

Details of these methods will be described in more detail in Chapter 11.

3.10.1 Laboratory Methods

Blood was collected from a free-flowing vein via a 21G Butterfly® needle and a Vacutainer® system with a luer adaptor after resting for at least 20 minutes, as described previously.(McCabe *et al*, 2004a) A 3ml lithium-heparin tube was taken first. The next four 2ml 3.2% sodium citrate-anticoagulated tubes were used for assessment of platelet function with modified light transmission aggregometry (VerifyNow Aspirin, P2Y12 and GpIIb/IIIa cartridges). The next two 3ml 3.2% sodium citrate tubes were used for assessment of platelet function at moderately high shear stress with the PFA-100, and for estimation of the platelet count, mean platelet volume (MPV) and platelet distribution width (PDW) in citrate-anticoagulated blood.

The VerifyNow is a cartridge-based platelet function analyser (Figure 3-12) employing a modified light transmission aggregometry paradigm to assess inhibition of platelet function in response to stimulation with different platelet agonists, depending on the cartridge employed: arachidonic acid in the 'Aspirin cartridge' [Accumetrics Inc. VerifyNow-ASA (aspirin assay) package insert. San Diego, CA. 2004 (Pamphlet)]; adenosine diphosphate (ADP), iso-thrombin receptor activating peptide (iso-TRAP), and PAR-4 activating peptide in the '$P2Y_{12}$ cartridge' [Accumetrics Inc. VerifyNow-P2Y12 (P2Y12 assay) package insert. San Diego, CA. 2006, (Pamphlet)]; and iso-TRAP alone which activates the platelet PAR–1 receptor in the 'GpIIb/IIIa assay' [Accumetrics Inc. VerifyNow-GP IIb/IIIa (GP IIb/IIIa assay) package insert. San Diego, CA. 2006, (Pamphlet)]. The PFA-100 activates platelets by exposure to moderately high shear stress (5000-6000 s^{-1}) and biochemical stimulation with collagen and epinephrine (C-EPI cartridge) or ADP (C-ADP cartridge).(Michelson, 2009;Kundu *et al*, 1995) This has already been described in section 3.4. Blood samples were analysed on the VerifyNow GpIIb/IIIa cartridge within 15 minutes of venepuncture. PFA-100 and VerifyNow Aspirin and P2Y12 assays were performed simultaneously between 2–3 hours after venepuncture.

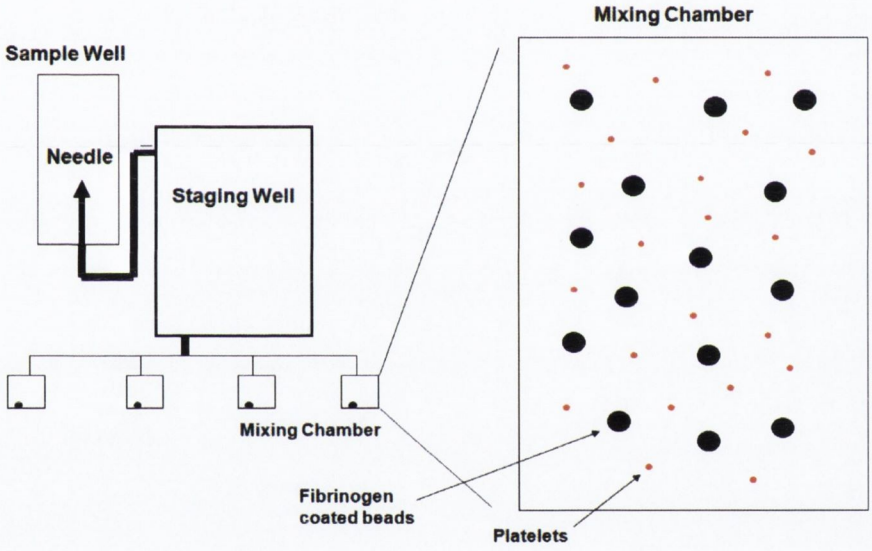
Figure 3-12 VerifyNow® Test Principle

A: A citratre-anticoagulated blood sample is loaded into a sample well at the start of the test and blood is aspirated into a staging well and distributed to mixing chambers containing fibrinogen coated beads and a metal ball to mix the sample.

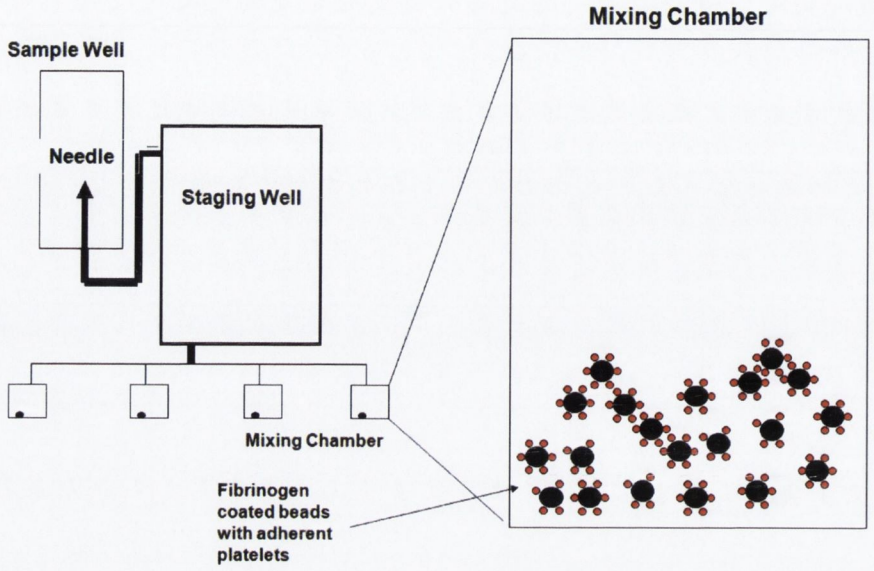
B: During the test, the platelets adhere to the fibrinogen coated beads depending on the degree of platelet function inhibition and fall out of solution.

C: Light from a source passes through the sample and the amount of light detected is dependent on the amount of platelet-fibrinogen bead complexes falling out of solution and is converted to Aspirin Reaction Units in the Aspirin cartridge, P2Y12 Reaction Units in the P2Y12 cartridge or Platelet Aggregation Units in the GP IIb/IIIa cartridge.

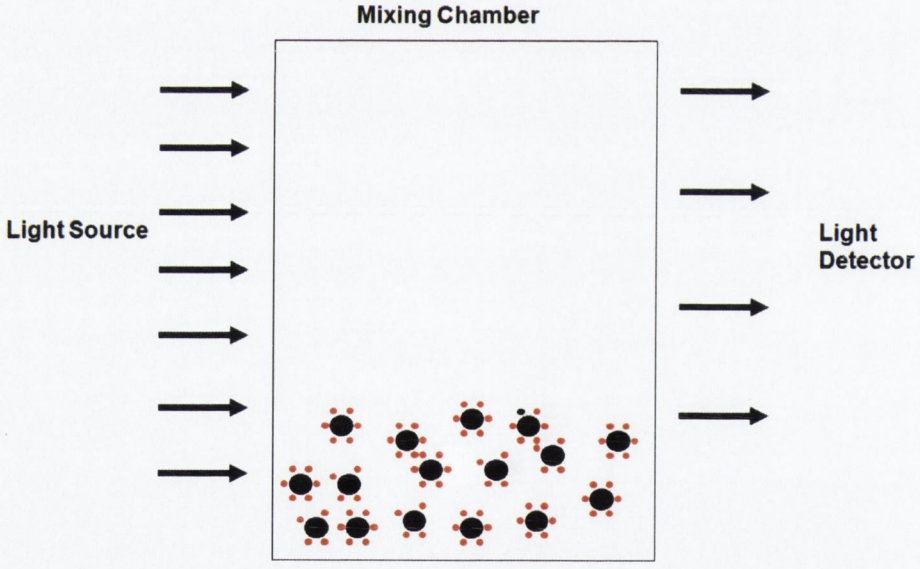
A



B


Mixing Chamber
Sample Well
Needle
Staging Well
Mixing Chamber
Fibrinogen
coated beads
with adherent
platelets

C


Mixing Chamber
Light Source
Light
Detector

3.11. Statistical Methods

The general statistical methods used in Chapters 4 to 10 were similar and are outlined below. Any other analyses specific to relevant chapters will be outlined in that chapter and in Chapter 11.

The primary analysis focused on unpaired comparisons between asymptomatic and early and late phase symptomatic carotid stenosis patients. Subgroup analyses where relevant, were pre-planned for patients on aspirin monotherapy, and for patients with asymptomatic and recently symptomatic severe (≥70%) carotid stenosis. We also performed paired, longitudinal comparisons in symptomatic patients followed up from the early to late phase after symptom onset or carotid intervention. Paired or unpaired t-tests were used for comparison of parametric variables. The Wilcoxon signed rank and Wilcoxon rank sum tests were used for comparison of paired and unpaired non-parametric variables, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. Chi-squared or Fisher exact tests were used to compare proportions between groups. Multiple linear regression analysis was performed to examine the potential influence of independent vascular risk factors on any observed differences between the groups. Based on previous data collected by McCabe et al, it was estimated that one would need 18 - 27 subjects in each group to have a 90% power to detect a 0.5% to 0.6% difference in the mean expression of a platelet activation marker of interest between patients with asymptomatic and symptomatic carotid artery stenosis. $P < 0.05$ was considered statistically significant. All calculations were performed with R version 2.9.1. (R Development Core Team, 2010)

4. Assessment of platelet activation in symptomatic and asymptomatic carotid stenosis subjects by flow cytometry

4.1. Introduction

The cellular mechanisms responsible for the disparity in stroke risk between asymptomatic and symptomatic carotid stenosis patients are not fully understood, but differences in the morphology and/or stability of the carotid plaque,(Golledge *et al*, 2000) interactions between platelets, white cells and the carotid plaque,(Matijevic *et al*, 2011) degrees of endothelial activation, or thrombogenicity of the circulating blood could all play a role.(McCabe *et al*, 2005b) Excessive platelet activation could predispose to carotid plaque 'activation' or thromboembolism from an activated carotid artery plaque, which could contribute to the high risk of recurrent TIA and stroke in recently symptomatic patients, but there are only limited pilot data available on this topic.(McCabe *et al*, 2005b;Cha *et al*, 2003;Jurk *et al*, 2010) It is possible that haemostatic factors, including excessive platelet activation, could predispose to TIA or stroke in a subset of asymptomatic patients who ultimately go on to develop cerebrovascular symptoms.

The aim of this component of the Platelets And Carotid Stenosis (PACS) study was to determine whether there is increased platelet production or activation in patients with recently symptomatic compared with asymptomatic moderate or severe carotid stenosis, and to longitudinally assess platelet activation status in symptomatic patients over time. We hypothesised that recently symptomatic carotid stenosis patients would have higher platelet counts and excessive platelet activation compared with their asymptomatic counterparts, and that platelet activation would decrease in symptomatic patients during follow up, especially following successful carotid intervention.

4.2. Methods

Chapter 3.1 outlines subject groups, inclusion and exclusion criteria for asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound examination (CDUS) using standardised velocity criteria,(Grant *et al*, 2003;Sidhu & Allan, 1997) who were invited to participate. Details regarding relevant clinical assessment and ethical approval are also outlined in that chapter.

4.2.1. Blood Sampling and Laboratory Tests

The blood sampling methods used are outlined in Chapter 3.2. Platelets were distinguished from white and red cells by their characteristic forward and side scatter pattern, and by ensuring that >95% of cells expressed GpIbα (CD42b).(McCabe *et al*, 2004a) Platelet activation was assessed by quantifying platelet surface activation markers (CD62P and CD63 expression) within 90 minutes of venepuncture,(McCabe *et al*, 2004a) and the percentages of circulating neutrophil–platelet, monocyte–platelet, and lymphocyte–platelet complexes within 3 hours of venepuncture.(McCabe *et al*, 2005b;McCabe *et al*, 2004a) using previously described (Shattil *et al*, 1987) and validated methodology.(McCabe *et al*, 2005b;McCabe *et al*, 2004a) The next four tubes were used to prepare platelet poor plasma (stored at -70°C for prospective studies). The seventh citrate-anticoagulated sample was processed to measure the platelet count, MPV and PDW in citrate-anticoagulated blood.

4.2.2. Statistical Methods

These are described in detail in Chapter 3.11.

4.3. Results

Thirty-one asymptomatic and 61 recently symptomatic carotid stenosis patients were initially recruited (Table 4.1). The following symptomatic patients were subsequently excluded: five with paroxysmal atrial fibrillation detected on subsequent ECG monitoring within one month of enrolment; 3 with acute infarction on diffusion-weighted MR brain imaging outside the vascular territory of the stenosed carotid artery of interest; 4 with < 50% internal carotid stenosis on another non-invasive imaging modality (MR or CT angiography) after their CDUS suggested ≥ 50% stenosis; 3 who had actually had TIA or stroke >4 weeks before enrolment. Thus, data from 46 early phase symptomatic patients were analysed, six of whom had symptomatic internal carotid artery occlusion. Of the remaining 40 symptomatic patients in whom intervention could be considered, 28 underwent carotid endarterectomy, one had endovascular treatment with carotid stenting, 6 declined surgical intervention, and the remaining 5 chose optimal medical management based on advice from their physicians.

Thirty-five symptomatic patients attended for late phase follow up. Two developed 50–69% carotid restenosis, and one developed >70% restenosis on repeat CDUS at least 3 months after endarterectomy, but none had experienced recurrent symptoms before follow-up. Eleven symptomatic patients did not have late phase data: 6 declined follow-up, one moved location and could not reattend, one developed active severe symptomatic peripheral vascular disease, and another ongoing active inflammatory gouty arthropathy, precluding laboratory reassessment; 2 died from unrelated causes (one from respiratory sepsis and one from cholecystitis).

There was a higher prevalence of current smokers and a lower prevalence of hypertension amongst symptomatic than asymptomatic patients, and a lower prevalence of statin use

amongst early symptomatic than asymptomatic patients. Otherwise, demographic and vascular risk profiles were similar in each group (Table 4.1).

FBC Parameters:

The median platelet count was significantly higher in citrate- (211 *vs.* 200 $x10^9$/L; p = 0.03), but not EDTA-anticoagulated blood (264 *vs.* 239 $x10^9$/L; p = 0.055) in early symptomatic than asymptomatic patients (Table 4.2). There were no differences in other FBC parameters between asymptomatic and symptomatic patients (Table 4.2).

Platelet activation status:

There were no significant differences in CD62P or CD63 expression, or % neutrophil-platelet or monocyte-platelet complexes between asymptomatic and symptomatic patients at any stage (Table 4.3). However, the median % lymphocyte-platelet complexes was higher in early symptomatic than asymptomatic patients (2.8 *vs.* 2.4%; p = 0.001) (Table 4.3).

After controlling for differences in the prevalence of hypertension, statin use and smoking between groups, the median platelet count in citrate-anticoagulated blood remained higher (p=0.045), and median % lymphocyte-platelet complexes remained higher (p=0.037) in early symptomatic than asymptomatic patients.

Subgroup analyses

Because different antiplatelet regimens could potentially have influenced the observed differences in platelet count or activation status between asymptomatic and symptomatic patients (Table 4.1), a pre-planned subgroup analysis was performed in asymptomatic and symptomatic patients on aspirin monotherapy. The differences in platelet count in

citrate-anticoagulated blood between asymptomatic and early symptomatic patients on aspirin monotherapy was not statistically significant (208 *vs* $194x10^9$/L; p=0.07). However, % lymphocyte-platelet complexes remained significantly higher in early symptomatic than asymptomatic patients on aspirin monotherapy (p = 0.013) (Table 4.4). CD63 expression in late symptomatic patients was lower than in the asymptomatic group on aspirin, but this comparison just reached statistical significance (p = 0.048). There were no other differences in FBC parameters or platelet activation status between groups on aspirin monotherapy.

We also compared FBC parameters and platelet activation between the subgroup of asymptomatic (n = 20) and early symptomatic patients (n = 33) who had ≥70% carotid stenosis. Median % lymphocyte-platelet complexes was elevated in early symptomatic than asymptomatic patients (2.86% vs 2.43%; p = 0.0005). Otherwise, there were no other significant differences in platelet activation or FBC parameters between early or late symptomatic versus asymptomatic patients ($p \geq 0.1$).

When we assessed the subgroup of early symptomatic patients who had their TIA or stroke within 7 days of study inclusion (n=23), median % lymphocyte-platelet complexes were higher in early symptomatic than asymptomatic patients (2.8 *vs*. 2.4%; p = 0.028). There were no other differences between the groups for the other measured markers of platelet function or activation ($p \geq 0.45$).

With the exception of a slight decrease in mean haemoglobin in the late compared with the early phase after symptom onset (13.53 *vs*. 13.85 g/dl, p = 0.041), there were no changes in FBC parameters (Table 4.5) or platelet activation over time, or after carotid intervention in the subgroup of symptomatic patients with follow-up data at each

timepoint. However, the number of subjects included in these subgroup analyses was limited, so these data must be interpreted with caution.

4.4. Discussion

To our knowledge, this is the largest multicentre, observational, study to simultaneously assess platelet, white cell and red cell parameters, and platelet activation status in moderate or severe asymptomatic versus symptomatic carotid stenosis in both a cross-sectional and longitudinal manner. These findings improve our understanding of the haemostatic and thrombotic profile in patients with moderate-severe carotid stenosis, and of the potential interaction between the 'activated carotid plaque' and circulating platelets and leucocytes.

The finding of a higher platelet count in early symptomatic than asymptomatic moderate or severe carotid stenosis indicates enhanced platelet production early after TIA or stroke, and is in keeping with the findings of a smaller pilot study in a similar patient population with severe carotid stenosis.(McCabe *et al*, 2005b) Although we did not confirm that the platelet count was elevated in our subgroup of patients with severe carotid stenosis compared with the asymptomatic group, this may reflect a type II error due to the smaller number of subjects included in this analysis. It is possible that carotid stenosis patients at risk of developing symptoms have a higher platelet count than those at lower risk, but this study was not prospectively designed to address this issue. The lack of significant differences in platelet counts between late phase symptomatic and asymptomatic patients could reflect resolution of the acute phase response during prolonged clinical follow-up or following intervention, or could also reflect a type II error because only 35/46 symptomatic patients had late phase follow-up data. Our longitudinal subgroup data suggest that this study was probably underpowered to detect changes between late symptomatic (n = 35) and asymptomatic patients (n = 31) because the platelet count did not fall significantly during follow-up in the subgroup of symptomatic patients assessed

in both the early and late phases after TIA or stroke onset, or before and after carotid intervention (Tables 4.2 and 4.5).

There was a small, statistically significant fall in haemoglobin in symptomatic patients in the late phase compared with the early phase after TIA or stroke. Although this may reflect an initial increased stimulus to red cell production in patients with recently symptomatic carotid stenosis, the 0.32 g/dl difference in haemoglobin concentration is unlikely to be of *clinical significance* in carotid stenosis patients.

Elevated leucocyte–platelet complexes, including neutrophil-platelet, monocyte-platelet, and lymphocyte-platelet complexes have previously been reported in a pilot study in severe symptomatic versus asymptomatic carotid stenosis.(McCabe *et al*, 2005b) In our subgroup analysis, lymphocyte-platelet complexes were elevated in early symptomatic versus asymptomatic severe carotid stenosis ($p = 0.0005$), consistent with these prior data.(McCabe *et al*, 2005b) The finding of an increased platelet count, taken together with increased circulating lymphocyte-platelet complexes, are consistent with enhanced platelet production and activation early after TIA or stroke in symptomatic carotid stenosis. Platelets and leucocytes may have excitatory effects on one another, with leucocyte-platelet complex formation facilitating interaction between the two cell types.(Li *et al*, 2000;Grau *et al*, 1994) The formation of leucocyte-complexes may therefore increase the risk of thrombus formation or plaque inflammation, and predispose to recurrent cerebrovascular events due to embolism from the thrombotic milieu of an activated plaque in patients with symptomatic carotid stenosis. Although platelet activation could arise in response to acute ocular or cerebral ischaemia or infarction, the lymphocyte-platelet complex data may also indicate that recently symptomatic patients have pre-existing excessive levels of platelet activation compared with their

asymptomatic counterparts. Further longitudinal studies in asymptomatic patients are warranted to address this issue.

Our study suggests that measurement of the % leucocyte-platelet complexes with unstimulated whole blood flow cytometry is a more sensitive method of assessing platelet activation status than measurement of % CD62P or CD63 expression alone in recently symptomatic carotid stenosis. However, a recent cross-sectional and case-control study found elevated soluble P-selectin, and surface CD62P and CD63 expression in symptomatic >70% compared with asymptomatic >50% carotid stenosis patients ($p < 0.05$).(Jurk *et al*, 2010) This apparent disparity in results between Jurk *et al.*(Jurk *et al*, 2010) and our group may reflect differences in demographic profiles, antithrombotic therapy, time from symptom onset and stenosis severity in the symptomatic groups, and differences in methodology employed to quantify leucocyte-platelet complexes. However, each study provides important evidence of enhanced platelet activation in symptomatic compared with asymptomatic carotid stenosis.

Despite trends towards increased levels, we did not find statistically significant increases in neutrophil-platelet or monocyte-platelet complexes in early symptomatic compared with asymptomatic patients. Only 23 (50%) of early symptomatic patients experienced symptoms within one week of enrolment, and the remainder were recruited over the subsequent 3 weeks after TIA or stroke. The % lymphocyte-platelet complexes may have been predominantly increased due to binding of activated platelets to more 'chronic inflammatory' leucocytes i.e. lymphocytes, with less pronounced binding to more 'hyperacute-phase' leucocytes, such as neutrophils or monocytes during the initial 4 week recruitment period, but this is speculative. There are no data available on the precise half-life of individual leucocyte-platelet complexes *in vivo* in humans. However, Marquardt *et*

al. comprehensively assessed leucocyte-platelet complexes in 45 patients within 24 hours of acute ischaemic stroke and compared these data with those from 30 healthy age- and sex- matched controls.(Marquardt *et al*, 2009) They found elevated neutrophil-platelet complexes from days 1 – 10 following stroke, and monocyte-platelet complexes on day 2 following stroke in patients versus controls. Lymphocyte-platelet complexes were not elevated at any time point up to 90 days after symptoms in their patients versus controls, although it must be emphasised that only 23% of their patients had stroke due to large artery atherosclerosis. Michelson *et al* reported that the half-life of circulating monocyte-platelet complexes (30 minutes) is longer than neutrophil-platelet complexes (5 minutes) *in vivo* in baboons; lymphocyte-platelet complexes were not assessed in that study.(Michelson *et al*, 2001)

Our late phase findings may reflect resolution of the acute phase response or successful removal of the stenosing plaque in a large proportion of symptomatic cases, but for the reasons mentioned above, it is possible that the lack of significant differences in % lymphocyte-platelet complexes between late symptomatic and asymptomatic patients reflects a type II error.

4.5. Conclusions

Larger, longitudinal studies are warranted to determine whether there is an ongoing stimulus to increased platelet production and activation in symptomatic carotid stenosis patients, and whether the platelet count and % leucocyte-platelet complexes could be used as potential biomarkers to risk-stratify carotid stenosis patients. Such findings could facilitate improved, targeted, individualised primary and secondary preventive treatment of patients with asymptomatic and symptomatic carotid stenosis.

Table 4.1: Demographic and vascular risk factor profile of patients. P values relate to chi-squared or Fisher exact testing between asymptomatic and symptomatic carotid stenosis groups. Values are means (±SD) or absolute values. Significant values are highlighted in bold.

Parameter	Asymptomatic Carotid Stenosis (n = 31)	Early Symptomatic Carotid Stenosis (n=46)	p	Late Symptomatic Carotid Stenosis (n=35)	p
Mean age (years)	68.2 [± 7.95]	65.0 [± 9.58]	0.78	65.0 [±9.9]	0.78
Gender (M; %)	18 (58%)	28 (61%)	0.8	20 (57%)	0.94
Median interval from symptom onset (days; range)	N/A	7.5 (0 - 27)		175 (99 – 360)	
Degree of Stenosis:					
(Moderate:≥50 – 69%)	11 (35%)	7 (15%)	**0.039**	15 (43%)	0.54
(Severe: ≥ 70 – 99%)	20 (65%)	33 (72%)	0.50	9 (26%)	**0.0015**
(Occlusion)	0	6 (13%)	**0.04**	4 (11%)	0.07
Antiplatelet therapy:					
Aspirin monotherapy	22 (71%)	35 (76%)	0.62	15 (43%)	**0.02**
Aspirin / Dipyridamole combination	2 (6%)	4 (9%)	0.54	11 (31%)	**0.01**
Clopidogrel monotherapy	5 (16%)	2 (4%)	0.09	6 (17%)	0.6
Aspirin /Clopidogrel combination	2 (6%)	5 (11%)	0.4	3 (9%)	0.6
Ischaemic heart disease	7 (23%)	10 (22%)	0.93	7 (20%)	0.8
Hypertension	27 (87%)	29 (63%)	**0.02**	23 (66%)	**0.04**
Diabetes Mellitus	7 (23%)	8 (17%)	0.57	6 (17%)	0.58
Prior TIA/Stroke before index event	8 (26%)	7 (15%)	0.25	6 (17%)	0.39
Prior venous thromboembolism	1 (3%)	0	0.4	0	0.5
Peripheral Vascular Disease	5 (16%)	5 (11%)	0.5	6 (17%)	0.91
Migraine (with or without aura)	6 (19%)	5 (11%)	0.3	5 (14%)	0.58
Family History Stroke	9 (29%)	16 (35%)	0.6	12 (34%)	0.65
Current smokers	5 (16%)	21 (46%)	**0.007**	14 (40%)	**0.03**
Ex-smoker	22 (71%)	17 (37%)	**0.003**	13 (37%)	**0.006**
Never smoker	4 (13%)	8 (17%)	0.59	8 (23%)	0.3
Statin therapy	28 (90%)	33 (72%)	**0.043**	27 (77%)	0.13

Table 4.2: FBC parameters. Values are means (±SD) or medians (25th - 75th percentile). P values refer to comparison between asymptomatic and symptomatic groups/subgroup.

	Asymptomatic (n=31)	Early Symptomatic (n=46)	p	Late Symptomatic (n=35)	p	Late Symptomatic Post-intervention Subgroup(n=23)	p
Platelet Count ($x10^9$/L) (EDTA)	239 (216 – 249)	264 (219 – 307)	0.055	251 (196 – 308)	0.12	251 (197 - 308)	0.21
Platelet Count ($x10^9$/L) (Citrate)	200 (179 – 217)	211 (187 – 273)	**0.03**	219 (167 – 255)	0.10	219 (170 – 249)	0.14
Platelet Distribution Width % (EDTA)	12.8 (±1.9)	12.9 (±1.9)	0.82	13.1 (±1.9)	0.6	13.4 (±1.7)	0.26
Platelet Distribution Width % (Citrate)	11.2 (±1.6)	10.9 (±1.6)	0.49	11.2 (±1.5)	0.88	11.4 (±1.5)	0.56
Mean Platelet Volume (fl) (EDTA)	10.7 (±0.9)	10.7 (±1.0)	0.77	10.8 (±0.9)	0.47	11.0 (±0.8)	0.19
Mean Platelet Volume (fl) (Citrate)	9.8 (±0.8)	9.7 (±0.9)	0.56	9.8 (±0.7)	0.83	9.9 (±0.7)	0.5
Haemoglobin (g/dl) (EDTA)	13.9(±1.4)	13.7 (±1.3)	0.59	13.5 (±1.1)	0.22	13.4 (±1.0)	0.11
WCC ($x10^9$/L) (EDTA)	7.4 (±1.8)	8.0 (±1.6)	0.17	7.6 (±2.0)	0.72	7.7 (±2.3)	0.70
Neutrophils ($x10^9$/L) (EDTA)	4.4 (3.7 – 4.9)	4.8 (3.9 – 5.6)	0.09	4.6 (3.6 – 5.7)	0.74	4.6 (3.6 – 5.7)	0.77
Monocytes ($x10^9$/L) (EDTA)	0.65 (±0.2)	0.7 (±0.2)	0.6	0.65 (±0.23)	0.97	0.64 (±0.3)	0.78
Lymphocytes ($x10^9$/L) (EDTA)	1.87 (1.5 – 2.5)	2.1 (1.5 – 2.6)	0.52	2.06 (1.6 – 2.4)	0.8	2.1 (1.6 – 2.5)	0.78

Table 4.3: Platelet activation markers. Values are medians (25th - 75th percentile). P values refer to comparison between asymptomatic and symptomatic groups/subgroup.

Marker	Asymptomatic (n=31)	Early Symptomatic (n=46)	p	Late Symptomatic (n=35)	p	Late Symptomatic Post-intervention Subgroup (n=23)	p
%CD62P	1.97 (1.19 – 2.44)	1.88 (1.29 – 2.5)	0.88	1.87 (1.31 – 2.76)	0.46	1.89 (1.39 – 2.74)	0.52
%CD63	11.0 (8.86 – 15.6)	9.78 (7.86 – 15.6)	0.64	10.1 (7.6 – 12.7)	0.1	8.95 (6.9 – 11.9)	0.051
% Neutrophil-Platelet Complexes	2.72 (2.4 – 3.1)	2.9 (2.5 – 3.4)	0.22	2.76 (2.4 – 3.4)	0.53	2.83 (2.42 – 3.36)	0.46
% Monocyte-Platelet Complexes	4.6 (4.3 – 5.6)	5.5 (4.0 – 6.8)	0.3	5.0 (4.1 – 5.9)	0.69	5.0 (4.5 – 6.5)	0.34
% Lymphocyte-Platelet Complexes	2.4 (1.8 – 2.7)	2.8 (2.4 – 3.3)	**0.001**	2.5 (2.2 – 3.0)	0.21	2.5 (2.1 – 2.8)	0.19

Table 4.4: Platelet activation status in patients on aspirin monotherapy. Values are medians (25th - 75th percentile). P values refer to comparison between asymptomatic and symptomatic groups/subgroup.

Marker	Asymptomatic (n=22)	Early Symptomatic (n=30)	p	Late Symptomatic (n=15)	p	Late Symptomatic Post-intervention Subgroup (n=10)	p
%CD62P	1.97 (1.2 – 2.4)	1.96 (1.4 – 2.7)	0.82	1.89 (1.4 – 2.5)	0.71	1.95 (1.57 – 2.67)	0.64
%CD63	11.15 (9.1 – 16.8)	9.16 (7.5 – 14.7)	0.087	8.95 (8.2 – 11.6)	**0.048**	8.9 (8.4 – 12.1)	0.06
% Monocyte-Platelet Complexes	4.5 (3.9 – 5.5)	5.35 (3.8 – 6.7)	0.3	5.0 (3.7 – 6.7)	0.76	5.0 (4.1 – 6.7)	0.42
% Neutrophil-Platelet Complexes	2.8 (2.3 – 3.2)	3.0 (2.6 – 3.4)	0.2	2.6 (2.4 – 3.5)	0.69	2.6 (2.4 – 3.5)	0.59
% Lymphocyte-Platelet Complexes	2.5 (1.7 – 2.8)	2.8 (2.4 – 3.4)	**0.013**	2.3 (2.2 – 2.6)	0.77	2.4 (2.2 – 2.6)	0.62

Table 4.5: Matched FBC parameters in early versus late symptomatic subjects with data at both time points. Values are means (±SD). Significant P values in bold.

	Early Symptomatic(n=35)	Late symptomatic (n=35)	p value
Platelet Count ($x10^9$/L) (EDTA)	260.1 (±67.2)	256.5(±63.9)	0.67
Platelet Count ($x10^9$/L) (Citrate)	221.5 (±57.1)	218 (±56.2)	0.55
Platelet Distribution Width % (EDTA)	13.2 (±1.6)	13.1 (±1.88)	0.57
Platelet Distribution Width % (Citrate)	11.15(±1.3)	11.24(±1.5)	0.54
Mean Platelet Volume (fl) (EDTA)	10.87(±0.85)	10.83(±0.91)	0.66
Mean Platelet Volume (fl) (Citrate)	9.77(±0.71)	9.81(±0.74)	0.62
Haemoglobin (g/dl) (EDTA)	13.85(±1.05)	13.53(±1.1)	**0.041**
WCC ($x10^9$/L) (EDTA)	7.99(±1.64)	7.61(±2.0)	0.27
Neutrophils ($x10^9$/L) (EDTA)	5.05(±1.51)	4.66(±1.5)	0.22
Monocytes ($x10^9$/L) (EDTA)	0.66(±0.2)	0.65(±0.23)	0.91
Lymphocytes ($x10^9$/L) (EDTA)	2.07(±0.68)	2.05(±0.68)	0.83

5. Assessment of on-treatment platelet reactivity in asymptomatic and symptomatic carotid artery stenosis using the PFA-100®

5.1. Introduction

There is good evidence that platelets are excessively activated/hyper-reactive in patients with TIA and ischaemic stroke compared with controls, (McCabe *et al*, 2004a;McCabe *et al*, 2004b;McCabe *et al*, 2005b;Jurk *et al*, 2010;Cha *et al*, 2003;Yip *et al*, 2006;Zeller *et al*, 1999) with very limited data in patients with recently symptomatic compared with asymptomatic moderate or severe carotid stenosis.(McCabe *et al*, 2005b;Jurk *et al*, 2010;Cha *et al*, 2003) Therefore, antiplatelet therapy has the potential to reduce the risk of subsequent vascular events in patients with ischaemic cerebrovascular disease, including in the important subgroup of patients with carotid stenosis.

85% of all patients with symptomatic carotid stenosis in NASCET were on antithrombotic medications, mostly aspirin, (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991) and all patients with asymptomatic >60% carotid stenosis in ACAS were on 325mg of aspirin daily during follow up.(Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995) Despite antiplatelet therapy in the majority of patients, the 2-year risk of ipsilateral stroke in patients with symptomatic severe carotid stenosis was as high as 26% in NASCET,(North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991) and 4% in asymptomatic patients in ACAS.(Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995)

There is some evidence that short-term treatment with aspirin-clopidogrel combination antiplatelet therapy appears more effective than aspirin monotherapy at preventing microemboli on TCD in patients with recently symptomatic extracranial carotid stenosis.(Markus *et al*, 2005) However, the incidence of subsequent TIA or stroke during short-term (7 day) follow-up was not significantly lower with aspirin and clopidogrel (7.8%) than with aspirin monotherapy (19.6%, $p > 0.05$). Recent data also suggest that combination treatment with aspirin and dipyridamole appears to be as effective at reducing microemboli on TCD as treatment with aspirin and clopidogrel, but the number of subjects included in this study was far too small to make any definitive conclusions about optimal antiplatelet therapy in this patient population.(King *et al*, 2011) The Asymptomatic Cervical Bruit Study investigated the outcome of treatment with aspirin compared with placebo in patients with asymptomatic ≥50% carotid stenosis.(Cote *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). There was no significant difference in the incidence of TIA, stroke, myocardial infarction (MI), unstable angina, or death between the aspirin and placebo groups over the 2.4 years of follow-up. Aspirin did not significantly reduce the overall risk of stroke versus placebo (5.8% *vs.* 5.4%), and the reduction in the rate of TIA with aspirin (8.5% *vs.* 12.5%) was not significant. 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. Because patients with asymptomatic carotid stenosis are at risk of subsequent vascular events, the majority of patients in clinical practice are still empirically treated with aspirin.

The use of platelet function monitoring is gaining momentum in patients with vascular disease. Coronary artery disease patients on antiplatelet therapy who are deemed to have 'high on-treatment platelet reactivity' (HTPR) or 'non-responsiveness' on an *ex vivo* test of platelet function have been shown to have a higher risk of adverse clinical outcome events than those without HTPR.(Breet *et al*, 2010b;Bonello *et al*, 2010;Price *et al*, 2011) However, the definition of HTPR on various platelet function devices varies between studies, and is also influenced by whether the tests are performed in whole blood or platelet rich plasma (PRP).(Price *et al*, 2011;Bonello *et al*, 2010;Breet *et al*, 2010b;Lordkipanidze *et al*, 2007;McCabe *et al*, 2005a;Alberts *et al*, 2004;Gengo *et al*, 2008;Harrison *et al*, 2005)

As outlined in Chapter 3, a number of studies have assessed 'peri-procedural' platelet function and HTPR around the time of carotid intervention in patients with predominantly symptomatic carotid stenosis.(Assadian *et al*, 2008;Webster *et al*, 2004;Robless *et al*, 2002;Payne *et al*, 2004;Vogten *et al*, 2008;Hayes *et al*, 2003) Preliminary, hypothesis-generating, subgroup data analysis from one study suggested that, compared with controls, the prevalence of HTPR reduced in patients with severe carotid stenosis who were followed up from the early (≤4 weeks) to late phase (≥ 3 months) after symptom onset or intervention.(McCabe *et al*, 2005a) Seven of 11 (64%) patients in the early phase, and 1/9 (11.1%) in the late phase had high on-treatment platelet reactivity / 'aspirin non-responsiveness' on the PFA-100. Six of these nine patients in the late phase had undergone carotid endarterectomy or stenting before repeat testing, potentially explaining in part why the vast majority of these patients subsequently became more responsive to aspirin *ex vivo*.(McCabe *et al*, 2005a) To date, no adequately powered studies or clinical trials have compared the prevalence of *ex vivo* HTPR in whole blood between asymptomatic and early and late phase symptomatic carotid

stenosis patients, or longitudinally assessed platelet reactivity during medium-term follow up in patients with symptomatic carotid stenosis who have and have not undergone intervention.

Ex vivo laboratory data also have the potential to enhance our understanding of the biological basis of recurrent vascular events in patients on antiplatelet therapy, thus facilitating optimisation of antiplatelet therapy and improvements in primary and secondary stroke prevention in patients with carotid stenosis.

Aims

The aims of this component of the Platelets And Carotid Stenosis (PACS) study was to determine whether HTPR on a moderately high shear stress test of platelet function was more common in patients with recently symptomatic than asymptomatic moderate or severe carotid stenosis, and to longitudinally assess HTPR in symptomatic patients over time.

Hypotheses

We hypothesised that recently symptomatic carotid stenosis patients would be more likely to have HTPR than their asymptomatic counterparts. We also hypothesised that the prevalence of HTPR would decrease in symptomatic patients during follow up, especially following successful carotid intervention and removal of the stenosing atherosclerotic plaque.

5.2. Methods

As described in Chapter 3.1, consecutive eligible patients > 18 years old with asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound using standardised velocity criteria.(Grant *et al*, 2003); (Sidhu & Allan, 1997) were recruited.

5.2.1 Patient Groups

As described in Chapter 3.1, patients were **included in the *'asymptomatic carotid stenosis'*** group if they were incidentally noted to have moderate (50 - 69%) or severe (≥ 70%) carotid stenosis on colour Doppler ultrasound imaging (CDUS), e.g. during a vascular work-up on a patient with peripheral or coronary arterial disease. Subjects were considered to be asymptomatic if they never had prior TIA or stroke, or had not had a TIA or stroke within the preceding three years. All demographic and vascular risk factors, and information regarding medication intake was recorded prospectively.

Patients were **included in the *'symptomatic carotid stenosis'* group** if they had a TIA or ischaemic stroke in the vascular territory supplied by a moderate or severe ipsilateral carotid stenosis or carotid occlusion within the preceding 4 weeks and the symptoms was attributed to the stenosed carotid artery of interest (early phase). These patients were reassessed at least three months after symptom onset or after surgical or endovascular intervention (late phase). If antiplatelet therapy was altered by their treating physician in the early phase after presentation, patients were invited to undergo repeat blood testing approximately 14 days later if they had not undergone carotid intervention by that stage. Details regarding antiplatelet regimens, dose and duration of therapy were also recorded prospectively.

Exclusion criteria for patients included: active infection, inflammation including vasculitis or neoplasia; platelet count < 120 or > 450 x 10^9/L; recurrent TIA, stroke, myocardial infarction, pulmonary embolism, deep vein thrombosis or major bleeding requiring transfusion or major surgery within the preceding 3 months; prior history of primary intracerebral haemorrhage; a known bleeding or clotting diathesis or platelet disorder; ongoing unstable coronary or peripheral arterial disease; renal impairment (urea > 10 mmol/l); or other nonsteroidal anti-inflammatory drug (NSAID) intake within 2 weeks (apart from aspirin in the patients on aspirin monotherapy or in combination with dipyridamole or clopidogrel). Patients were also excluded if there was evidence of a potential cardioembolic source detected within 3 months of recruitment in to the study. This included paroxysmal or persistent atrial fibrillation detected on ECG or holter monitoring.

5.2.2 Control Subjects

As outlined in Chapter 3.1, to establish the normal range of platelet function assays in the laboratory, a group of healthy controls of similar age and gender, with no history of known cerebrovascular disease, were recruited from the local population and from amongst the family members of the participating subjects. Control subjects were clinically assessed on one occasion and had CDUS screening to exclude asymptomatic ≥ 50% carotid or vertebral artery stenosis prior to inclusion into the study. Subjects were also excluded from the control group if they were on antiplatelet therapy or NSAIDs, or had any other exclusion criteria that applied to the patients, as outlined above.

5.2.3 Clinical Assessment

All patients underwent a detailed general and neurological assessment by the Clinical Neurology Research Registrars (JAK or WOT) or Consultant Vascular Neurologist (DJHM) participating in the study, to confirm that the asymptomatic patients met inclusion criteria, and to confirm a diagnosis of atherothrombotic TIA or ischaemic stroke in the symptomatic cohort. Information regarding vascular risk factors, medication intake (including anti-thrombotic therapy - Chapter 4), and the degree of disability following ischaemic stroke was quantified by using the modified Rankin scale (Farrell *et al*, 1991). Results of routine haematological, coagulation, biochemical and blood glucose testing were collected prospectively. CT and/or MRI brain was performed in all symptomatic patients and magnetic resonance angiography (MRA) or CT angiography (CTA) was performed where deemed appropriate by the treating physician to establish concordance between CDUS and another imaging modality. Chest radiograph, electrocardiograph (ECG), 24-hour ECG recording and transthoracic or transoesophageal echocardiography were also obtained in symptomatic patients.

All late stage symptomatic carotid stenosis patients were phoned before their appointment to stress the importance of medication adherence in the week prior to assessment. Adherence in this group was assessed by history taking alone. Patients who were not adherent to their antithrombotic regimen were invited back for reassessment after 14 days.

5.2.4 Blood sampling and laboratory tests

Blood sampling was performed in the same manner as described in Chapter 3.2. In brief, venepuncture was performed using a standardised manner as described previously (McCabe *et al*, 2004a). Full blood counts were performed between 2 and 4 hours in

EDTA and citrate anticoagulated tubes, primarily to assess platelet and white cell parameters. Platelet function was assessed using the PFA-100®, to measure C-ADP and C-EPI closure times in citrate-anticoagulated whole blood between 2 and 2.5 hours after venepuncture. The PFA-100 activates platelets by exposure to moderately high shear stress (5000-6000 s^{-1}) and biochemical stimulation with collagen and either epinephrine (C-EPI cartridge) or ADP (C-ADP cartridge).(Michelson, 2009;Kundu *et al*, 1995) The time taken for activated platelets to occlude an aperture in the cartridge is called the closure time; the maximum closure time recorded by the device is 300 s, and we arbitrarily defined closure times above 300 s as 301 s for statistical analyses, using non-parametric tests. We chose to study platelet function in whole blood with the PFA-100 because of our extensive experience with this device in our lab, and because of the potential of the PFA-100 to provide clinically informative data on *ex vivo* platelet reactivity in patients with moderate or severe carotid stenosis whose platelets should be exposed to moderate or high levels of shear stress *in vivo*.

5.2.5 Statistical Methods

Descriptive statistical calculations were performed to calculate the percentage of patients on different antiplatelet regimens. Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively. The Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, and the Kruskal-Wallis rank sum test for comparison of multiple non-parametric variables, where appropriate. Chi-squared or Fisher exact tests were used to compare changes in proportions between subject groups. Multiple linear regression analysis was performed to examine the potential influence of independent vascular risk

factors on any observed differences in FBC parameters between the groups, where appropriate.

We established laboratory normal ranges for the PFA-100 assays in 18 healthy controls (mean age 62 years; 72% male), as outlined above. For the purpose of this study, we considered patients to have *ex-vivo* HTPR on the PFA-100 if they had evidence of platelet 'hyper-reactivity' on the relevant PFA-100 cartridge despite treatment with their prescribed antiplatelet regimen. Therefore, (a) *ex-vivo* HTPR on aspirin was defined as **failure to prolong the C-EPI closure time beyond** the mean + 2 standard deviations of our control range **(162s)** in patients on **aspirin** monotherapy, aspirin-dipyridamole or aspirin-clopidogrel combination therapy. (**b**) *Ex-vivo* HTPR on clopidogrel was defined as **failure to prolong the CADP closure time beyond** the mean + 2 standard deviations of our control range **(165s)** in patients on **clopidogrel** monotherapy, or clopidogrel in combination with aspirin as per usual 'cross sectional, case-control' definitions in the literature.(Grau *et al*, 2003;McCabe *et al*, 2005a). P <0·05 was considered to be statistically significant. All statistical calculations were performed using R, version 2.9.1.(R Development Core Team, 2010)

5.3. Results

The same patient population described in Chapter 4 was initially assessed. 31 asymptomatic and 46 patients with early (≤4 weeks) symptomatic carotid stenosis or occlusion had platelet function data available for analysis; 35 of these symptomatic patients had follow up data at the late stage after symptom onset or carotid intervention (Table 4.1). Of the 46 early symptomatic patients, 35 were on aspirin monotherapy (median dose 300mg daily, median duration of treatment 11 days), 5 were on combination therapy with aspirin (75mg - 300mg daily) and dipyridamole MR (200mg BD), 2 on clopidogrel monotherapy (75mg or 150mg daily), and 4 were on aspirin (75mg or 300mg daily) and clopidogrel (75mg daily) combination therapy. The demographic and vascular risk factors and antiplatelet regimens in the asymptomatic and early and late symptomatic carotid stenosis patients are outlined in Table 4.1.

Assessment of Platelet Reactivity:

There were no significant differences in median C-EPI or C-ADP closure times between the entire asymptomatic *vs.* the early or late symptomatic groups, when one analysed the data on all patients regardless of their prescribed antiplatelet treatment regimen (Table 5.1). Furthermore, there were no differences in the prevalence of HTPR between the subgroups of asymptomatic *vs.* early or late phase symptomatic patients who were on aspirin overall (either alone or in combination with dipyridamole or clopidogrel) or clopidogrel (either monotherapy or in combination with aspirin; Table 5.1). However, the prevalence of HTPR on the C-EPI cartridge was lower in the **late symptomatic post-intervention subgroup** than in the asymptomatic carotid stenosis subgroup on aspirin monotherapy (10% vs. 50%, p = 0.03; Table 5.1). There were no significant differences in vascular risk factors between these subgroups.

Amongst **symptomatic patients** who had longitudinal data at both the early and late phases after symptom onset or intervention, regardless of their prescribed antiplatelet regimen, the median C-EPI closure time was significantly longer (203s *vs.* 143s, p = 0.03), but the median C-ADP closure time was similar in the late phase compared with the early phase after symptom onset (Table 5.2; Figures 5.1 & 5.2). Amongst symptomatic patients on aspirin, alone or in combination with dipyridamole or clopidogrel, the median C-EPI closure time was prolonged (p = 0.023), and the proportion of patients with HTPR was lower in the late than in the early phase after symptom onset or intervention (p = 0.01). Similar results were seen in symptomatic patients on **aspirin monotherapy**, with a significant reduction in the prevalence of HTPR on the C-EPI cartridge during follow up (p = 0.016).

There was a non-significant trend towards a reduction in the prevalence of HTPR in symptomatic patients on aspirin monotherapy or combination therapy who were followed from the early phase to the late post-intervention phase (63% vs. 32%; p = 0.051; Table 5.3). However, there was a significant reduction in HTPR between the early and late post-intervention phases in symptomatic patients who were on aspirin monotherapy (50% vs. 0%; p = 0.02), but the number of subjects in this subgroup analysis was also very limited.

FBC Parameters:

The median platelet count was higher in citrate-anticoagulated whole blood in early symptomatic than asymptomatic patients (211 vs. 200 $x10^9$/L; p = 0.03; Table 4.2). Having controlled for differences in the prevalence of hypertension, statin use and smoking between the two groups, the median platelet count in citrate-anticoagulated blood remained higher (p=0.045) in early symptomatic versus asymptomatic patients.

There were no significant differences in other platelet or white cell parameters between early symptomatic versus late symptomatic or late symptomatic post-intervention groups (Tables 4.5 & 5.4). As stated before (Chapter 4), there was a small statistically significant reduction in haemoglobin levels as symptomatic patients were followed from the early to late phase after symptom onset.

5.4. Discussion

This novel study has revealed a number of interesting findings. There were no significant differences in platelet adhesion and aggregation in response to moderately high shear stress and biochemical stimulation with either collagen-epinephrine or collagen-ADP between patients with asymptomatic and recently symptomatic carotid stenosis. This may be explained by the fact that stenosing atherosclerotic carotid plaques exposed circulating platelets to similar levels of shear stress *in vivo* in both patient groups (Kulkarni *et al*, 2000;Berndt *et al*, 2001;McCabe *et al*, 2005a;Ruggeri, 1997;Al-Tamimi *et al*, 2012) and thus, the PFA-100 could not easily differentiate between asymptomatic and symptomatic carotid stenosis patients. This hypothesis is supported by a number of other findings in the study: the fact that the median C-EPI closure time increased over time in symptomatic patients who had data at both the early and late phases after symptom onset, and that there was a non-significant increase in C-EPI closure times between patients with data in the early phase compared with the late post-intervention phase, when the majority of symptomatic patients had undergone successful carotid intervention. Furthermore, the prevalence of HTPR on the C-EPI cartridge was lower in the late symptomatic post-intervention subgroup than in the asymptomatic carotid stenosis subgroup on aspirin monotherapy (10% vs. 50%, $p = 0.03$), but the number of subjects included in this latter subgroup analysis was far too small to make any definitive conclusions. Although larger studies are warranted to confirm or refute these findings, we feel it is less likely that the lack of differences between the main groups reflects a type I error, but further studies will further inform this debate.

The novel longitudinal data in the symptomatic patient group are also interesting, and indicate that the prevalence of HTPR tends to fall as one is followed from the early to the late phase after symptom onset or intervention (Tables 5.4 and 5.6). These results may

again partly reflect the effects of removal of the stenosing carotid plaque, but may also be influenced by the addition of other antiplatelet agents (dipyridamole or clopidogrel) to the treatment regimen.(King *et al*, 2011;Markus *et al*, 2005) as well as resolution of the acute phase response over time. Larger, longitudinal studies assessing the same patients before and after changing antiplatelet therapy (Tobin *et al*, 2011) are needed to adequately assess the impact of changing antiplatelet therapy on HTPR in patients with carotid stenosis. Such studies will allow one to determine whether patients who exhibit a reduction in on-treatment platelet reactivity in response to a treatment change will have a lower risk of recurrent vascular events than patients who do not exhibit such a dynamic change. The results of the PFA-100 have been clearly shown to be significantly influenced by VWF antigen levels in the blood (Chakroun *et al*, 2004;McCabe *et al*, 2005a) and further analysis of our data is planned to assess the potential impact of VWF:Ag levels on our inter-group and intra-group comparisons.

As discussed in Chapter 4, the increased platelet count in patients with recently symptomatic compared with asymptomatic carotid stenosis may indicate increased platelet production and / or turnover in the setting of recently symptomatic moderate and severe carotid artery stenosis. Differences in platelet counts between groups could, in theory, influence *ex vivo* on-treatment platelet reactivity, but did not appear to influence the results of the PFA-100 in this study because overall platelet reactivity on the C-EPI and C-ADP cartridges was similar in the entire asymptomatic *vs.* symptomatic patient groups. As stated before (Chapter 4), the slight reduction in haemoglobin levels in symptomatic patients as they were followed from the early to late phase after symptom onset is not likely to be clinically significant, or influence the results of the PFA-100.(Harrison *et al*, 1999)

5.5. Conclusions

As stated in the Methods section above, we chose to assess platelet function in this thesis with the PFA-100, in part because platelets in patients with ≥ 50% carotid stenosis are believed to be exposed to at least moderate levels of shear stress *in vivo*.(Berndt *et al*, 2001;Ruggeri, 1997) It is possible that one might derive more informative data on HTPR if one were to use an *ex vivo* test of platelet function that exposed platelets to low shear stress, variable levels of shear stress, or simply stirred the platelets in solution, to avoid excessive exposure of platelets to high shear stress both *in vivo* and *ex vivo*. Consequently, we have designed further experiments to simultaneously assess on-treatment platelet reactivity in patients with asymptomatic and symptomatic carotid stenosis with a panel of platelet function tests that expose platelets to different levels of shear stress *ex vivo*. These assays include the PFA-100, VerifyNow (Chapter 11), multiplate platelet function analyser,(Jaitner *et al*, 2011;Reece *et al*, 2011) and other novel tests of platelet function that are being developed by national and international collaborators. These results will be analysed in conjunction with clinical outcome measures to determine whether there is a relationship between HTPR and the risk of subsequent cerebrovascular events in patients with asymptomatic or symptomatic carotid stenosis, with a view to tailoring antiplatelet therapy to suit individual patients.

Table 5.1: Comparison of platelet reactivity in asymptomatic versus early symptomatic, late stage symptomatic and late stage symptomatic post-intervention carotid stenosis patients. Values are medians (25th - 75th percentile). Significant p values in bold. HTPR = high on treatment platelet reactivity.

PFA-100 Results	Asymptomatic (n=31)	Early Symptomatic (n=46)	p	Late Symptomatic (n=35)	p	Late Symptomatic post-intervention (n=23)	p
Entire Cohort							
C-EPI closure time (sec)	145 (124 – 229)	146 (112 – 240)	0.83	203 (123 – 301)	0.22	189 (139 – 253)	0.36
C-ADP closure time (sec)	95 (80 – 103)	90 (78 – 100)	0.56	93 (74 – 109)	0.94	97 (79 – 113)	0.62
	Asymptomatic (n=20)	**Early Symptomatic (n=33)**		**Late Symptomatic (n=9)**			
Severe Stenosis Subgroup							
C-EPI closure time (sec)	139 (124 – 290)	146 (121 – 230)	0.88	205 (123 – 301)	0.53		
C-ADP closure time (sec)	97 (72 – 104)	92 (81 – 103)	0.81	98 (85 – 107)	0.86		
	Asymptomatic (n=26)	**Early Symptomatic (n=40)**		**Late Symptomatic (n=29)**		**Late Symptomatic post-intervention (n=19)**	
Subgroup on Aspirin (Alone or in Combination with Dipyridamole or Clopidogrel)							
C-EPI closure time (sec)	164 (131 – 278)	152 (115 – 295)	0.55	223 (165 – 301)	0.27	204 (166 – 289)	0.56
Number (%) with HTPR on C-EPI cartridge	12 (46%)	22 (55%)	0.48	8 (28%)	0.15	4 (21%)	0.08
	Asymptomatic (n=22)	**Early Symptomatic (n=30)**		**Late Symptomatic (n=15)**		**Late Symptomatic post-intervention (n=10)**	
Aspirin Monotherapy Subgroup							
C-EPI closure time (sec)	148 (131 – 221)	169 (121 – 301)	0.66	205 (166 – 301)	0.17	203 (167 – 205)	0.27
Number (%) with HTPR on C-EPI cartridge	11 (50%)	15 (50%)	1.0	3 (20%)	0.09	1 (10%)	**0.03**
	Asymptomatic (n=7)	**Early Symptomatic (n=6)**		**Late Symptomatic (n=9)**		**Late Symptomatic post-intervention (n=6)**	
Clopidogrel Subgroup (Alone or in Combination with Aspirin)							
C-ADP closure time (sec)	97 (78 – 142)	93 (82 – 106)	1.0	120 (100 – 130)	0.46	128 (119 – 145)	0.25
P value							
Number (%) with HTPR on C-ADP cartridge	5 (71%)	5 (83%)	1.0	8 (89%)	0.55	5 (83%)	1.0
P value							

Table 5.2: Comparison of platelet reactivity between early and late phase symptomatic carotid stenosis patients with longitudinal data at both time points regardless of their prescribed antiplatelet regimen; those on aspirin alone or in combination with dipyridamole or clopidogrel; or aspirin monotherapy. Values are medians (25th - 75th percentile). Significant p values in bold.

PFA-100 Results	Early Symptomatic (n=35)	Late Symptomatic (n=35)	P value
Entire Symptomatic Subgroup with Longitudinal Data			
C-EPI closure time (sec)	143 (113 – 223)	203 (123 – 301)	**0.03**
C-ADP closure time (sec)	89 (78 – 100)	93 (74 – 109)	0.48
Symptomatic Subgroup on Aspirin (Alone or in Combination with Dipyridamole or Clopidogrel)	**Early Symptomatic (n=27)**	**Late Symptomatic (n=27)**	**P value**
C-EPI closure time (sec)	149 (115 – 263)	205 (150 – 301)	**0.023**
Number (%) with HTPR on C-EPI cartridge	16 (59%)	7 (26%)	**0.01**
C-ADP closure time (sec)	89 (78 – 96)	92 (73 – 99)	0.8
Symptomatic Subgroup on Aspirin Monotherapy	**Early Symptomatic (n=13)**	**Late Symptomatic (n=13)**	**P value**
C-EPI closure time (sec)	152 (118 – 301)	205 (167 – 301)	0.15
Number (%) with HTPR on C-EPI cartridge	8 (62%)	2 (15%)	**0.016**

Table 5.3: Comparison of 'matched' platelet reactivity data between early and late phase symptomatic carotid stenosis patients who underwent carotid intervention. Values are medians (25th - 75th percentile). Significant p values in bold.

PFA-100 Results	**Early Symptomatic (n=23)**	**Late Symptomatic Post-intervention (n=23)**	**P value**
Symptomatic Subgroup with Longitudinal Data pre- and post-intervention			
C-EPI closure time (sec)	146 (125 – 254)	191 (143 – 277)	0.32
C-ADP closure time (sec)	92 (80 – 104)	97 (79 – 113)	0.53
Symptomatic Subgroup on Aspirin (Alone or in Combination with Dipyridamole or Clopidogrel) PFA-100	**Early Symptomatic (n=19)**	**Late Symptomatic Post-intervention (n=19)**	**P value**
C-EPI closure time (sec)	152 (132 – 301)	189 (155 – 240)	0.59
Number (%) with HTPR on C-EPI cartridge	12 (63%)	6 (32%)	0.051
C-ADP closure time (sec)	92 (80 – 100)	93 (73 – 108)	0.63
Symptomatic Subgroup on Aspirin Monotherapy PFA-100	**Early Symptomatic (n=8)**	**Late Symptomatic Post-intervention (n=8)**	**P value**
C-EPI closure time (sec)	231 (147 – 301)	204 (181 – 264)	1
Number (%) with HTPR on C-EPI cartridge	4 (50%)	0 (0%)	**0.02**
C-ADP closure time (sec)	89 (77 – 133)	99 (69 – 138)	0.69

Table 5.4: FBC parameters in early versus late symptomatic subjects who underwent carotid intervention. Values are either means (±SD) or medians (25th - 75th percentile). Significant p values in bold

	Early Symptomatic (n=23)	Late symptomatic post-intervention (n=23)	P value
Platelet Count ($x10^9$/L) (EDTA)	251.1 (±55.3)	253(±60.0)	0.85
Platelet Count ($x10^9$/L) (Citrate)	214.5 (±47.0)	215.5 (±52.1)	0.91
Platelet Distribution Width % (EDTA)	13.56 (±1.4)	13.4 (±1.7)	0.51
Platelet Distribution Width % (Citrate)	11.39(±1.1)	11.43(±1.5)	0.82
Mean Platelet Volume (fl) (EDTA)	11.1(±0.74)	10.98(±0.84)	0.37
Mean Platelet Volume (fl) (Citrate)	9.9(±0.60)	9.9(±0.70)	0.92
Haemoglobin (g/dl) (EDTA)	13.76(±0.98)	13.39(±1.04)	0.07
WCC ($x10^9$/L) (EDTA)	7.68(±1.49)	7.66(±2.3)	0.98
Neutrophils ($x10^9$/L) (EDTA)	4.8(±1.3)	4.7(±1.6)	0.78
Monocytes ($x10^9$/L) (EDTA)	0.63(±0.2)	0.64(±0.25)	0.82
Lymphocytes ($x10^9$/L) (EDTA)	2.01(±0.74)	2.07(±0.75)	0.58

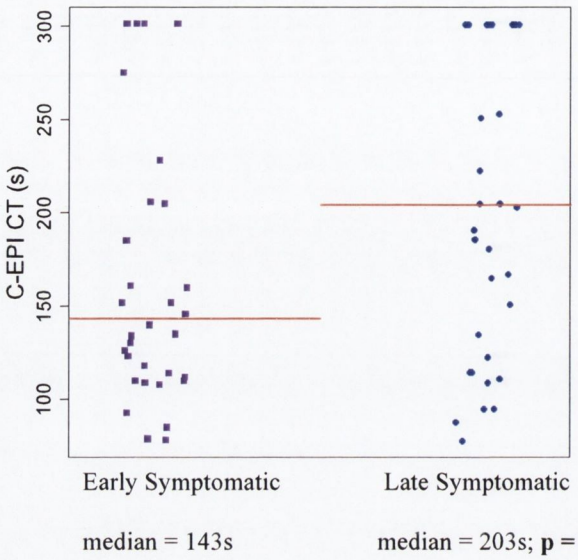


Figure 5.1: PFA-100 C-EPI cartridge closure times (CT) in early symptomatic (n = 35) versus late symptomatic carotid stenosis (n = 35) subjects. Each point represents data from an individual patient, and horizontal limes represent medians. * represents a significant p value.

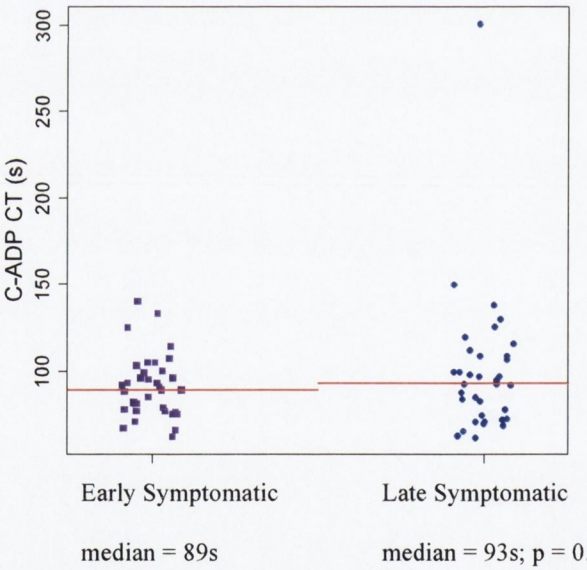


Figure 5.2: PFA-100 C-ADP cartridge closure times (CT) in early symptomatic (n = 35) versus late symptomatic carotid stenosis (n = 35) patients. Each point represents data from an individual patient, and horizontal lines represent medians.

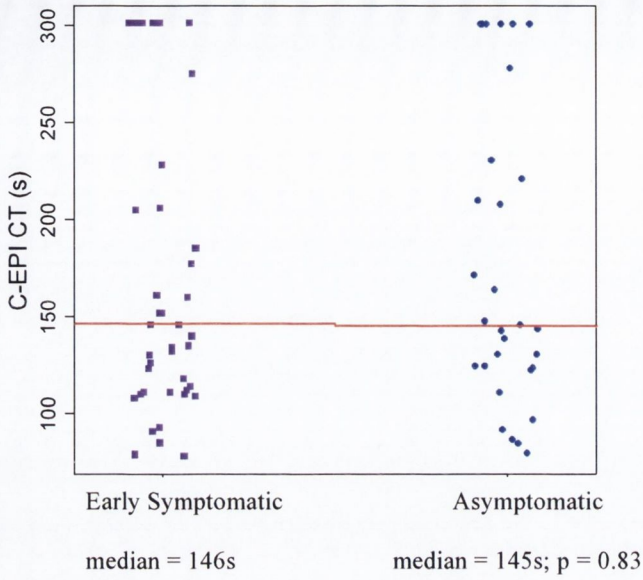


Figure 5.3: PFA-100 C-EPI cartridge closure times (CT) in early symptomatic (n = 46) versus asymptomatic carotid stenosis (n = 31) patients. Each point represents data from an individual patient, and horizontal lines represent medians.

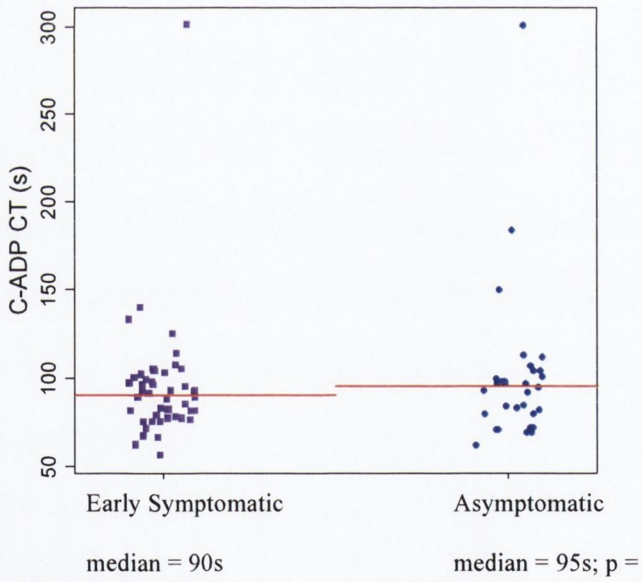


Figure 5.4: PFA-100 C-ADP cartridge closure times (CT) in early symptomatic (n = 46) *vs.* asymptomatic carotid stenosis (n = 31) patients. Each point represents data from an individual patient, and horizontal lines represent medians.

6. Reticulated platelets and full blood count parameters in asymptomatic and symptomatic moderate-severe carotid artery stenosis

6.1. Introduction

Platelets are released into the peripheral blood following megakaryocyte fragmentation within the bone marrow and/or pulmonary circulation.(Harrison *et al*, 1997;Lunetta & Penttila, 1997) Young platelets that have been recently released into the circulation contain a residual amount of megakaryocyte-derived mRNA and were first identified by Ingram and Coopersmith in 1969.(Ingram & Coopersmith, 1969) They were termed 'reticulated platelets' because of the analogy with red cell reticulocytes. Reticulated platelets have been 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. Studies using washed, fixed platelets, obtained from platelet rich plasma (PRP), have shown that the circulating reticulated platelets may increase when thrombopoiesis is increased, and may be normal or decreased when platelet production is suppressed.(Ault *et al*, 1992). However, reticulated platelets may not be increased in ischaemic CVD unless the stimulus to platelet activation also promotes thrombopoiesis.

Three previous studies have investigated circulating reticulated platelets in patients following ischaemic stroke.(McCabe *et al*, 2004b); (Smith *et al*, 2002); (Nakamura *et al*, 2002) Circulating reticulated platelets were found to be elevated very early following stroke onset (<3 days) when compared with controls in one small study (Smith *et al*, 2002), and also in the early (<28 days) and late (>78 days) phases following TIA or stroke only after adjusting for age in a larger study.(McCabe *et al*, 2004b) A further study that compared 68 stroke patients with "neurological controls" found that circulating reticulated platelets were only elevated in the subgroup of patients with cardioembolic stroke compared with controls.(Nakamura *et al*, 2002)

To our knowledge, no study has assessed whether there are differences in circulating reticulated platelets between asymptomatic and symptomatic moderate or severe carotid stenosis patients, or longitudinally assessed reticulated platelets in symptomatic patients who are followed up over time. This deserves study because the reticulated platelets may act a marker of enhanced platelet turnover and/or production following acute carotid plaque activation or rupture. Measurement of circulating reticulated platelets may also facilitate a better understanding of the mechanisms that mediate 'high on-treatment platelet reactivity' in some CVD patients with recently symptomatic carotid stenosis, because COX-2 expression may be upregulated in newly released, reticulated platelets (Rocca *et al*, 2002), and functionally active COX-2 could contribute to thromboxane A_2 formation in platelets, despite inhibition of COX-1 by aspirin therapy.(Guthikonda *et al*, 2007).

Aims

The aim of this pilot study was to determine whether there was evidence of increased platelet turnover (circulating reticulated platelets) or production (platelet count) in

patients with recently symptomatic versus asymptomatic carotid stenosis. We also aimed to determine whether circulating reticulated platelets changed over time in patients with recently symptomatic carotid stenosis who were followed up to the late phase after symptom onset regardless of whether they underwent carotid intervention or not.

Hypotheses

We hypothesised that patients with recently symptomatic carotid stenosis would have excessive platelet turnover or production compared with patients with asymptomatic carotid stenosis. We hypothesised that platelet turnover or production would reduce in patients with recently symptomatic carotid stenosis who were followed up from the early to late phase after symptom onset or intervention. We also hypothesised that the mean platelet volume (MPV) and platelet distribution width (PDW) would be higher in patients with elevated levels of circulating reticulated platelets, consistent with prior findings in an overall ischaemic CVD population that indicated that reticulated platelets are the largest in the circulation.(McCabe *et al*, 2004b)

6.2. Methods

Chapter 3.1 outlines subject groups, and inclusion and exclusion criteria for patinest with asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound examination (CDUS) using standardised velocity criteria,(Grant *et al*, 2003;Sidhu & Allan, 1997) who were invited to participate. Details regarding relevant clinical assessment and ethical approval are also outlined there. Only patients with complete data on reticulated platelets were included in the daya analysis for this chapter.

6.2.1. Blood sampling and laboratory tests

All subjects were rested for at least 20 min, and careful venepuncture was performed from a free-flowing vein using a sterile 21G Butterfly needle (Venisystems™, Abbott, Ireland) and a Vacutainer® system with a luer adaptor (Becton Dickinson Vacutainer Systems, UK). Blood sampling was performed as outlined in Chapter 3 and as described previously.(McCabe *et al*, 2004a) Full blood count was performed from EDTA Vacutainer® tubes including the mean platelet volume (MPV) and platelet distribution width (PDW), between 2 and 4 hours after venepuncture using a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd, Milton Keynes, UK). This was to standardize the effect of the delay between venepuncture and sample analysis on the results obtained, because the MPV increases and the platelet count decreases over time with both anticoagulants.(Bath, 1993)

The whole blood flow cytometry method used in this study was adapted from a previously described (Shattil *et al*, 1987) and validated protocol.(McCabe *et al*, 2004a) 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 results obtained can be compared (Harrison *et al*, 1997); (Robinson *et al*, 2000b). It has been shown that high concentrations of TO can non-specifically label dense granules and possibly mitochondrial DNA within platelets, whereas low concentrations of the dye do not result in non-specific labelling. (Robinson *et al*, 2000a);(Robinson *et al*, 2000b);(Robinson *et al*, 1998) Therefore, a low concentration of TO was used in this assay.

1 ml of Isoton® II alone was aliquoted into the control tube, and a 1:10 dilution of Retic-COUNT™ (Becton Dickinson, San Jose, USA) 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 minutes. The supernatant was discarded to prevent further incubation of the test sample with Retic-COUNT™, and the remaining pellet was re-suspended in 1 ml of Isoton® II before being analysed on the flow cytometer within an hour of re-suspension.

Whole blood was initially stained for GpIb, as outlined in Chapter 3, to allow accurate identification of the platelet cloud on the flow cytometer. A manual gate was positioned around the platelet cloud, and 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.

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, thus facilitating single labelling of platelets with Thiazole orange (Retic-COUNT™).

The platelet cloud in the reticulated platelets control sample tube was identified on a scatterplot of log FS versus log SS that had been saved on the flow cytometer. The non-specific fluorescence of the control sample was calculated, and the % of TO-positive (reticulated) platelets in the test sample was then measured in FL1 (see Figure 3.8).

The reticulated platelet assays were performed as part of a panel set-up on the flow cytometer, and the % RP was calculated by measuring the test sample fluorescence in FL1. Previous experiments in the laboratory have shown that the uptake of TO by platelets is stable and reproducible if sample processing begins within 30 to 60 minutes after venepuncture. Subsequent experiments that were performed in the laboratory also showed that the percentage of reticulated platelets (%RP) 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.

6.2.2. Statistical Methods

These are described in detail in Chapter 3.11. The Pearson product-moment correlation coefficient was calculated to assess the relationship between reticulated platelets and FBC components. $P < 0{\cdot}05$ was considered to be statistically significant. All statistical calculations were performed using R version 2.9.1. (R Development Core Team, 2010)

6.3. Results

Information regarding screening of the overall population of patients with asymptomatic and symptomatic carotid stenosis for the Platelets and Carotid Stenosis study is outlined in Chapter 3.1.

For the reticulated platelets component of our study, 25 patients with asymptomatic ≥ 50% carotid stenosis, and 36 patients with recently symptomatic ≥ 50% carotid stenosis were recruited within 4 weeks of symptom onset. Twenty-nine of these symptomatic patients were followed up to the late phase after symptom onset, 19 of whom had undergone carotid intervention; none had a recurrent vascular event in the interval between presentation and late-phase follow up. Demographic data on the study subjects, including the degree of carotid stenosis, vascular risk factor profile and antiplatelet therapy are outlined in Table 6.1.

Reticulated Platelets:

There were no differences in the %RP between asymptomatic (18.2%) and either early symptomatic (17.3%, p = 0.7) or late symptomatic carotid stenosis patients (17.8%; p = 0.86). The % RP was also similar in the asymptomatic and 19 late phase symptomatic stenosis patients who had undergone carotid intervention (18.2% vs. 18.8% p = 0.82) (Table 6.2 & Figure 6.1).

During longitudinal follow up in the symptomatic cohort who had data at each time point, there were no differences in % RP between early symptomatic and late symptomatic patients (17.2% vs 17.4%; p = 0.89), or between the subgroup of early symptomatic

stenosis patients who underwent carotid intervention and were followed up to the late phase after intervention (17.8% vs. 18.2%; p = 0.82. Table 6.3).

FBC Parameters:

The platelet count in EDTA-anticoagulated blood was significantly higher in early symptomatic than asymptomatic patients (269 $x10^9$/L *vs.* 235 $x10^9$/L; p = 0.02). Otherwise, there were no significant differences in FBC parameters between asymptomatic and symptomatic patients, or within the symptomatic carotid stenosis group at any stage during follow up or intervention (Tables 6.4, 6.5, and 6.6).

Correlation Assessments:

There was a significant positive correlation between the mean platelet volume (MPV) and %RP, and between the platelet distribution width (PDW) and %RP in the asymptomatic, and the early and late phase symptomatic carotid stenosis groups (Table 6.7, Figures 6.2 & 6.3).

6.4. Discussion

This comprehensive, novel, pilot study did not reveal any evidence of increased circulating reticulated platelets in a well-defined cohort of patients with early or late phase symptomatic versus asymptomatic moderate or severe carotid artery stenosis or occlusion. Furthermore, we did not find any evidence that the %RP decreases over time after symptom onset or carotid intervention in patients with recently symptomatic carotid stenosis. Although one cannot completely exclude the possibility of a type II error contributing to our negative results, these data do not provide any evidence that platelet turnover is higher in patients with symptomatic than asymptomatic carotid stenosis. Therefore, measurement of the %RP is not likely to be a sensitive biomarker of increased platelet turnover that will allow one to risk stratify patients with moderate or severe carotid stenosis, and identify those at highest risk of early recurrent symptoms up to the 3 months after symptom onset.

On the contrary, in keeping with the findings from other studies in this thesis, the platelet count in EDTA-anticoagulated whole blood was higher in early symptomatic than asymptomatic patients. This finding is supportive of increased platelet production in the early phase after TIA or ischaemic stroke in association with moderate or severe carotid stenosis. Measurement of the platelet count on a routine haematology analyser appears to be a more sensitive biomarker of platelet production than measurement of the %RP on whole blood flow cytometry. However, further studies are warranted to quantify the 'immature platelet fraction' in patients with carotid stenosis using newer, user-friendly techniques, including automated measurements on a Sysmex XE 2100 analyser.(Pons *et al*, 2010)

The lack of significant differences in platelet count between asymptomatic and late phase symptomatic patients may reflect resolution of the acute phase response, but may also represent a type II error as there were no significant differences in platelet count between the early and late phase symptomatic patients who were followed up over time, regardless of whether they underwent carotid intervention (Tables 6.5 and 6.6).

We found a significant positive correlation between platelet size (mean platelet volume and platelet distribution width) and the % RP in asymptomatic and symptomatic carotid stenosis patients. These findings are in keeping with prior data in an overall ischaemic CVD population, and are consistent with the hypothesis that reticulated platelets are larger than more mature 'non-reticulated' platelets in the circulation.(McCabe *et al*, 2004b)

It is important to note that these findings do not contradict the limited available data on reticulated platelets in an overall patient population with different subtypes of cerebrovascular disease, because none of the prior studies were designed to compare the %RP between patients with asymptomatic and symptomatic carotid stenosis.(Smith *et al*, 2002);(Nakamura *et al*, 2002);(McCabe *et al*, 2004b)

6.5. Conclusions

One must acknowledge that the number of subjects included in this study was relatively small. Therefore, larger studies are warranted to assess the impact of circulating reticulated platelets on *ex vivo* platelet reactivity in patients with carotid stenosis to determine whether the %RP influences the prevalence of high on treatment platelet reactivity (HTPR) in this patient population.(McCabe *et al*, 2005a;Assadian *et al*, 2008)

Table 6.1: Demographic data and vascular risk factor profile of study patients. P values relate to chi-squared and Fisher exact testing in asymptomatic versus relevant symptomatic carotid stenosis patient groups. Values are means (±SD) or absolute counts. Significant p values in bold.

Parameter	Asymptomatic Carotid Stenosis (n=25)	Early Symptomatic Carotid Stenosis (n=36)	p	Late Symptomatic Carotid Stenosis (n=29)	p
Mean Age (years)	68.4 (±7.02)	65.5 (±9.2)	0.8	66.1 (±9.3)	0.84
Gender (M; %)	14 (56%)	20 (56%)	1	16 (55%)	0.97
Degree of Stenosis					
(moderate, 50 – 69%)	10 (40%)	5 (14%)	**0.03**	12 (41%)	0.92
(severe, 70 – 99%)	15 (60%)	27 (75%)	0.21	10 (34%)	0.06
(occlusion)	0 (0%)	4 (11%)	0.14	3 (10%)	0.24
Antiplatelet therapy					
-Aspirin monotherapy	18 (72%)	27 (75%)	0.92	12 (41%)	0.23
-Aspirin /Dipyridamole combination	1 (4%)	3 (8%)	0.53	9 (31%)	**0.03**
-Clopidogrel monotherapy	4 (16%)	2 (6%)	0.23	6 (21%)	0.71
-Aspirin /Clopidogrel combination	2 (8%)	4 (11%)	0.72	2 (7%)	0.88
IHD	6 (24%)	9 (25%)	0.94	6 (21%)	0.81
Hypertension	24 (96%)	24 (67%)	0.34	19 (66%)	0.35
Diabetes Mellitus	6 (24%)	5 (14%)	0.4	3 (10%)	0.26
Prior Stroke/TIA	7 (28%)	5 (14%)	0.27	4 (14%)	0.29
Family History Stroke	7 (28%)	15 (42%)	0.45	12 (41%)	0.48
Prior DVT/PE	0	0	1.0	0	1.0
Peripheral Vascular Disease	4 (16%)	7 (19%)	0.77	5 (17%)	0.92
Migraine (with or without aura)	4 (16%)	5 (14%)	0.84	4 (14%)	0.85
Current smokers (at enrolment)	4 (16%)	17 (47%)	0.07	11 (38%)	0.17
Ex-smoker	19 (76%)	13 (36%)	0.09	11 (38%)	0.13
Never smoker	2 (8%)	6 (17%)	0.38	7 (24%)	0.18
Statin therapy	22 (88%)	26 (72%)	0.61	27 (93%)	0.89

Table 6.2: Comparison of %RP between asymptomatic, early symptomatic and late phase symptomatic subjects in the entire cohort, and the cohort with severe (≥70%) carotid stenosis only. Values are means (±SD). Significant p values in bold.

	Asymptomatic (n=25)	Early Symptomatic (n=36)	p	Late Symptomatic (n=29)	p	Late Symptomatic post-intervention (n=19)	p
% Reticulated Platelets	18.2 (±9.6)	17.3 (±8.3)	0.7	17.8 (±7.6)	0.86	18.8 (±7.5)	0.82

Severe (≥70%) stenosis	Asymptomatic (n=15)	Early Symptomatic (n=27)		Late Symptomatic (n=10)	
% Reticulated Platelets	16.2 (±9.8)	17.1 (±9.2)	0.77	16.0 (±8.3)	0.97

Table 6.3a: Comparison of %RP between symptomatic carotid stenosis patients followed up from the early to the late phase after symptom onset or intervention; values are means (±SD). Significant p values in bold.

Table 6.3b: Comparison of %RP between symptomatic patients followed up from the early pre-intervention to the late post-intervention phase; values are means (±SD). Significant p values in bold.

	Early Symptomatic (n=28)	Late Symptomatic (n=28)	P value
a. % Reticulated Platelets	17.2 (±8.4)	17.4 (±7.4)	0.89
	Early Symptomatic pre-intervention (n=18)	**Late Symptomatic post-intervention (n=18)**	**P value**
b. % Reticulated Platelets	17.76 (±8.9)	18.2 (±7.3)	0.82

Table 6.4: FBC parameters in asymptomatic *vs.* early symptomatic, late symptomatic, and late phase post-intervention symptomatic patients. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold

	Asymptomatic (n=25)	Early Symptomatic (n=36)	p	Late Symptomatic (n=29)	p	Late Symptomatic post-intervention (n=19)
Platelet Count ($x10^9$/L) (EDTA)	235.4 (±38.0)	269.3 (±71.9)	**0.02**	256.8 (±68.3)	0.15	253.6 (±63.4)
Platelet Count ($x10^9$/L) (citrate)	200 (182.2 – 216.7)	208.3 (186.7 – 276.7)	0.06	220.0 (166.7 – 255.6)	0.24	220.0 (170 – 249.4)
Platelet Distribution Width % (EDTA)	13.1 (±1.95)	12.8 (±1.74)	0.5	12.9 (±1.86)	0.78	13.2 (±1.57)
Platelet Distribution Width % (citrate)	11.4 (±1.72)	10.86 (±1.61)	0.22	11.25 (±1.54)	0.74	11.49 (±1.43)
Mean Platelet Volume (fl) (EDTA)	10.78 (±0.92)	10.66 (±0.96)	0.62	10.75 (±0.9)	0.90	10.87 (±0.8)
Mean Platelet Volume (fl) (citrate)	9.88 (±0.79)	9.64 (±0.86)	0.26	9.83 (±0.75)	0.80	9.92 (±0.69)
Haemoglobin (g/dl) (EDTA)	13.7 (13.0 – 15.0)	13.85 (12.85 – 14.63)	0.8	13.7 (12.9 – 14.2)	0.51	13.4 (12.9 – 14.2)
WCC ($x10^9$/L) (EDTA)	7.70 (±1.69)	7.92 (±1.55)	0.62	7.65 (±2.04)	0.92	7.88 (±2.4)
Neutrophils ($x10^9$/L) (EDTA)	4.56 (±1.18)	4.8 (±1.36)	0.42	4.6 (±1.5)	0.91	4.8 (±1.7)
Monocytes ($x10^9$/L) (EDTA)	0.69 (±0.2)	0.68 (±0.2)	0.76	0.66 (±0.2)	0.57	0.67 (±0.25)
Lymphocytes ($x10^9$/L) (EDTA) p-value	2.04 (1.68 – 2.55)	2.24 (1.5 – 2.7)	0.74	2.08 (1.6 – 2.5)	0.88	2.08 (1.6 – 2.6)

Table 6.5: FBC parameters in symptomatic carotid stenosis patients followed up from the early to the late phase after symptom onset or intervention. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Early Symptomatic (n=28)	Late Symptomatic (n=28)	P value
Platelet Count ($x10^9$/L) (EDTA)	260.7 (±71.1)	259.4 (±68.2)	0.9
Platelet Count ($x10^9$/L) (citrate)	206 (186.0 – 275)	228 (167.5 – 255.6)	0.56
Platelet Distribution Width % (EDTA)	13.0 (±1.58)	12.8 (±1.8)	0.29
Platelet Distribution Width % (citrate)	11.1 (±1.41)	11.2 (±1.52)	0.6
Mean Platelet Volume (fl) (EDTA)	10.8 (±0.87)	10.72 (±0.90)	0.39
Mean Platelet Volume (fl) (citrate)	9.76 (±0.75)	9.81 (±0.76)	0.54
Haemoglobin (g/dl) (EDTA)	13.95 (13.2 – 14.6)	13.65 (12.9 – 14.2)	0.15
WCC ($x10^9$/L) (EDTA)	7.8 (±1.59)	7.70 (±2.1)	0.82
Neutrophils ($x10^9$/L) (EDTA)	4.8 (±1.33)	4.6 (±1.54)	0.69
Monocytes ($x10^9$/L) (EDTA)	0.66 (±0.2)	0.67 (±0.2)	0.81
Lymphocytes ($x10^9$/L) (EDTA)	2.2 (1.5 – 2.7)	2.13 (1.63 – 2.55)	0.98

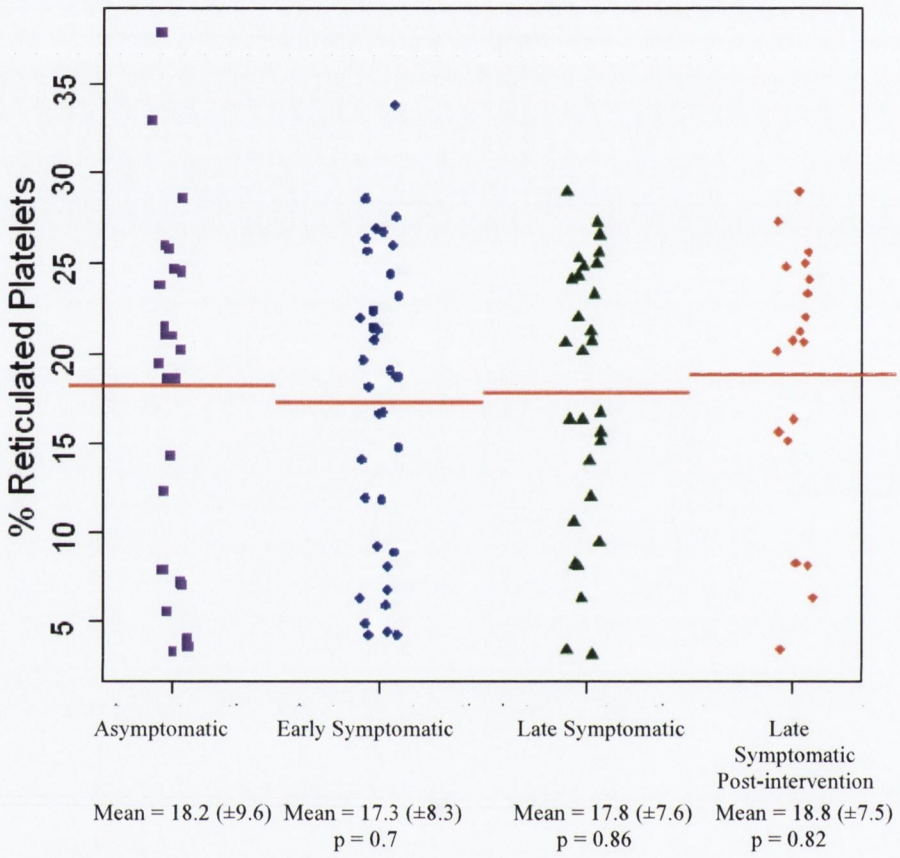
Table 6.6: FBC parameters in symptomatic patients followed up from the early pre-intervention to the late post-intervention phase; values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Early Symptomatic pre-intervention (n=18)	Late Symptomatic post-intervention (n=18)	P value
Platelet Count ($x10^9$/L) (EDTA)	254 (±58.6)	257.4 (±63.0)	0.8
Platelet Count ($x10^9$/L) (citrate)	200 (187.2 – 259.7)	228 (176.4 – 250.8)	0.96
Platelet Distribution Width % (EDTA)	13.4 (±1.26)	13.0 (±1.5)	0.24
Platelet Distribution Width % (citrate)	11.3 (±1.11)	11.4 (±1.41)	0.77
Mean Platelet Volume (fl) (EDTA)	11 (±0.7)	10.8 (±0.8)	0.16
Mean Platelet Volume (fl) (citrate)	9.9 (±0.63)	9.9 (±0.7)	1
Haemoglobin (g/dl) (EDTA)	13.85 (13.25 – 14.5)	13.4 (12.9 – 14.0)	0.31
WCC ($x10^9$/L) (EDTA)	7.5 (±1.1)	7.98 (±2.4)	0.41
Neutrophils ($x10^9$/L) (EDTA)	4.8 (±1.33)	4.8 (±1.73)	0.45
Monocytes ($x10^9$/L) (EDTA)	0.65 (±0.2)	0.68 (±0.3)	0.61
Lymphocytes ($x10^9$/L) (EDTA)	2.12 (1.5 – 2.8)	2.13 (1.6 – 2.6)	0.81

Table 6.7: Correlation between the MPV and %RP and between the PDW and %RP in the asymptomatic, early and late phase symptomatic carotid stenosis groups. Significant p values in bold.

Subject Group	Correlation Comparison	Pearson product-moment coefficient (ρ)	P value
Asymptomatic (n=25)			
	MPV and % RP		
	EDTA-anticoagulated sample	0.53	**0.006**
	Citrate-anticoagulated sample	0.57	**0.003**
	PDW and % RP		
	EDTA-anticoagulated sample	0.63	**0.0008**
	Citrate-anticoagulated sample	0.51	**0.0096**
Early Symptomatic (n=36)			
	MPV and % RP		
	EDTA-anticoagulated sample	0.41	**0.013**
	Citrate-anticoagulated sample	0.36	**0.03**
	PDW and % RP		
	EDTA-anticoagulated sample	0.38	**0.023**
	Citrate-anticoagulated sample	0.36	**0.033**
Late Symptomatic (n=29)			
	MPV and % RP		
	EDTA-anticoagulated sample	0.46	**0.01**
	Citrate-anticoagulated sample	0.37	**0.048**
	PDW and % RP		
	EDTA-anticoagulated sample	0.39	**0.036**
	Citrate-anticoagulated sample	0.38	**0.045**

Figure 6.1: % Reticulated platelets in asymptomatic versus early symptomatic, late symptomatic and late symptomatic carotid stenosis patients post-intervention. Horizontal lines are means; each dot represents individual patient data. Values below are mean (±SD).



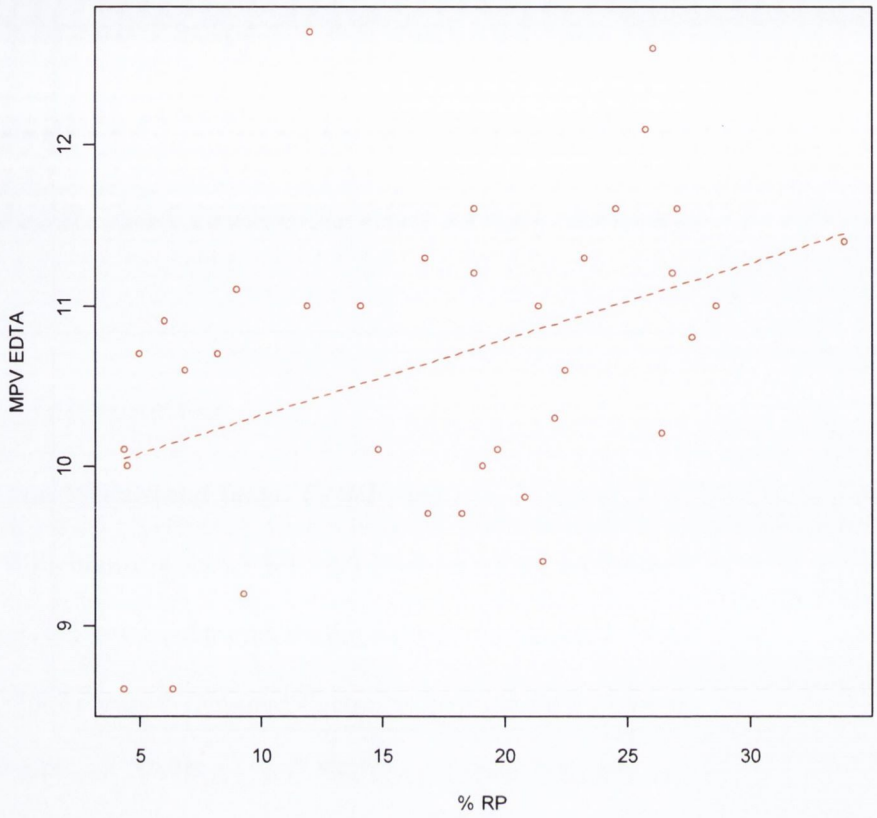


Figure 6.2: Correlation between mean platelet volume (MPV) in EDTA-anticoagulated blood versus % RP in early symptomatic carotid stenosis patients. Pearson rho = 0.41; p = 0.013.

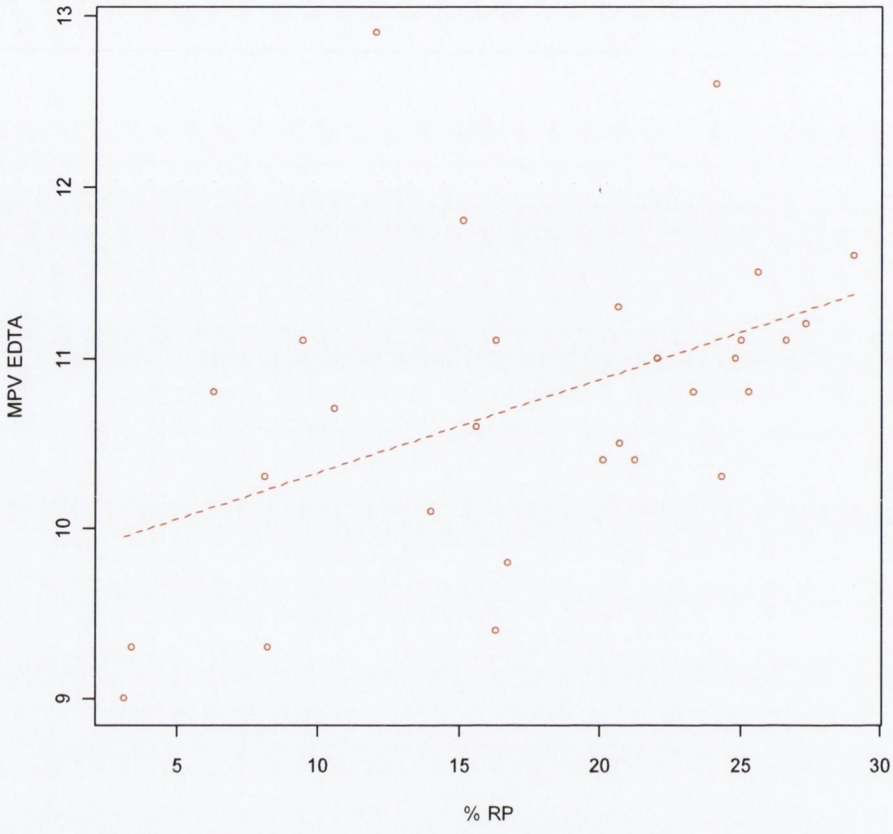


Figure 6.3: Correlation between mean platelet volume (MPV) in EDTA-anticoagulated blood versus % RP in late symptomatic carotid stenosis patients. Pearson rho = 0.46; p = 0.01.

7. Assessment of endothelial activation and the role of von Willebrand Factor (VWF:Ag) and von Willebrand Factor propeptide (VWF:Ag II) in symptomatic and asymptomatic moderate and severe carotid artery stenosis

7.1. Introduction

7.1.1 von Willebrand factor (VWF:Ag)

Von Willebrand factor (VWF:Ag) is an adhesive multimeric plasma glycoprotein that is synthesised in vascular endothelial cells and megakaryocytes.(Bongers *et al*, 2006;Nishio *et al*, 2004) Von Willebrand Factor propeptide (VWF:Ag II) is produced by cleavage of pro-VWF into VWF:Ag and VWF:Ag II.(van Mourik *et al*, 1999;Frijns *et al*, 2006) Endothelial cells secrete VWF constitutively into the circulating blood or into the subendothelial matrix, or may store VWF in endothelial Weibel-Palade bodies.(Ruggeri, 1997)

VWF stored in Weibel-Palade bodies may undergo multimerisation to form ultralarge VWF multimers.(Nishio *et al*, 2004) Ultra large VWF multimers are also secreted from activated endothelial cells and may be anchored via P-selectin to the endothelial cell surface.(Nishio *et al*, 2004) Under normal circumstances, ADAMTS-13 (A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif no 13) then cleaves ultra-large VWF multimers into smaller multimers; these smaller multimers have a lower propensity to activate platelets than ultra large VWF multimers.(Nishio *et al*, 2004)

After secretion, VWF may circulate in plasma in a noncovalent complex with coagulation factor VIII.(van Mourik *et al*, 1999). von Willebrand factor propeptide (VWF:Ag II) undergoes endoproteolytic cleavage to form VWF:Ag in endothelial cells. VWF:Ag II is also produced by endothelial cells and stored in Weibel-Palade bodies and released from Wiebel Palade bodies upon endothelial activation, as well as directly from endothelial cells by the constitutive pathway in a molar concentration in normal plasma one tenth of that of VWF:Ag.(van Mourik *et al*, 1999) VWF:Ag II is believed to be a more sensitive marker of *acute* endothelial cell activation than von Willebrand factor antigen (VWF:Ag), because VWF:Ag II has a shorter plasma half-life and returns to baseline concentration briskly after the endothelial insult has been removed.(van Mourik *et al*, 1999); (Frijns *et al*, 2006)

In the following section, I have summarised the literature on VWF in TIA and ischaemic stroke, and will then concentrate on the limited data in patients with carotid stenosis.

VWF:Ag in Stroke and TIA:

Several studies have demonstrated elevated levels of plasma VWF in patients with ischaemic cerebrovascular disease compared with controls. Catto *et al*. performed a longitudinal study and found elevated VWF antigen levels in the acute and convalescent phases following ischaemic stroke compared with age and gender matched controls.(Catto *et al*, 1997) The authors also found lower VWF antigen levels in the convalescent phase compared with the acute phase after symptom onset. McCabe *et al* performed a prospective, observational analytical study in patients following ischaemic stroke or TIA, and found that mean plasma VWF antigen levels were higher in the early

(< 4 weeks) and late (> 3 months) phases after symptom onset compared with controls without vascular disease ($p < 0.001$).(McCabe *et al*, 2005a)

Other studies have also demonstrated elevated plasma VWF levels in the acute (Lynch *et al*, 2004); (Bongers *et al*, 2006); (Kozuka *et al*, 2002); and convalescent (Kain *et al*, 2002) phases following TIA or ischaemic stroke compared with healthy controls ($p \leq 0.006$).

VWF:Ag in Carotid Stenosis:

There have been very few studies assessing plasma VWF levels specifically in the patients with symptomatic or asymptomatic carotid artery stenosis.

One cross-sectional, population-based study based on a population from the Tromso study investigated the relationship between carotid plaque morphology and echogenicity on carotid ultrasound and endothelial dysfunction.(With Noto *et al*, 2006) Plasma VWF antigen levels were measured in 133 subjects with carotid stenosis and 138 controls without carotid stenosis. Plasma VWF antigen levels were higher in carotid stenosis patients than controls ($p \leq 0.048$).

A prospective, single-centre, cohort study, that investigated progression of atherosclerosis in 258 subjects with carotid plaques, found a small positive association between plaque area on carotid ultrasound with VWF levels.(Nilsson *et al*, 2002) Another cross-sectional study assessed plasma VWF levels in 53 patients with TIA and ipsi- or contralateral carotid stenosis on ultrasound (mean degree of stenosis 85%; range 40–100%, with occlusion confirmed on digital subtraction angiography).(Blann *et al*, 2000) The authors reported that VWF antigen levels were unrelated to the degree of carotid artery stenosis ($r = 0.11$; $p = 0.43$), but an exact breakdown of the degree of stenosis was not provided.

The Progetto Lombardo Atero-Trombosi (PLAT) Study was an observational, prospective, multicentre study of clinical outcomes (documented recurrent atherothrombotic events up to 2 years after inclusion) in patients with vascular disease i.e. previous TIA, symptomatic coronary artery disease or peripheral arterial disease. 38% of all TIA patients had carotid stenosis, but classification of TIA subtypes in all patients was not provided. The authors did not find any association between plasma VWF:Ag levels and the occurrence of recurrent vascular events at 2 years follow-up in all patients with previous TIA.(Cortellaro *et al*, 1992)

In a recent study of 67 carotid stenosis patients undergoing carotid stenting (CAS), plasma levels of VWF:Ag were increased immediately after versus immediately before CAS ($p < 0.01$).(Xia *et al*, 2011) The authors also found that plasma VWF:Ag levels 24 hours after stenting were higher in patients who subsequently developed carotid restenosis than in those who did not have restenosis at 1 year follow-up ($p < 0.05$).

7.1.2. von Willebrand factor propeptide (VWF:Ag II)

To our knowledge, quantification of plasma VWF:Ag II or VWF:Ag/VWF:Ag II ratio in patients with asymptomatic versus symptomatic carotid stenosis has not been performed previously. Such studies could provide a more sensitive indicator of acute endothelial activation in patients with carotid stenosis than measurement of VWF:Ag levels alone.

Overall, plasma VWF levels are significantly elevated in the early and late phases following TIA or stroke compared with healthy controls. Plasma VWF levels may reduce following the acute phase after ischaemic stroke or TIA, but remain elevated compared with healthy controls, suggesting ongoing endothelial activation in these patients. Data on VWF:Ag levels in patients with carotid stenosis are limited, and prior to the commencement of this thesis, there were no well designed studies comparing VWF:Ag

levels or the VWF:Ag/VWF:Ag II ratio in asymptomatic *vs.* symptomatic moderate or severe carotid stenosis.

Aims

The aim of this part of the study was to determine whether there was evidence of increased endothelial activation in patients with recently symptomatic versus asymptomatic moderate or severe carotid stenosis. We also investigated whether endothelial activation reduces over time in patients with recently symptomatic carotid stenosis who were followed up to the late phase after symptom onset or carotid intervention.

Hypotheses

We hypothesised that patients with recently symptomatic carotid stenosis would have excessive endothelial cell activation compared with patients with asymptomatic carotid stenosis. We also hypothesised that endothelial cell activation would decrease during longitudinal follow-up in patients with recently symptomatic carotid stenosis. We planned subgroup analyses in symptomatic and asymptomatic patients on aspirin monotherapy, and in patients with severe (≥70%) carotid stenosis.

7.2. Methods

As detailed in Chapter 3.1, consecutive eligible patients > 18 years old with asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound using standardised velocity criteria (Sidhu & Allan, 1997)(Grant *et al*, 2003) were recruited. Patient groups, ethical approval, clinical assessment and inclusion and exclusion criteria, are described in detail in Chapter 3.1. The patient population in this chapter was identical to that outlined in Chapter 4.

7.2.1 Blood sampling and laboratory tests

Venepuncture was performed using a standardised method, as described previously (Chapter 3 "General Methods"). Full blood count was measured between 2 and 4 hours after venepuncture in EDTA- and citrate-anticoagulated whole blood. FBC data have been presented in Chapter 3 and will not be represented here.

Platelet Poor Plasma (PPP)

PPP was obtained from fresh citrate-anticoagulated whole blood by centrifugation as described earlier and stored at -70°C (Chapter 3, General Methods). Samples were thawed once at 37°C for 20 minutes before analysis in a VWF:Ag ELISA assay. Samples were then refrozen and stored at -70°C, until they were thawed once more for the VWF:Ag II ELISA assays. Because VWF:Ag II may be endothelium- or platelet-derived, as outlined above, measurement of soluble E-selectin levels, as an additional specific measure of endothelial activation, has been prospectively planned in our laboratory, but had not been performed at the time of data analysis for this thesis.

Enzyme Linked Immunosorbant Assay (ELISA)

The concentration of VWF:Ag and VWF:Ag II in each PPP sample was quantified, as previously described,(O'Donnell *et al*, 2005); (Preston *et al*, 2009) to assess endothelial activation status (the VWF methodology has already been described in detail in the general methods section in Chapter 3).

In brief, polyclonal Rabbit Anti-Human VWF antibody (DAKO) was used as coating antibody, and polyclonal rabbit Anti-Human VWF/HRP antibody (DAKO) as detection antibody for the VWF:Ag ELISA. M193902 CLB-Pro 35 coating antibody (Plesmanlaan 125) and M103904HRP CLB-Pro 14.3 detection antibody (Plesmanlaan 125) were used for VWF:Ag II quantification. The ELISA assay result was measured by spectrophotometry at 490nm, using a VERSA Max Tuneable Microplate Reader. VWF:Ag and VWF:Ag II levels were measured in units of mg/mL.

7.2.2 Statistical Methods

These are described in detail in Chapter 3.11. All statistical calculations were performed with R version 2.9.1. (R Development Core Team, 2010)

7.3. Results

As described in Chapter 4, 31 patients with asymptomatic carotid stenosis, and 61 recently symptomatic carotid stenosis patients were initially recruited to the study. For reasons outlined in chapter 4, data from 46 early phase symptomatic patients were analysed, six of whom had symptomatic internal carotid artery occlusion on CDUS, 3 of whom had this confirmed on MRA or CTA. Thirty-five symptomatic patients attended for late phase follow up. Eleven symptomatic patients did not have available late phase laboratory data: 6 declined follow-up, one moved location and could not reattend, one developed active severe symptomatic peripheral vascular disease, and another ongoing active inflammatory gouty arthropathy, precluding laboratory reassessment; 2 died from unrelated causes (one from respiratory sepsis and one from cholecystitis).

The patient demographics are presented in Table 4.1. There was a higher prevalence of current smokers, but a lower prevalence of hypertension amongst symptomatic patients, and a lower prevalence of statin use amongst the early symptomatic versus asymptomatic patients. Otherwise, the demographic and vascular risk profiles were similar in the asymptomatic and symptomatic patients (Table 4.1).

Endothelial activation markers:

There was no significant difference in unadjusted VWF:Ag levels, or in the VWF:Ag/VWF:Ag II ratio between the overall population of asymptomatic patients and symptomatic patients at any stage after symptom onset or intervention ($p \geq 0.056$). However, VWF:Ag II levels were higher in early (12.8 mg/mL; $p < 0.001$), late (10.6 mg/mL; $p = 0.01$) and late post-intervention symptomatic (10.6 mg/mL; $p = 0.038$) than

asymptomatic carotid stenosis patients (8.9 mg/mL; Table 7.1). During longitudinal follow-up in the symptomatic group, VWF:Ag levels were elevated in early versus late symptomatic carotid stenosis patients (16.5 *vs.* 14.8 mg/mL; p = 0.048. Table 7.4). Otherwise, there were no differences in other markers of endothelial activation between the early symptomatic and late phase symptomatic patients, regardless of whether they underwent intervention (Tables 7.4 & 7.5). Having controlled for the independent influence of smoking status, hypertension and statin use with multiple linear regression, the difference in VWF:Ag levels between early symptomatic versus asymptomatic patients two groups became significant (p = 0.029) and VWF:Ag II levels remained significantly elevated in early symptomatic patients (p = 0.0007). The difference in VWF:Ag II levels between the late symptomatic and asymptomatic patients remained significant (p = 0.035).

Pre-planned Subgroup Analyses:

Because differences in prescribed antiplatelet regimens could potentially have influenced the observed differences between asymptomatic and symptomatic patients (Table 4.1), a pre-planned subgroup analysis was performed in patients on aspirin monotherapy. VWF:Ag (15.4 *vs.* 12.1 mg/mL; p = 0.045) and VWF:Ag II levels (12.8 *vs.* 8.7 mg/mL; p = 0.003) were both higher in early symptomatic than asymptomatic patients on aspirin.(Table 7.2) Otherwise, there were no other differences between symptomatic and asymptomatic carotid stenosis subjects on aspirin monotherapy, but it must be emphasised that smaller numbers of subjects were included in this subgroup analysis than in our main study.

In a further pre-planned, subgroup analysis, VWF:Ag II levels were elevated in early (12.6 mg/mL; p = 0.004) and late (10.9 mg/mL; p = 0.023) symptomatic severe (≥70%)

versus asymptomatic severe carotid stenosis (8.8 mg/mL; Table 7.5). There were no other differences in endothelial activation markers between patients with symptomatic and asymptomatic severe carotid stenosis.

FBC Parameters, Platelet number and size:

These data have already been described elsewhere (Chapter 4 Results), and are not re-presented in this chapter.

7.4. Discussion

This well-designed, observational, longitudinal study is the first to assess plasma markers of endothelial activation in a clinically well-defined cohort of asymptomatic versus symptomatic carotid stenosis patients, with simultaneous assessments of platelet activation and function.

This study has revealed several interesting findings, including evidence of excessive endothelial activation (elevated 'unadjusted' VWF:Ag II levels) in early, late, and late post-intervention symptomatic versus asymptomatic carotid stenosis patients. The persistent changes during follow up indicate that the findings in the early symptomatic group are not simply reactive of an acute phase response to recent ocular or cerebral ischaemia or infarction. There was no elevation in unadjusted VWF:Ag levels, but the difference in VWF:Ag levels between early symptomatic and asymptomatic patients became significant ($p = 0.029$), and the differences in VWF:Ag II levels remained significant after adjusting for the differences in vascular and demographic risk factors between groups. These data highlight the role of VWF:Ag II and also VWF:Ag levels as potential markers of increased risk of cerebrovascular events in patients with carotid stenosis, and enhance our understanding of the importance of endothelial activation in recently symptomatic compared with asymptomatic carotid stenosis. The fact that VWF:Ag II is thought to act as a marker of acute rather than chronic endothelial cell activation (van Mourik *et al*, 1999);(Frijns *et al*, 2006) indicates ongoing endothelial activation in symptomatic carotid stenosis patients compared with their asymptomatic counterparts for at least 3 months after TIA or stroke onset. Furthermore, our data confirm that VWF:Ag II levels are a more sensitive marker of acute endothelial activation than measurement of VWF:Ag levels in symptomatic carotid stenosis patients.

We also found higher VWF:Ag and VWF:Ag II levels in early symptomatic than asymptomatic patients on aspirin monotherapy, indicating that excessive endothelial activation is seen in symptomatic compared with asymptomatic patients despite low-medium dose aspirin therapy. These subgroup data also support the hypothesis that the differences in endothelial activation status between early symptomatic and asymptomatic patients are not simply explained by differences in antiplatelet regimens between the groups. However, because of the relatively small numbers of subjects included in our subgroup analyses, the effects of different antiplatelet regimens on markers of endothelial activation in patients with carotid stenosis deserves further formal study in a larger patient population. This was not the primary aim of this part of our study.

We also found elevated VWF:Ag II levels in early and late symptomatic versus asymptomatic subjects with severe carotid stenosis indicating ongoing excessive endothelial activation levels in this subgroup of symptomatic patients. The levels of plasma VWF:Ag II in these subgroups were similar to those in the entire symptomatic and asymptomatic cohorts, but our study was not designed to specifically comment on the influence of increasing degrees of carotid stenosis on levels of VWF:Ag II released from the endothelium.

Our longitudinal study in symptomatic patients revealed that endothelial activation status, as measured by VWF:Ag levels in early symptomatic subjects decreased as patients were followed up to the late stage after symptom onset or carotid intervention. These findings may partly reflect resolution of the acute phase response over time, the effects of removal of the stenosing atherosclerotic plaque, and perhaps alteration of antiplatelet therapy in some symptomatic patients by the late stage of follow up. These data, when interpreted in conjunction with our 'cross-sectional', comparative data on VWF:Ag II levels in

symptomatic versus asymptomatic patients, indicate that endothelial activation is increased and remains higher in symptomatic patients throughout the follow up phase.

7.5. Conclusions

These findings enhance our understanding of the potential role of endothelial activation in the pathogenesis of TIA or stroke in patients with symptomatic versus asymptomatic carotid stenosis. If one could identify a subgroup of patients with elevated biomarkers of endothelial activation at increased risk of subsequent cerebrovascular events, one could utilise these data to risk-stratify carotid stenosis patients who might benefit from earlier, intensive medical or interventional treatment to prevent subsequent TIA or stroke.

Table 7.1: Comparison of Endothelial System activation as measured by von Willebrand Factor (VWF:Ag), von Willebrand Factor propeptide (VWF:Ag II) and VWF:Ag/VWF:Ag II ratio in asymptomatic versus early symptomatic, late phase symptomatic, and post-intervention late phase symptomatic subjects. Values are medians (25th - 75th percentile). Significant p values in bold.

Marker	Asymptomatic (n=31)	Early Symptomatic (n=46)	p	Late Symptomatic (n=35)	p	Late Symptomatic post-intervention (n=23)	p
VWF:Ag (mg/mL)	13.3 (9.2 – 16.1)	15.4 (11.9 – 21.0)	0.056	12.8 (9.9 – 19.9)	0.6	12.4 (9.9 – 17.7)	0.
VWF:Ag II (mg/mL)	8.9 (3.4 – 10.4)	12.8 (8.6 – 17.0)	**<0.001**	10.6 (7.2 – 17.0)	**0.01**	10.6 (7.1 – 17.0)	**0.**
VWF:Ag/VWF:Ag II ratio	1.6 (1.2 – 3.3)	1.3 (0.8 – 2.3)	0.096	1.3 (1.1 – 1.9)	0.5	1.2 (0.9 – 2.1)	0.

Table 7.2: Comparison of von Willebrand Factor (VWF:Ag), von Willebrand Factor propeptide (VWF:Ag II) and VWF:Ag/VWF:Ag II ratio in asymptomatic versus early symptomatic, late phase symptomatic, and post-intervention late phase symptomatic subjects on aspirin monotherapy. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

Marker	Asymptomatic (n=22)	Early Symptomatic (n=30)	p	Late Symptomatic (n=15)	p	Late Symptomatic post-intervention (n=10)	p
VWF:Ag (mg/mL)	12.1 (9.1 – 16.5)	15.4 (12.2 – 20.2)	**0.045**	10.9 (8.9 – 17.2)	0.8	10.7 (7.9 – 14.7)	0.52
VWF:Ag II (mg/mL)	8.7 (3.5 – 10.5)	12.8 (8.6 – 17.6)	**0.003**	9.4 (6.6 – 15.7)	0.38	10.6 (6.6 – 18.2)	0.35
VWF:Ag/VWF:Ag II ratio	2.0 (1.1 – 3.5)	1.2 (0.8 – 1.8)	0.07	1.4 (1.1 – 1.8)	0.28	1.1 (0.8 – 1.9)	0.21

Table 7.3: Comparison of Endothelial activation in asymptomatic versus early symptomatic and late phase symptomatic subjects with ≥70% carotid stenosis. Values are medians (25th - 75th percentile). Significant p values in bold.

Marker	Asymptomatic (n=20)	Early Symptomatic (n=33)	p	Late Symptomatic (n=9)	p
VWF:Ag (mg/mL)	13.8 (9.4 – 16.9)	15.1 (10.2 – 19.3)	0.31	12.6 (10.5 – 15.9)	0.84
VWF:Ag II (mg/mL)	8.8 (3.2 – 10.0)	12.6 (7.8 – 16.9)	**0.004**	10.9 (7.7 – 18.2)	**0.023**
VWF:Ag/VWF:Ag II ratio	1.8 (1.4 – 3.9)	1.2 (0.8 – 1.9)	0.052	1.3 (1.1 – 1.8)	0.055

Table 7.4: Endothelial activation data in early versus late symptomatic carotid stenosis patients with data at each time point. Values are either means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Early Symptomatic (n=35)	Late symptomatic (n=35)	P value
VWF:Ag (mg/mL)	16.5 (±6.4)	14.8 (±6.4)	**0.048**
VWF:Ag II (mg/mL)	13.0 (±6.0)	11.7 (±6.0)	0.33
VWF:Ag/VWF:Ag II ratio	1.2 (0.8 – 1.8)	1.3 (1.1 – 1.9)	1.0

Table 7.5: Endothelial activation markers in early symptomatic pre-intervention versus late symptomatic post-intervention carotid stenosis patients. Values are means (±SD) or medians (25th - 75th percentile).

	Early Symptomatic(n=23)	Late symptomatic (n=23)	P value
VWF:Ag (mg/mL)	16.0 (±6.7)	13.7 (±5.7)	0.054
VWF:Ag II (mg/mL)	11.9 (±5.5)	11.2 (±5.8)	0.67
VWF:Ag/VWF:Ag II ratio	1.4 (0.9 – 2.1)	1.2 (0.9 – 2.1)	0.28

8. Assessment of Coagulation System Potential in Asymptomatic and Symptomatic moderate and severe Carotid Artery Stenosis

8.1. Introduction

The process of platelet activation is intimately linked to activation of the coagulation system.(Monroe & Hoffman, 2006);(Monroe *et al*, 2002). Activation of platelets and the coagulation system may occur simultaneously, and may be initiated by the same agonists in some instances (Figure 8.1).(Monroe *et al*, 2002)

As outlined in Chapter 4, platelet activation is increased in patients in both the early and late phases after TIA or ischaemic stroke.(McCabe *et al*, 2004a) Elevation in cytosolic calcium during platelet activation induces exposure of procoagulant phosphatidylserine (PS) on the platelet surface, thus promoting assembly of tenase and prothrombin complexes, and subsequent accelerated thrombin generation.(van der Meijden *et al*, 2005) Thrombin is a potent platelet agonist, and during thrombus formation, activates platelets by binding to the GpIb-IX-V complex.(Berndt *et al*, 2001) However ADP-induced activation of the $P2Y_{12}$ ADP receptor on platelets actually increases the time taken to achieve peak thrombin formation, and may serve a type of 'negative feedback mechanism' to slow down thrombin generation.(van der Meijden *et al*, 2005)

There have only been two studies investigating endogenous thrombin potential in the late phase after TIA or ischaemic stroke,(Faber *et al*, 2003);(van der Meijden *et al*, 2005) but none specifically assessing thrombin generation potential in a carotid stenosis patient cohort. Furthermore, the contribution of platelet activation to overall thrombin generation in both the early and late phases after ischaemic stroke or TIA in patients with carotid stenosis is uncertain.

8.1.1 Review of literature on thrombin generation potential in ischaemic stroke

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

Subsequently, Van der Meijden *et al.* investigated peak thrombin, time-to-peak thrombin, and ETP in PRP from 11 young (32 - 51 years) patients and 9 older stroke patients (60-74 years) within 3-12 months of ischaemic stroke, and also in 12 patients with type-II diabetes mellitus (26 - 73 years) and 11 healthy controls whose age was not outlined in the paper.(van der Meijden *et al*, 2005) The 9 older stroke patients were also re-assessed 2 weeks following the introduction of clopidogrel monotherapy (75mg daily) The authors did not specify the proportion of patients on aspirin, or the prescribed aspirin dose at study entry. Peak thrombin generation rate was higher in PRP in the older stroke and diabetic patients ($p \leq 0.05$) than in healthy controls. The authors comment that the presence of platelets in PRP may contribute to elevated thrombin generation potential in patients compared to measurements from PPP samples. There were no significant

differences in any thrombin generation markers between young stroke patients and controls. However, the numbers of subjects in each subgroup was far too small to make any definite conclusions.

Therefore, the limited data available to date suggest that endogenous thrombin potential in PRP may be elevated in young (Faber *et al*, 2003) or older patients (van der Meijden *et al*, 2005) following ischaemic stroke, although the findings in young patients have not been replicated to date. However, thrombin generation potential has not been assessed in the same patients in the both the early and late phases following TIA or ischaemic stroke, or in patients with asymptomatic and symptomatic carotid stenosis.

8.1.2. Thrombin Generation in Carotid Stenosis

To our knowledge, no studies have specifically investigated thrombin generation potential in a pure carotid stenosis cohort, but studies assessing other markers of thrombin generation in carotid stenosis patients have been performed.

One recent case-control study investigated the relationship between carotid plaque morphology on ultrasound and markers of thrombin generation in 128 subjects with ≥ 35% carotid stenosis and 136 age- and gender-matched controls without stenosis.(With Noto et al, 2008) Thrombin-antithrombin complexes (TAT), which are formed by the binding of thrombin to antithrombin following thrombin generation, and prothrombin fragments 1 and 2 (F_1 and F_2), which are generated during the conversion of prothrombin to thrombin,(Fareed *et al*, 1998) were measured in these subjects. Data on antiplatelet regimens and proportions of the study population on antiplatelet treatment were not provided. Patients with echogenic plaques [n = 63] had higher levels of TAT (5.24 μg/l) than those with echolucent plaques (3.44 μg/l, n = 65; $p < 0.001$) and controls (3.33 μg/l; n = 136; $p < 0.001$). In addition, patients with echogenic plaques had higher levels of F_1 and F_2 (2.14 nM) than those with echolucent plaques (1.54 nM; $p < 0.001$) or controls (1.49 nM; $p < 0.001$). TAT ($p = 0.002$) and F_1 and F_2 levels ($p = 0.001$) increased linearly with plaque echogenicity, independent of the degree of stenosis. These findings suggested that patients with echogenic carotid plaques, which are traditionally felt to be more stable and less likely to undergo rupture and cause symptoms, are more likely to have increased thrombin generation markers than patients with echolucent plaques. One could interpret these data to suggest that thrombin generation is not important in the genesis of symptoms in patients with carotid stenosis. However, well designed studies in patients with asymptomatic compared with recently symptomatic carotid stenosis are

warranted to investigate this issue further, as are studies in patients with more severe degrees of carotid stenosis.

Soinne *et al.* performed a cross-sectional study to compare plasma markers of 'haemostasis or fibrinolysis' between asymptomatic and symptomatic severe carotid stenosis subjects.(Soinne *et al*, 2005) 92 consecutive patients with ≥70% carotid stenosis (NASCET criteria) were assessed. 18 patients were asymptomatic, 54 patients were assessed within a median interval of 41 days of onset of TIA or stroke in the vascular territory supplied by the stenosed carotid artery of interest, and 20 patients had symptoms in another vascular territory, rendering the plaque of interest "asymptomatic" (median duration from symptom onset of 106 days). In the "symptomatic plaque group", 19 patients were on anticoagulation, as was 1 patient who had a TIA or stroke in another vascular territory. Plasma levels of prothrombin fragments F_1 and F_2, TAT complexes, plasminogen activator inhibitor-1 (PAI-1), and tissue-type plasminogen activator (tPA) antigen and activity were measured. Plasma TAT levels were reduced in the 54 symptomatic compared with the 18 asymptomatic carotid stenosis subjects overall (2.2 vs 4.0 μg/l; $p = 0.006$). However, when the authors confined the analysis to the subset of symptomatic patients who were not on anticoagulants (mainly warfarin; $n = 35$), the differences between the symptomatic and asymptomatic carotid stenosis groups were no longer statistically significant (2.6 vs 4.0 μg/l; $p = 0.05$) possibly as a result of type II error. There were no other differences in plasma markers between the symptomatic and asymptomatic groups. The indication for warfarin therapy was unclear as patients with a cardioembolic source of embolism were excluded from the study.

Another study assessed the concentration of TAT complexes and antithrombin (AT) in carotid plaques by means of ELISA techniques and plasma (PPP) from patients with symptomatic ($n = 20$) and asymptomatic ($n = 18$) carotid stenosis (mean degree of

stenosis = 75% (range 50 – 95%).(Migdalski *et al*, 2004) The investigators found that symptomatic plaques contained more TAT complexes than asymptomatic plaques (20.35 vs 11.08 ng/mg of protein; $p < 0.04$). There was no difference in plasma TAT complex concentration between symptomatic and asymptomatic patients ($p > 0.05$). Interestingly, the finding of increased TAT complex concentrations only within symptomatic severe carotid plaques potentially indicates that plaque rupture, and perhaps intramural haemorrhage or thrombus formation, may lead to local thrombin generation in the plaques of such patients that may not be detected with standard plasma assays.

Cote *et al*. prospectively measured plasma TAT complex and F_1 and F_2 levels in 82 symptomatic patients within 7 days of a TIA (41% of whom had ≥50% carotid stenosis – no further breakdown on degree of carotid stenosis provided), 157 asymptomatic individuals with a cervical bruit (56% of whom had ≥50% carotid stenosis), and 65 healthy control subjects,(Cote *et al*, 2000). There were no data provided on antiplatelet use other than mentioning that a 'substantial proportion' of patients at baseline were taking aspirin. There were no differences in TAT complexes or F_1 and F_2 levels between symptomatic and asymptomatic subjects or controls ($p \geq 0.093$). However, F_1 and F_2 levels were found to be independent predictors of subsequent cerebral or cardiac ischaemic events in asymptomatic individuals with cervical bruits (Relative risk (RR) = 1.70; 95% CI 1.14-2.53), and of MI, ischaemic stroke and vascular death in TIA patients (RR = 2.36; 95% CI 1.19-4.68) over a mean follow-up period of 2.83 years. These data suggest a potential role for the coagulation system in the pathogenesis of vascular events in patients with prior TIAs, or asymptomatic carotid stenosis (some of whom had ≥50% carotid stenosis), but larger prospective studies with careful patient phenotyping would be needed to address this issue further.

Therefore, prior to the commencement of this thesis, it was unclear whether plasma markers of thrombin generation might be of use in potentially risk-stratifying patients with carotid stenosis. Furthermore, there were no well designed studies comparing a comprehensive panel of markers of thrombin generation potential in a pure population of patients with asymptomatic versus symptomatic moderate or severe carotid stenosis.

Aims

The aim of this pilot study was to determine whether there was evidence of increased thrombin generation potential in patients with recently symptomatic versus asymptomatic carotid stenosis. We also aimed to determine whether there was any evidence of increased thrombin generation potential in symptomatic patients in the early compared with the late phase after TIA or stroke in association with moderate or severe carotid stenosis.

Hypotheses

We hypothesised that patients with recently symptomatic carotid stenosis would have increased thrombin generation potential compared with patients with asymptomatic carotid stenosis. We also hypothesised that thrombin generation potential would decrease over time in patients with recently symptomatic carotid stenosis who were followed up to the late phase after symptom onset or carotid intervention.

8.2. Methods

As detailed in Chapter 3.1, consecutive eligible patients > 18 years old with asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound using standardised velocity criteria (Sidhu & Allan, 1997)(Grant *et al*, 2003) were recruited. Patient groups, ethical approval, clinical assessment and inclusion and exclusion criteria, are described in detail in Chapter 3.1. The patient population in this chapter was identical to that in Chapter 4. The diagnosis of TIA or ischaemic stroke was confirmed in all cases by Dr Kinsella and/or Dr McCabe, and patients were not included if Dr Kinsella or Dr McCabe had any doubt about the working diagnosis.

8.2.1 Blood sampling and laboratory tests

Venepuncture was performed using a standardised method, as described previously (Chapter 3 "General Methods"). Full blood counts were performed between 2 and 4 hours in EDTA and citrate anticoagulated tubes. FBC data have been presented in Chapter 3 and will not be represented here.

Platelet Poor Plasma (PPP)

PPP was obtained from fresh citrate-anticoagulated whole blood by centrifugation as described earlier and stored at -70°C (Chapter 3 "General Methods"). Samples were thawed once at 37°C for 20 minutes before analysis in the thrombin generation assay.

Thrombin Generation Assay: Assessment of coagulation system potential

As described in Chapter 3 coagulation system potential was measured as previously described.(Faber *et al*, 2003) 20 µl of 5pM PPP-Reagent, containing a mixture of

phospholipids and Tissue Factor, (Thrombinoscope BV, Netherlands) was added to 80 µl of platelet poor plasma in a polystyrene 96-well plate, in triplicate. In a fourth well per patient, 20 µml of Thrombin Calibrator (Thrombinoscope BV, Netherlands) was added to 80 µl of platelet poor plasma from the same patient. No thrombin production took place in this well, and it served as an internal control from which thrombin production in the other 3 wells were calculated.

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

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

'Lag time' to thrombin spark (min), 'peak thrombin' generated (nM), 'time to peak thrombin' (ttPeak; min), 'start-tail' time to end of thrombin generation (min), and endogenous thrombin potential (ETP, nM*min) were quantified for each patient plasma

sample (Figure 8.2). Lag time represents the time to initiation of thrombin generation and happens to correspond to the clotting time because clot appears when roughly 1% of thrombin is formed; a shorter lag time corresponds with a more pro-coagulant sample. Peak thrombin represents the maximum concentration of thrombin that a given sample is capable of generating. The time to peak thrombin is a measure of how quickly maximal thrombin generation can be reached; shorter time to peak thrombin represents a more pro-coagulant sample. The start-tail represents the total time taken to generate thrombin; longer start-tail times indicate that a more persistent stimulus to thrombin generation is present, and therefore, samples with longer start-tail times are believed to be more pro-coagulant. Endogenous thrombin generation potential corresponds to the total amount of thrombin that is produced.

8.2.2 Statistical Methods

These are described in detail in Chapter 3.11. Because a study of thrombin generation potential has never been done before, power calculations were not possible prior to commencement of this study. All statistical calculations were performed with R version 2.9.1. (R Development Core Team, 2010)

8.3. Results

The number of patients who were recruited, including those initially screened and subsequently excluded, has been outlined in detail in Chapter 4. For this part of our study, data from 31 patients with asymptomatic carotid stenosis, 46 early phase symptomatic, and 35 late phase symptomatic patients (23 of whom had undergone carotid intervention) were analysed (Table 4.1). There was a higher prevalence of current smokers, but a lower prevalence of hypertension amongst symptomatic patients, and a lower prevalence of statin use amongst the early symptomatic versus asymptomatic patients. Otherwise, the demographic and vascular risk profiles were similar in the asymptomatic and symptomatic patients (Table 4.1).

Thrombin Generation Potential Parameters

Peak thrombin production was significantly elevated in early symptomatic compared with asymptomatic carotid stenosis patients (344.2 *vs.* 305.3 nM; p = 0.01; Table 8.1). ETP was also higher in early symptomatic than asymptomatic carotid stenosis patients (1772.4 *vs.* 1589.7 nM*min; p = 0.047; Table 8.1), indicative of enhanced thrombin generation potential in early symptomatic patients. Otherwise, there were no significant differences in thrombin generation potential parameters between the symptomatic and asymptomatic groups. When we performed multiple linear regression analysis to control for the potential independent influence of smoking status, hypertension and statin use on the observed differences between the groups, all of the above differences between the early symptomatic and asymptomatic patients remained significant.

In the longitudinal component of the study in the symptomatic group, peak thrombin production was elevated in the early compared with the late phase after symptom onset in patients who had data at each timepoint (339.7 *vs.* 308.6 nM; p = 0.02; Table 8.4). There were no other differences in other markers of thrombin generation potential in this group or between the early and late phase symptomatic patients who underwent intervention (Table 8.4 & 8.5). However, in the subgroup of symptomatic carotid stenosis patients who did **not** undergo intervention, ETP was elevated in the early compared with late phase (1887.3 *vs.* 1617.9 nM; p = 0.048; Table 8.6). There were no other differences in other markers of thrombin generation potential between the early and late phase symptomatic patients who did not have intervention (Table 8.6).

Subgroup analyses

In the subgroup of patients on aspirin monotherapy, there were no differences in markers of thrombin generation potential between early symptomatic (n = 30), late symptomatic (n = 15) and asymptomatic carotid stenosis patients (n = 22; Table 8.2). However, peak thrombin production (338.3 *vs.* 299.5 nM; p = 0.02) and ETP were higher in patients with early severe symptomatic carotid stenosis than in patients with severe asymptomatic carotid stenosis (1708.4 *vs.* 1528.6 nM x min; p = 0.04; Table 8.3), consistent with the results seen in the overall study patient population.

8.4. Discussion

To our knowledge, this novel, well-designed longitudinal observational pilot study is the first to assess a panel of plasma markers of thrombin generation potential in a clinically well-defined cohort of patients with asymptomatic compared with symptomatic carotid stenosis.

We found evidence of enhanced coagulation system potential (peak thrombin and ETP) in early symptomatic compared with asymptomatic moderate and severe carotid stenosis patients, as well as in the subgroup with severe early symptomatic versus severe asymptomatic carotid stenosis. Other markers were not increased in symptomatic versus asymptomatic patients, indicating that measurement of peak thrombin and ETP appear to be more sensitive markers of thrombin generation potential than lag time, time to peak thrombin, or start-tail time. It is possible that the lack of significant differences in the other markers of thrombin generation potential between groups represents a type II error because the numbers of patients included in our study was relatively small. This issue is currently being addressed in ongoing studies in larger groups of patients at our institution. The finding of increased peak thrombin production and ETP in early symptomatic compared with asymptomatic patients may reflect 'inherent' enhanced coagulation system potential in patients with carotid stenosis who subsequently go on to develop symptoms. These findings may also reflect activation of the coagulation system secondary to recent carotid plaque activation, with subsequent ocular or cerebral ischaemia/infarction. This could partly explain why recently symptomatic patients are at high risk of recurrent symptoms despite treatment with antiplatelet therapy early after symptom onset compared with their asymptomatic counterparts. Furthermore, because we have already shown that platelet production and activation (Chapter 4), and endothelial activation (Chapter 7) are enhanced in the early phase after symptom onset,

increased platelet or endothelial activation could lead to enhanced coagulation system potential/activation and vice versa. Because all of these patients were treated with antiplatelet therapy, and none were treated with therapeutic anticoagulation, further studies are needed to determine whether patients with enhanced coagulation system potential are at higher risk of recurrent symptoms than those without, and whether targeting these patients with anticoagulation or more hyperacute carotid intervention is warranted. We cannot make any definitive conclusions about the value of these biomarkers in predicting the risk of recurrent vascular events in patients with carotid stenosis as none of the patients had recurrent vascular events during medium-term follow up in this study.

In our interesting longitudinal study in the symptomatic carotid stenosis group, peak thrombin production was significantly higher in the early compared with late phase after TIA or stroke onset (N = 35). This may reflect resolution of the acute phase response over time, or may have been impacted upon by 'carotid interventional treatment' in the majority of symptomatic patients before late phase follow up. Although one did not see statistically significant differences in peak thrombin production between early and late phase symptomatic patients who underwent intervention, and in whom data were available at each time point (n = 23), there was a non-significant fall in peak thrombin production over time (329.5 vs. 304 nM; p = 0.14; Table 8.5). Therefore, this lack of difference may potentially reflect a type II error, and further studies are warranted to assess the impact of carotid intervention on thrombin generation over time. The finding of elevated ETP in the early versus late symptomatic patients who did **not** undergo intervention (1887.3 *vs.* 1617.9 nM; p = 0.048; Table 8.6) could represent a type I error as the result was just statistically significant, and only 12 subjects were included in this subgroup analysis. One could also interpret these findings as indicating that initial plaque

activation / rupture exposes the circulating blood to a stimulus to excessive thrombin generation that resolves in association with plaque healing and resolution of the acute phase response by the 3 month follow-up stage. It is possible that the carotid plaque is not the primary stimulus to elevated thrombin generation in patients with carotid stenosis, and that thrombin generation falls even though the plaque remains *in situ*. Other factors, such as excessive endothelial or platelet activation, or ocular or cerebral ischaemia or infarction could also promote thrombin generation in the early phase after TIA or stroke in these patients, with a reduction in the impact of these stimuli over time.

Although the differences between symptomatic and asymptomatic patients on aspirin monotherapy were not statistically significant, this may also represent a type II error invoked by subgroup analysis in a smaller number of patients, because there was also a non-significant trend towards higher peak thrombin production in early symptomatic than asymptomatic patients (Table 8.2). This study was not designed to address the impact of altering antiplatelet regimens on coagulation system potential, but this is the subject of another collaborative study in our laboratory that is nearing completion.

We did not simultaneously measure thrombin generation potential and TAT complexes in our study. However, if one considers our findings in conjunction with those of Migdalski *et al.*, our data suggest that measurement of 'overall thrombin generation potential' appears to be a more sensitive and comprehensive indicator of enhanced coagulation system activation/potential in plasma than measurement of TAT complexes (Migdalski *et al*, 2004). We did not perform simultaneous histopathological or biochemical carotid plaque assessments in our study, so we cannot comment further in this regard. In addition, because the clinical characteristics, antithrombotic regimens, and precise stenosis categories in our patient population were not directly comparable with those in two prior studies (Cote *et al*, 2000); (Soinne *et al*, 2005), one cannot reliably comment on

the relative sensitivity of our assays compared with assays of F_1 and F_2. These issues would need to be addressed in larger longitudinal studies simultaneously quantifying plasma markers of thrombin generation potential, TAT complexes, F1 and F2 fragments, and carotid plaque imaging and morphology.

8.5. Conclusions

These findings enhance our understanding of the potential role of thrombin generation in the pathogenesis of TIA or stroke in patients with symptomatic versus asymptomatic carotid stenosis. If one could identify a subgroup of patients with elevated biomarkers of coagulation system potential/activation with an increased risk of subsequent cerebrovascular events, this could lead to improvements in primary and secondary stroke prevention.

Table 8.1: Comparison of Coagulation System Potential in asymptomatic versus early symptomatic, late phase symptomatic, and post-intervention late phase symptomatic subjects. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

Marker	Asymptomatic (n=31)	Early Symptomatic (n=46)	p	Late Symptomatic (n=35)	p	Late Symptomatic post-intervention (n=23)	p
Lag Time (min)	3.1 (2.6 – 3.5)	3.0 (2.7 – 3.3)	0.59	3.0 (2.7 – 3.3)	0.52	3.0 (2.7 – 3.2)	0.56
Peak Thrombin (nM)	305.3 (±66.9)	344.2 (±55.2)	**0.01**	308.6 (±68.7)	0.85	304.0 (±72.6)	0.95
Time to Peak Thrombin (min)	5.72 (5.1 – 6.3)	5.5 (5.2 – 6.3)	0.69	5.67 (5.0 – 6.2)	0.75	5.6 (5.1 – 6.3)	0.87
Start Tail (min)	20.3 (18.0 – 22.7)	20.0 (18.0 – 22.9)	0.7	20.5 (17.3 – 22.6)	0.86	20.3 (17.6 – 22.9)	0.77
ETP (nM x min)	1589.7 (±380.1)	1772.4 (±389.1)	**0.047**	1586.0 (±427.0)	0.97	1568.6 (±447.4)	0.86

Table 8.2: Comparison of Coagulation System Potential in asymptomatic versus early symptomatic, late phase symptomatic, and post-intervention late phase symptomatic subjects on aspirin monotherapy. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

Marker	Asymptomatic (n=22)	Early Symptomatic (n=30)	p	Late Symptomatic (n=15)	p	Late Symptomatic post-intervention (n=10)	p
Lag Time (min)	3.4 (2.9 – 3.6)	3.0 (2.7 – 3.3)	0.25	3.1 (2.8 – 3.6)	0.94	3.1 (3.0 – 3.5)	0.77
Peak Thrombin (nM)	299.4 (±67.5)	328.8 (±55.7)	0.1	287.1 (±68.4)	0.32	299.1 (±72.5)	0.67
Time to Peak Thrombin (min)	6.0 (5.1 – 6.4)	5.6 (5.2 – 6.3)	0.44	5.9 (5.1 – 6.7)	0.57	6.0 (5.3 – 6.8)	0.41
Start Tail (min)	21.0 (18.0 – 22.7)	19.5 (18.1 – 22.8)	0.96	19.7 (17.7 – 22.0)	0.57	20.5 (18.7 – 22.4)	0.98
ETP (nM x min)	1566.4 (±369.7)	1692.6 (±401.7)	0.25	1516.9 (±433.6)	0.47	1605.0 (±421.8)	0.93

Table 8.3: Comparison of Coagulation System Potential in asymptomatic versus early symptomatic and late phase symptomatic subjects with ≥70% carotid stenosis. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

Marker	Asymptomatic (n=20)	Early Symptomatic (n=33)	p	Late Symptomatic (n=9)	p
Lag Time (min)	3.2 (2.3 – 3.5)	3.0 (2.8 – 3.3)	0.66	2.9 (2.6 – 3.3)	0.94
Peak Thrombin (nM)	299.5 (±58.7)	338.3 (±50.1)	**0.02**	306.6 (±56.3)	0.76
Time to Peak Thrombin (min)	5.7 (5.1 – 6.2)	5.4 (5.2 – 6.3)	0.96	5.7 (5.1 – 6.2)	0.81
Start Tail (min)	19.5 (18.0 – 22.7)	19.7 (18.0 – 22.7)	0.86	21.0 (17.0 – 22.0)	0.96
ETP (nM x min)	1528.6 (±295.9)	1708.4 (±306.7)	**0.04**	1620.3 (±336.1)	0.49

Table 8.4: Coagulation System Potential in early versus late symptomatic carotid stenosis patients with data at each time point. Values are either means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Early Symptomatic (n=35)	Late symptomatic (n=35)	P value
Lag Time (min)	3.0 (2.7 – 3.3)	3.0 (2.7 – 3.3)	0.15
Peak Thrombin (nM)	339.7 (±58.1)	308.6 (±68.7)	**0.02**
Time to Peak Thrombin (min)	5.54 (5.2 – 6.3)	5.7 (5.0 – 6.2)	0.46
Start Tail (min)	19.3 (18.0 – 22.8)	20.5 (17.3 22.6)	0.69
ETP (nM x min)	1733.9 (±390.3)	1586.0 (±427.2)	0.06

Table 8.5: Assessing the impact of carotid intervention on Coagulation System Potential in early versus late symptomatic carotid stenosis patients who underwent intervention. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold

	Early Symptomatic(n=23)	Late symptomatic (n=23)	P value
Lag Time (min)	3.0 (2.7 – 3.2)	3.0 (2.7 – 3.2)	0.43
Peak Thrombin (nM)	329.5 (±53.5)	304.0 (±72.6)	0.14
Time to Peak Thrombin (min)	5.4 (5.2 – 6.0)	5.6 (5.1 – 6.3)	0.85
Start Tail (min)	19.3 (18.0 – 22.2)	20.3 (17.6 – 22.9)	0.83
ETP (nM x min)	1653.8 (±326.2)	1568.6 (±447.4)	0.39

Table 8.6: Assessing the impact of carotid intervention on Coagulation System Potential in early versus late symptomatic carotid stenosis patients who did not have intervention. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold

	Early Symptomatic(n=12)	Late symptomatic (n=12)	P value
Lag Time (min)	3.3 (2.8 – 3.3)	3.2 (2.8 – 3.3)	0.18
Peak Thrombin (nM)	359.2 (±63.9)	317.1 (±63.1)	0.07
Time to Peak Thrombin (min)	5.8 (5.2 – 6.3)	5.8 (5.0 – 6.0)	0.14
Start Tail (min)	19.5 (18.8 – 24.2)	20.5 (17.2 – 22.1)	0.27
ETP (nM x min)	1887.3 (±467.9)	1617.9 (±404.6)	**0.048**

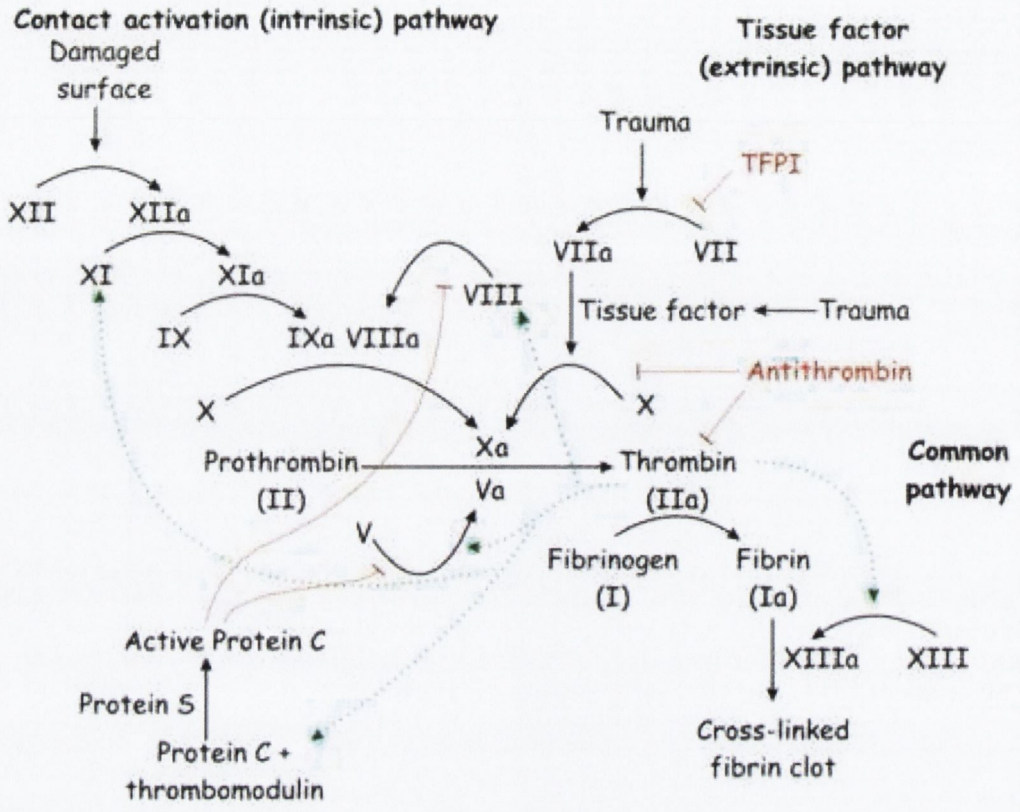


Figure 8.1 Intrinsic and extrinsic coagulation pathways / cascade.

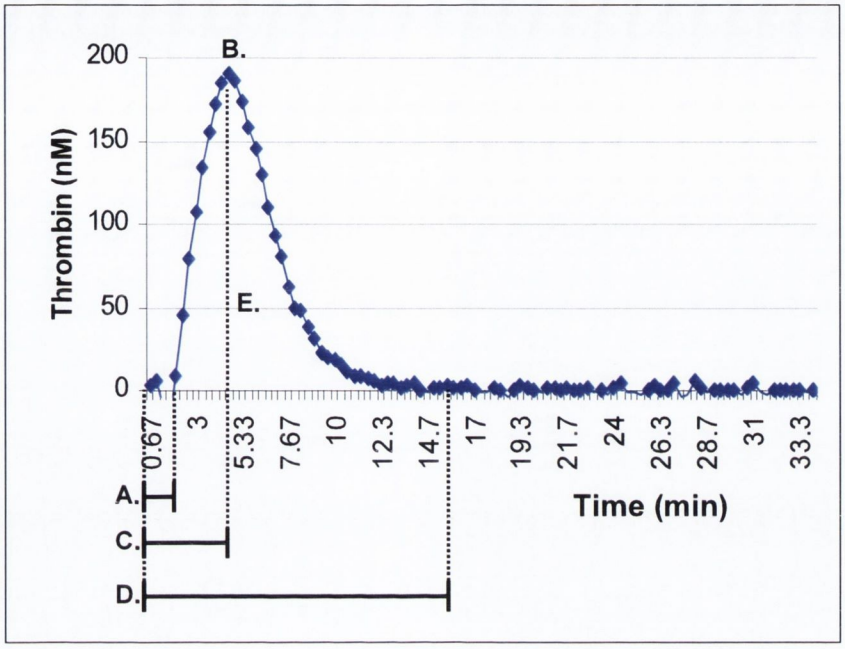


Figure 8.2 Example of Thrombin Generation Curve. A: **Lag-time: Time to initiation of thrombin generation;** B: **Peak thrombin generated;** C: **ttPeak: Time to Peak thrombin generated;** D: **Start-tail: Time to end point of thrombin generation;** E: **ETP: Area under thrombin generation curve. All times measured from t=0 min.**

9. Transcranial Doppler ultrasound of intracranial vessels for microembolic signal detection in symptomatic and asymptomatic carotid artery stenosis and relationship to platelet activation

9.1. Introduction

As stated in Chapter 2, historical data suggest that the two year risk of stroke in the arterial territory supplied by a severely stenosed (≥ 70%) internal carotid artery is approximately 4% in asymptomatic patients in whom the stenosis is incidentally picked up,(The European Carotid Surgery Trialists Collaborative Group, 1995) but this rises to 26% in patients with recent TIA or stroke treated with medical therapy alone.(North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991) This disparity in risk may relate to differences in the morphology and / or stability of the atherosclerotic plaque,(Golledge *et al*, 2000) different degrees of endothelial and / or platelet activation, or differences in thrombogenicity of the circulating blood. (McCabe *et al*, 2005a).

Transcranial Doppler ultrasound (TCD) is a non-invasive imaging technique whereby patients undergo Doppler ultrasound of their intracranial basal arteries through naturally occurring bony 'windows' in the skull. For detection of microembolic signals (MES) in patients with extracranial carotid artery stenosis, one typically insonates the middle cerebral artery (MCA) through the 'temporal windows' of the skull. Previous studies have shown that optimal scan time varies, but the general consensus is that monitoring for carotid plaque-derived MES in the setting of symptomatic or asymptomatic carotid artery stenosis should typically be performed for 1 hour. (Blaser *et al*, 2004;Bernd Ringelstein *et al*, 1998)

Several studies have illustrated the potential role of MES detection and carotid plaque imaging in identifying asymptomatic carotid stenosis patients who may derive most benefit from best medical rather than surgical therapy.(Spence *et al*, 2005) A recent study in patients with > 60% asymptomatic carotid stenosis, suggested that tighter vascular risk factor control with intensive medical therapy, including plasma lipid, blood pressure and glycaemic control, leads to reduction in MES detection on TCD, and a reduction in cardiovascular events after > 1 year follow up.(Spence *et al*, 2010) The authors concluded that patients with > 60% asymptomatic carotid stenosis should only be considered for carotid intervention if they had MES detected on TCD, and otherwise, should receive best medical therapy, as MES represent a surrogate marker of future stroke risk. Another more recent study in patients with > 60% asymptomatic carotid stenosis showed that the 3-year risk of stroke or death was 20% in patients with MES detected on TCD versus 2% in those without MES ($p = 0.003$).(Madani *et al*, 2011) Results from the much anticipated ACES Study were published recently. (Markus *et al*, 2010) The study found that in patients with ≥ 70% asymptomatic carotid stenosis, detection of asymptomatic emboli on TCD could be used to identify patients at a higher risk of stroke or TIA. The authors concluded that assessment for the presence of embolic signals on TCD might be useful in the selection of patients with asymptomatic carotid stenosis who are likely to benefit most from endarterectomy. An accompanying editorial suggested that the use of diffusion weighted imaging (DWI) in association with TCD may be even more useful in selecting those patients who would benefit most from intervention.(Amarenco *et al*, 2010) A further study from the ACES group reported that the combination of plaque echolucency on colour Doppler ultrasound of the carotid arteries and MES-positivity on TCD in patients with ≥ 70% asymptomatic carotid

stenosis was associated with a markedly increased risk of ipsilateral stroke (Hazard Ratio of 10.61 if both these factors were present; p = 0.0003).(Topakian *et al*, 2011)

Molloy and Markus reported that there was a trend towards a higher incidence of MES in the ipsilateral middle cerebral artery in symptomatic patients with TIA or stroke within the preceding 12 months in association with > 60% carotid stenosis (42%) compared with asymptomatic patients with > 60% carotid stenosis (29%; p = 0.15).(Molloy & Markus, 1999). The authors also reported that MES were significantly more common in the subgroup of symptomatic patients with symptoms within the preceding 3 months (14/26 [54%]) compared with their asymptomatic counterparts (12/42 [29%], p = 0.037), and that the occurrence of cerebral emboli may be predictive of an increased risk of clinical TIA or stroke syndromes during follow-up.(Molloy & Markus, 1999).

TCD has also been used to assess the impact of different antiplatelet regimens on the incidence of MES in patients with extracranial or intracranial arterial stenosis. The CARESS study demonstrated that in patients with recently symptomatic ≥50% carotid stenosis, the combination of short-term aspirin and clopidogrel reduced the incidence of MES on TCD compared with aspirin alone (relative risk reduction = 39.8%; P=0.0046).(Markus *et al*, 2005) The recent 'Clopidogrel plus aspirin versus aspirin alone for reducing embolisation in patients with acute symptomatic cerebral or carotid artery stenosis (CLAIR) study revealed that therapy with clopidogrel and aspirin was more effective than aspirin alone in reducing MES in patients with predominantly intracranial symptomatic stenosis (RR reduction = 42.4%, p = 0.025).(Wong *et al*, 2010) Vogten *et al*. assessed 27 patients who were scheduled to undergo carotid endarterectomy on either aspirin monotherapy (N = 18) or aspirin and clopidogrel combination therapy (N = 9). Neither the exact stenosis severity nor the proportion of symptomatic patients were specified.(Vogten *et al*, 2008) All patients underwent TCD monitoring (unclear if ipsi-

/unilateral or bilateral) from the beginning of CEA until 2 hours postoperatively. The authors found a lower mean number of microemboli in the aspirin/clopidogrel than in the aspirin monotherapy group (4.1 *vs.* 17.6; $p<0.01$). Similarly, Payne *et al.* performed continuous intra-operative and immediate postoperative TCD for 3 hours on 100 patients undergoing CEA, and found that patients who were on aspirin monotherapy had a significantly increased risk of having > 20 MES detected on TCD versus patients on aspirin / clopidogrel combination therapy (odds ratio = 10.23; $p = 0.01$).(Payne *et al*, 2004)

Overall, these studies illustrate that data from TCD monitoring may help identify patients with asymptomatic carotid stenosis who warrant carotid intervention, and the limited available data suggest that TCD has the potential to help predict the response to different antiplatelet regimens in the clinical setting. Furthermore, the finding of more frequent MES in patients with recently symptomatic than asymptomatic carotid stenosis provides some insight into the reasons why recently symptomatic patients have a higher stroke risk than patients with asymptomatic carotid stenosis, but does not fully explain all of the differences in risk between the groups. A better understanding of the cellular mechanisms responsible for the disparity in stroke risk and the incidence of MES between symptomatic and asymptomatic patients may improve risk-stratification in this patient population, and facilitate improvements in TIA and stroke prevention in future. To our knowledge, prior to the commencement of this thesis, no studies had systematically and simultaneously compared FBC parameters and platelet activation status and the incidence of MES in patients with asymptomatic versus recently symptomatic carotid stenosis.

Aims

This study aimed to determine:

- whether patients with early symptomatic carotid stenosis had more MES on TCD than those with asymptomatic carotid stenosis;
- whether any differences between these groups reduced as symptomatic patients were followed up to the late phase after symptom onset or carotid intervention;
- whether symptomatic patients with MES on TCD (MES +) had differences in FBC parameters or excessive platelet activation compared with asymptomatic MES + patients;
- whether symptomatic patients without MES on TCD (MES -) had differences in FBC parameters or excessive platelet activation compared with asymptomatic MES - patients;
- within the symptomatic subgroup, whether FBC parameters and platelet activation status varied according to the 'MES status' of these patients at both early and late phases after symptom onset.

Hypotheses

We hypothesised that:

- patients with early symptomatic carotid stenosis would have more MES on TCD than those with asymptomatic carotid stenosis;
- the differences between these groups would fall as symptomatic patients were followed up to the late phase after symptom onset or carotid intervention;
- symptomatic patients with MES on TCD (MES +) would have an increased platelet count and / or excessive platelet activation compared with asymptomatic MES + patients.

- symptomatic patients without MES on TCD (MES -) would have an increased platelet count and / or excessive platelet activation compared with asymptomatic MES - patients.
- within the symptomatic subgroup, MES + patients would have excessive platelet activation compared with MES - patients at the early or late phases after symptom onset.

9.2. Methods

9.2.1. Patient Groups

The definition of asymptomatic and symptomatic patient groups and clinical assessment protocols, as well as ethical approval and inclusion criteria have been described in Chapter 3.1.

9.2.2 Transcranial Doppler

Transcranial Doppler ultrasound (TCD) recordings were performed on all study subjects at a dedicated Vascular Laboratory at our institution by one of two highly-experienced operators (Dr Kinsella or Dr Tobin) using the same settings and criteria for the duration of the study. The number of spontaneous cerebral microembolic signals (MES) detected in the ipsilateral and contralateral middle cerebral arteries (MCAs) during a 1-hour recording period were recorded. (Molloy *et al*, 1998);(Mackinnon *et al*, 2004) Asymptomatic or symptomatic patients undergoing antiplatelet changes were assessed immediately before and at least 2 weeks after the change in antiplatelet treatment, once they had not undergone carotid intervention in the interim. If symptomatic patients did not undergo surgical or endovascular intervention, they were reassessed > 3 months after symptom onset. Any patients who underwent carotid intervention were reassessed at least 3 months following intervention.(Molloy *et al*, 1998;Mackinnon *et al*, 2004)

TCD recordings were performed with the same device for the entire study (Viassys Pioneer TC8080) from the MCA ipsilateral and contralateral to the carotid stenosis of interest via the transtemporal route (mainly the posterior or middle portion of the temporal window) as per published criteria.(Bernd Ringelstein *et al*, 1998) All recordings were performed with 2-MHz probes held firmly in position by a specially-designed rigid external fixation device when the patients were lying supine. Standard settings were used,

with a sample volume (SV) between 5 and 8mm for the majority of assessments, although in some instances, an SV of 8 - 15 mm was used. We also used a constant sweep speed of 5.1 seconds, and 128-point fast-Fourier transform (FFT) spectral analysis was performed, giving an overlap of > 50%. Depth of insonation was 50 - 60 mm in an attempt to record flow in the M1 main stem of the middle cerebral artery. Each patient underwent recording for 60 minutes, although 5 patients (9%) could only tolerate 30 - 45 minutes due to discomfort from the headband that necessitated premature termination of the TCD recording. The Doppler audio signal, which incorporated automated embolic signal detection, (Cullinane *et al*, 2002) was recorded directly onto the system's hard drive. However, entire TCD recordings were subsequently analysed 'off-line' at a later date by the same experienced investigator blinded to patient details (JAK). Embolic signals were identified by their typical visual unidirectional appearance within the spectral display (Figure 9.2 & 9.3) and their characteristic high-pitched chirping, clicking or moaning sound, using an intensity threshold of > 7 dB. Signal intensity was measured by a standard method that calculated the difference in gain between the MES and the background signal in the spectral waveform.(Markus & Molloy, 1997).

Symptomatic patients were classified as being 'MES +' if they had MES detected on TCD ipsilateral to the stenosed carotid artery of interest. Asymptomatic patients were considered to be 'MES +' if they had MES detected on TCD ipsilateral to a moderately or severely stenosed carotid artery. Other patients were deemed to be 'MES -'.

9.2.3 Blood Sampling and Laboratory Tests

Full blood count parameters in EDTA and citrate, and a comprehensive panel of platelet activation markers were measured within 24 hours of TCD measurement, as outlined in Chapter 3.

9.2.4 Statistical Methods

These are described in detail in Chapter 3.11. Approximately 10% of all recordings (including data from symptomatic, asymptomatic and control subjects) were also analysed by an experienced, independent observer (Dr Maher Saqqur, University of Alberta, Canada) who was blinded to the clinical details, symptomatic status, and 'emboli status' of the study subjects, to 'validate' the accuracy of the TCD data that we collected at our centre. Inter-observer agreement between Dr Kinsella and Dr Saqqur was calculated for (a) the actual number of emboli detected by each observer, and (b) for the number of 'emboli-positive' patients (those with ≥ 1 embolus) recorded by each observer using Cohen's unweighted kappa statistic (κ). A kappa value of 0–0.20 indicates poor agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement, and 0.81–1.00 very good/excellent agreement.(Cohen, 1968) $P < 0.05$ was considered statistically significant. All statistical calculations were performed with R version 2.9.1. (R Development Core Team, 2010)

9.3. Results

Forty-six patients with early symptomatic carotid artery stenosis were recruited to the study, 31 of whom had complete transcranial Doppler ultrasound (TCD) data available for analysis. Four declined investigation, 5 had no trans-temporal windows, and 6 did not have complete data recorded for offline analysis due to an error in TCD data recording settings by the company representative at the start of the study. Thirty-five patients were followed up from the early to the late stage after symptom onset or carotid intervention, and 27 of these had TCD data available for analysis; 3 had no temporal windows, 3 declined repeat assessment, and 2 did not have recordings available for offline analysis due to an error in TCD data recording settings. Twenty-two symptomatic patients had longitudinal TCD data available at both the early and late phases after symptom onset. Thirty-one patients with asymptomatic carotid artery stenosis were enrolled in the study, and 25 had TCD data available for analysis; 3 had no temporal windows, 2 declined TCD investigations, and 1 did not have TCD recordings available for offline analysis, as outlined above. The study subject characteristics are presented in Table 9.1 and reasons for exclusion summarised in Figure 9.1.

The inter-observer agreement in the calculation of the number of MES (83% complete concordance, $\kappa = 0.85$), and the presence of MES (93% concordance, $\kappa = 0.89$) between Dr Kinsella and Dr Saqqur was excellent. These findings strongly supported the validity of our on-site data analysis at AMNCH. For the purpose of this thesis, we focused on the TCD data pertaining to the presence or absence of MES rather than on the number of MES, and off-line analysis of all remaining TCD data, and their relationship with markers of platelet activation was performed by Dr Kinsella on site.

In the early symptomatic carotid stenosis group, 10/31 (32%) subjects had $\geq$1 MES (actual MES numbers: 1 – 8) in the MCA ipsilateral to the $\geq$50% symptomatic carotid

stenosis of interest. Interestingly, 5 patients had MES detected in the contralateral MCA, and 3 patients had bilateral MES detected. In the late symptomatic cohort, 5/27 (19%) subjects had ≥1 MES (actual MES numbers: 1 – 3) in the MCA ipsilateral to the symptomatic carotid artery of interest; 1 of these carotid arteries was still moderately stenosed in a patient who had not undergone intervention, 1 was occluded, and the other 3 had < 50% stenosis after carotid intervention. In this late symptomatic cohort, one patient had bilateral MES (< 50% stenosis on either side), and another 3 had MES detected contralateral to the symptomatic stenosis (one who had moderate and another with severe carotid stenosis on the contralateral side). Amongst the asymptomatic carotid stenosis cohort, 3/25 (12%) subjects had MES detected; 2 had >50% stenosis, and the third had complete carotid occlusion with contralateral severe stenosis with MES detected distal to the occlusion.

When one compared the proportion of MES + patients between the asymptomatic and symptomatic groups, 3/25 (12%) asymptomatic versus 10/31 early symptomatic (32%, p = 0.02), and 5/27 (19%, p = 0.2) late symptomatic patients were MES +.

When one compared MES data from asymptomatic patients with the subgroups of late phase symptomatic patients who did and did not undergo carotid intervention, 3/25 asymptomatic patients *vs.* 3/19 late phase symptomatic patients who underwent carotid intervention (12 *vs.* 16%, p = 0.35), and 2/8 late phase symptomatic patients who had not undergone intervention (12 *vs.* 25%, p = 0.19) were MES +. *Post-hoc* analysis revealed that the proportion of patients who were MES positive was not significantly different between late phase symptomatic patients who had undergone intervention compared with those who had not undergone intervention (16 vs. 25%, p = 0.62).

Amongst the symptomatic patients who had longitudinal data at both the early and late phases after symptom onset, although there was a trend, the proportion of patients who

were MES + did not significantly decrease between the early (8/22 [36%]) and late phases (5/22 [23%]; p = 0.17) after symptom onset, regardless of whether or not they underwent intervention.

There were no significant differences in FBC parameters or platelet activation markers between early symptomatic MES + subjects and asymptomatic MES + subjects (Table 9.2a; FBC data not shown). However, lymphocyte-platelet complex formation was significantly higher in early symptomatic MES - patients than in asymptomatic MES - subjects (2.78 vs 2.29, p = 0.0085; Table 9.2b). There were no other significant differences in FBC parameters or markers of platelet activation between these groups. Having controlled for differences in the prevalence of smoking between the early symptomatic MES - patients and asymptomatic MES – patients, the differences in the % lymphocyte-platelet complexes between the subgroups persisted (p = 0.02).

We performed a pre-planned subgroup analysis within the symptomatic patient cohort to compare FBC parameters and platelet activation markers between MES + and MES – patients. The platelet distribution width in EDTA (13.81 *vs.* 12.21; p = 0.016) and mean platelet volume in EDTA (11.05 *vs.* 10.38, p = 0.043; Table 9.3) were higher in early symptomatic MES+ than MES – patients. These differences in FBC parameters were not significant between late symptomatic MES + *vs.* MES – patients, but the numbers included in this subgroup analysis was very small (Table 9.4). Platelet surface CD63 expression was lower in early symptomatic MES + than MES - patients (5.88% *vs.* 14.5%; p = 0.0028). However, in contrast to the early phase, CD63 expression was increased in late symptomatic MES+ than MES - patients (12.8% *vs.* 10.02%; p = 0.023). Otherwise, there were no significant differences in CD62P expression, % reticulated platelets or leucocyte-platelet complexes between MES + and MES - symptomatic patients in the early or late phases after symptom onset (Tables 9.5 & 9.6).

9.4. Discussion

Prior to the commencement of this thesis, published data supported the concept that the presence of microembolic signals predicts subsequent TIA or ischaemic stroke risk in patients with > 60% (Molloy & Markus, 1999);(Spence *et al*, 2010);(Madani *et al*, 2011) and ≥ 70% asymptomatic carotid artery stenosis (Markus *et al*, 2010); (Topakian *et al*, 2011). However, to our knowledge, no studies had compared both MES and platelet activation status in a well-characterised population of patients with asymptomatic versus recently symptomatic ≥ 50% carotid stenosis who had TIA or stroke within the preceding 4 weeks, and who had longitudinal follow up data at the late phase after symptom onset or intervention. There are no data, to our knowledge, comparing MES status in late phase symptomatic versus asymptomatic carotid stenosis patients. The longitudinal component of this study has the potential to provide some insight into the impact of surgical or medical intervention, or time from symptom onset on MES status in symptomatic versus asymptomatic patients.

Ritter *et al*. found higher soluble P-selectin levels in patients with >50% asymptomatic carotid stenosis with MES than in those without MES (122 ng/ml versus 80 ng/ml; p = 0.0007).(Ritter *et al*, 2009) The authors also reported that patients with recent atherothrombotic stroke (proportion with moderate or severe carotid stenosis not specified) who were MES positive had higher soluble P-selectin levels than MES negative subjects (178 ng/ml versus 121 ng/ml; p = 0.02). However, platelet surface CD62P expression was actually lower in MES + than MES - patients with recent atherothrombotic stroke (9 versus 4.5 arbitrary units; p = 0.004). There were no other differences in platelet surface thrombospondin expression, or monocyte-platelet or neutrophil-platelet complexes between MES + and MES - patients in either the

asymptomatic or atherothrombotic carotid stenosis groups. However, data from asymptomatic and symptomatic patients were not compared with one another.

In our study, MES were significantly more common in early symptomatic than asymptomatic carotid stenosis patients ($p = 0.02$), but the difference between late symptomatic and asymptomatic patients was not significant. These early phase data are in keeping with the subgroup analysis in an earlier study in asymptomatic versus symptomatic patients within 3 months of TIA or stroke onset.(Molloy & Markus, 1999) Our longitudinal, late phase data may reflect successful treatment of the stenosing atheromatous plaque in 21/31 (68%) of symptomatic patients during follow up, with alteration of antiplatelet therapy (Markus *et al*, 2005;Wong *et al*, 2010;King *et al*, 2011) or 'plaque healing' in other cases. At the discretion of the treating physicians who referred patients for recruitment to this study, 12/27 of late phase symptomatic patients were treated with aspirin monotherapy (44%), whereas others were treated with aspirin and dipyridamole combination therapy (30%), clopidogrel monotherapy (19%), and two patients were on aspirin and clopidogrel combination therapy (7%; Table 9.1). Because the principal researchers or researcher supervisor (JK, WOT, DM) did not alter treatment regimens in patients who were not directly under our care, we could not formally assess the impact of altering antiplatelet regimens on MES status; this deserves further study.

The absolute percentage of patients who were MES + was slightly lower in late symptomatic patients who did undergo carotid interventional treatment (3/19 [16%]) than in those who did not have intervention (2/8 [25%]), but the differences between these symptomatic subgroups was not significant. However, the number of subjects included in this *post* hoc analysis was far too small to make any definitive conclusions about the impact of endarterectomy or time on MES status. Larger longitudinal studies in the same patients followed up from the early to late phase after symptom onset or intervention are

warranted to further assess the impact of endarterectomy, endovascular treatment, or of altering antiplatelet therapy on MES status in patients with either asymptomatic or symptomatic carotid stenosis. Such data could guide clinical care in future. For example, if altering antiplatelet therapy were shown to markedly reduce MES signals in a patient with recently symptomatic carotid stenosis to levels below those seen in the asymptomatic cohort, and if this translates into a lower stroke risk during follow up, it might be possible be avoid or defer endarterectomy in some cases. Conversely, if some asymptomatic patients are found to be continuously embolising despite changes in antiplatelet therapy, then those patients could be selected for early carotid intervention if their peri-operative risk is deemed acceptable.

The presence of contralateral or bilateral MES in some patients, even in the absence of ≥50% carotid stenosis, may suggest embolisation from a non-stenotic contralateral carotid plaque, from an atheromatous plaque in the aortic arch, or potential embolism from an occult source in the heart, although no patients included in the study had any evidence of a cardioembolic source on thorough cardiac and neurovascular workup. There is some evidence that 'diffuse inflammation' may occur in the vascular system, perhaps secondary to atherosclerotic plaque activation associated with a systemic inflammatory response,(Lutgens *et al*, 2003) in patients with symptomatic carotid stenosis who have contralateral < 50% or >50% 'clinically asymptomatic' carotid stenosis.(Tang *et al*, 2006) Activation of these 'apparently clinically silent plaques' could lead to distal embolisation and the detection of contralateral MES in some study subjects.

Lymphocyte-platelet complex formation was higher in early symptomatic than in asymptomatic MES negative patients, indicative of increased platelet activation in this early symptomatic subgroup. After controlling for differences in demographic and vascular risk factors between groups, the % lymphocyte-platelet complexes remained

higher in early symptomatic than asymptomatic MES – patients. These data confirm that platelets may be excessively activated in subgroups of symptomatic patients who do not have any detectable emboli on TCD. As discussed in Chapter 4, platelets and leucocytes may have excitatory effects on one another, with leucocyte-platelet complex formation facilitating interaction between the two cell types. The formation of leucocyte-complexes may therefore increase the risk of thrombus formation or plaque inflammation, and predispose to recurrent cerebrovascular events due to embolism from an activated plaque in patients with recently symptomatic carotid stenosis. If one could show that individual patients with increased platelet activation are at a higher risk of stroke than patients with lower levels of activation, measurement of leucocyte-platelet complexes may help to further risk-stratify symptomatic patients, either independently or in conjunction with data on their MES status. There was a non-significant trend towards higher monocyte-platelet complexes in early symptomatic compared with asymptomatic MES + patients, but the differences between these subgroups were not significant (Table 9.2a). In this part of our study, 42% of patients were recruited within 1 week, 32% within 1 - 2 weeks, and the remaining 26% between 2 weeks and 4 weeks after TIA or stroke onset. The lack of significant differences in neutrophil-platelet and monocyte-complexes between the early symptomatic and asymptomatic subgroups may reflect a type II error, or the fact that 58% of patients were recruited more than 1 week after symptom onset when binding of more 'chronic phase' leucocytes to platelets may have become predominant.

The lower CD63 levels in early symptomatic MES+ subjects versus MES- subjects somewhat argues against the theory that increased platelet activation leads to microemboli formation in the acute phase after symptom onset. These findings could be partly explained by the shedding of expressed activation markers from the platelet surface following enhanced platelet activation, or the phenomenon of 'platelet exhaustion',

whereby platelets which have been in a maximal activation state, have degranulated and lost most of their reserves of CD63 from the lysosomes or dense granules of the platelet.(McCabe *et al*, 2005b) This may also explain the findings of Ritter et al who found that CD62P levels were lower in MES + than in MES – patients after atherothrombotic stroke.(Ritter *et al*, 2009). However, in the late symptomatic phase in our study, MES + patients had higher CD63 expression than MES - subjects, which could be explained by increased platelet activation among patients with ongoing embolisation either from a carotid artery that had been treated with surgical or endovascular intervention or from another 'non-carotid artery source'. However numbers were too small to make definitive conclusions re this issue, and these findings may simply represent a type 1 error. At present, one cannot consider platelet surface CD63 to be a reliable marker of platelet activation that will help differentiate MES + from MES – patients in patients with carotid stenosis.

Further exploratory data analysis did not reveal any significant differences between MES+ and MES- groups in relation to time to recruitment after symptom onset, or the proportion of subjects on statin or antiplatelet therapy. More intensive antiplatelet regimens may have contributed to the absence of MES in some subjects, but the numbers are too small to make definitive conclusions about the impact of antiplatelet therapy on the presence or absence of MES.

The increased mean platelet volume (MPV) and platelet distribution width (PDW) in early symptomatic MES + versus MES - subjects may be an additional marker of increased platelet activation and/or turnover in these patients. However, this may also simply represent a type 1 error as the numbers involved in this subgroup analysis were relatively small.

9.5. Conclusions

Microembolic signals were more frequently detected in early symptomatic than asymptomatic patients, but the differences between the late symptomatic and asymptomatic groups were not significant. Increased lymphocyte-platelet complex formation in recently symptomatic compared with asymptomatic MES negative patients indicates enhanced platelet activation status in this early symptomatic subgroup. Use of a multimodal risk-stratification model in patients with carotid stenosis, that includes MES detection and concurrent platelet activation analysis, especially with quantification of leucocyte-platelet complexes, may help identify patients with asymptomatic and early symptomatic carotid stenosis at highest risk of recurrent events who should be considered for urgent medical or surgical intervention. Based on the results of this thesis, such a study has been designed and is currently ongoing in our laboratory under the supervision of Dr McCabe.

Table 9.1: Demographic data and risk factor profiles of study patients. P values relate to chi-squared or Fisher exact tests between asymptomatic versus early or late phase symptomatic patients. Values are means (±SD) or absolute counts, with percentages in square parentheses, where appropriate.

Parameter	Asymptomatic Carotid Stenosis (n = 25)	Early Symptomatic Carotid Stenosis (n=31)	p	Late Symptomatic Carotid Stenosis (n=27)	p
Mean age (years)	69 (±7.8)	65.6 (±9.8)	0.2	64.9 (±10.4)	0.14
Gender (Male [%])	17 (68%)	23 (74%)	0.61	19 (70%)	0.85
Degree of Stenosis:					
Moderate, 50 – 69%	8 (32%)	4 (13%)	0.11	5 (19%)	0.34
Severe, 70 – 99%	17 (68%)	24 (77%)	0.43	2 (7%)	**< 0.001**
Occlusion	0	3 (10%)	0.25	3 (11%)	0.24
No of patients who were MES+ (%)					
-Ipsilateral to carotid stenosis of interest:	3	10 (32%)	**0.02**	5 (19%)	0.2
<50% stenosis	2	0	0.2	3(11%)	1
Moderate stenosis	0	0	1.0	1(4%)	1
Severe stenosis	0	9 (29%)	**0.003**	0	1
Occlusion	1	1(3%)	1	1(4%)	1
-Contralateral to carotid stenosis of interest	2 (8%)	8 (26%)	0.08	4 (15%)	0.44
-Bilateral HITS	0	3 (10%)	0.11	1 (4%)	0.33
Antiplatelet therapy					
-Aspirin monotherapy	16 (64%)	22 (71%)	0.58	12 (44%)	0.16
-Aspirin /Dipyridamole combination	2 (8%)	4 (13%)	0.56	8 (30%)	**0.048**
-Clopidogrel monotherapy	5 (20%)	1 (3%)	**0.044**	5 (19%)	0.89
-Aspirin /Clopidogrel combination	2 (8%)	1 (3%)	0.43	2 (7%)	0.94
IHD	6 (24%)	7 (23%)	0.9	5 (19%)	0.63
Hypertension	22 (88%)	19 (61%)	**0.025**	17 (63%)	**0.037**
Diabetes Mellitus	5 (20%)	5 (16%)	0.71	3 (11%)	0.37
Prior Stroke/TIA	8 (32%)	5 (16%)	0.16	5 (19%)	0.26
Family History Stroke	8 (32%)	8 26%)	0.61	10 (37%)	0.7
Prior DVT/PE	0	0	1.0	0	1.0

Peripheral Vascular Disease	4 (16%)	6 (19%)	0.74	5 (19%)	0.81
Migraine (with or without aura)	5 (20%)	4 (13%)	0.49	3 (11%)	0.46
Current smoker	4 (16%)	15 (48%)	**0.013**	5 (19%)	1.0
Ex-smoker	18 (72%)	12 (39%)	**0.013**	18 (67%)	0.68
Never smoker	3 (12%)	4 (13%)	0.92	4 (15%)	0.77
Statin therapy	24 (96%)	22 (71%)	**0.015**	26 (96%)	0.96

Table 9.2a: Markers of platelet activation and turnover in early symptomatic MES positive versus asymptomatic MES positive carotid stenosis patients

Table 9.2b: Markers of platelet activation and turnover in early symptomatic MES negative versus asymptomatic MES negative carotid stenosis patients. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

9.2a	Early Symptomatic MES positive (n=10)	Asymptomatic MES positive (n=3)	P value
CD62P %	1.93 (1.17 – 2.46)	2.59 (2.19 – 3.22)	0.22
CD63 %	5.88 (5.76 – 9.11)	10.9 (9.53 – 13.45)	0.1
Reticulated platelets %	21.3 (18.7 – 23.2)	24.5 (22.0 – 24.6)	0.29
Lymphocyte-platelet complexes %	2.81 (2.44 – 3.36)	2.82 (2.0 – 2.83)	0.47
Monocyte – platelet complexes %	5.45 (4.45 – 6.45)	3.7 (3.6 – 3.85)	0.052
Neutrophil – platelet complexes %	3.21 (2.7 – 3.3)	3.07 (2.55 – 3.2)	0.37

9.2b	Early Symptomatic MES negative (n=21)	Asymptomatic MES negative (n=22)	P value
CD62P %	1.87 (1.57 – 2.25)	1.81 (1.05 – 2.31)	0.47
CD63 %	14.5 (8.84 – 15.7)	10.8 (8.54 – 13.93)	0.14
Reticulated platelets %	20.8 (11.9 – 26.0)	20.9 (7.38 – 25.95)	1.0
Lymphocyte-platelet complexes %	2.78 (2.49 – 3.36)	2.29 (1.8 – 2.6)	**0.0085**
Monocyte – platelet complexes %	4.95 (3.84 – 6.55)	4.6 (4.4 – 5.5)	0.99
Neutrophil – platelet complexes %	3.11 (2.26 – 3.54)	2.7 (2.4 – 3.1)	0.26

Table 9.3: FBC parameters in the MES positive versus negative early symptomatic carotid stenosis subjects. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Early Symptomatic MES positive (n=10)	Early Symptomatic MES negative (n=21)	P value
Platelet Count ($x10^9$/L) (EDTA)	252.5 (205.5 - 326)	270 (228 – 303)	0.72
Platelet Count ($x10^9$/L) (citrate)	209 (181 – 274.7)	212 (192 – 260)	0.8
Platelet Distribution Width % (EDTA)	13.81 (±1.39)	12.21 (±1.99)	**0.016**
Platelet Distribution Width % (citrate)	11.41(±1.28)	10.38(±1.78)	0.079
Mean Platelet Volume (fl) (EDTA)	11.05(±0.62)	10.38(±1.14)	**0.043**
Mean Platelet Volume (fl) (citrate)	9.85(±0.57)	9.4(±1.0)	0.12
WCC ($x10^9$/L) (EDTA)	8.15 (±1.4)	8.4 (±1.44)	0.65
Neutrophils ($x10^9$/L) (EDTA)	5.5 (4.4 – 6.0)	5.06 (4.57 – 5.64)	0.63
Monocytes ($x10^9$/L) (EDTA)	0.61 (±0.2)	0.75 (±0.2)	0.1
Lymphocytes ($x10^9$/L) (EDTA)	1.85 (±0.72)	2.11 (±0.59)	0.32

Table 9.4: FBC parameters in the MES + versus MES- late symptomatic carotid stenosis subjects. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Late Symptomatic MES positive (n=5)	Late Symptomatic MES negative (n=22)	P value
Platelet Count ($x10^9$/L) (EDTA)	265 (250 - 276)	251.5 (195 – 308)	0.76
Platelet Count ($x10^9$/L) (citrate)	220 (219 – 247)	205 (161 – 255)	0.63
Platelet Distribution Width % (EDTA)	13.66 (±1.7)	12.58 (±1.53)	0.26
Platelet Distribution Width % (citrate)	11.1(±0.58)	10.96(±1.38)	0.81
Mean Platelet Volume (fl) (EDTA)	11.2(±0.8)	10.57(±0.8)	0.18
Mean Platelet Volume (fl) (citrate)	9.64(±0.21)	9.7(±0.7)	0.82
WCC ($x10^9$/L) (EDTA)	8.37 (±1.42)	7.67 (±2.23)	0.4
Neutrophils ($x10^9$/L) (EDTA)	4.9 (4.7 – 5.9)	4.49 (3.5 – 5.7)	0.62
Monocytes ($x10^9$/L) (EDTA)	0.83 (±0.4)	0.66 (±0.2)	0.38
Lymphocytes ($x10^9$/L) (EDTA)	2.25 (±0.35)	2.01 (±0.78)	0.3

Table 9.5: Platelet activation data in early symptomatic MES positive versus MES negative carotid stenosis subjects. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Early Symptomatic MES positive (n=10)	Early Symptomatic MES negative (n=21)	P value
CD62P %	1.93 (1.17 – 2.46)	1.87 (1.57 – 2.25)	0.77
CD63 %	5.88(5.76 – 9.11)	14.5 (8.84 – 15.7)	**0.0028**
Reticulated platelets %	21.3 (18.7 – 23.2)	20.8 (11.9 – 26.0)	0.94
Lymphocyte-platelet complexes %	2.81 (2.44 – 3.36)	2.78 (2.5 – 3.36)	0.93
Monocyte – platelet complexs %	5.45 (4.45 – 6.45)	4.95 (3.84 – 6.55)	0.69
Neutrophil – platelet complexes %	3.21 (2.7 – 3.31)	3.11 (2.26 – 3.54)	0.98

Table 9.6: Platelet activation data in late symptomatic MES + versus MES- subjects. Values are means (±SD) or medians (25th - 75th percentile). Significant p values in bold.

	Late Symptomatic MES positive (n=5)	Late Symptomatic MES negative (n=22)	P value
CD62P %	2.23 (2.01 – 4.56)	1.84 (1.24 – 2.24)	0.12
CD63 %	12.8 (12.5 – 18.9)	10.02 (7.85 – 11.0)	**0.023**
Reticulated platelets %	20.7 (18.2 – 23.7)	20.4 (9.7 – 24.0)	0.6
Lymphocyte-platelet complexes %	2.46 (2.39 – 2.72)	2.59 (2.1 – 3.02)	0.63
Monocyte – platelet complexs %	5.85 (4.7 – 7.4)	5 (4.5 – 5.8)	0.39
Neutrophil – platelet complexes %	3.2 (2.77 – 3.64)	2.69 (2.4 – 3.24)	0.37

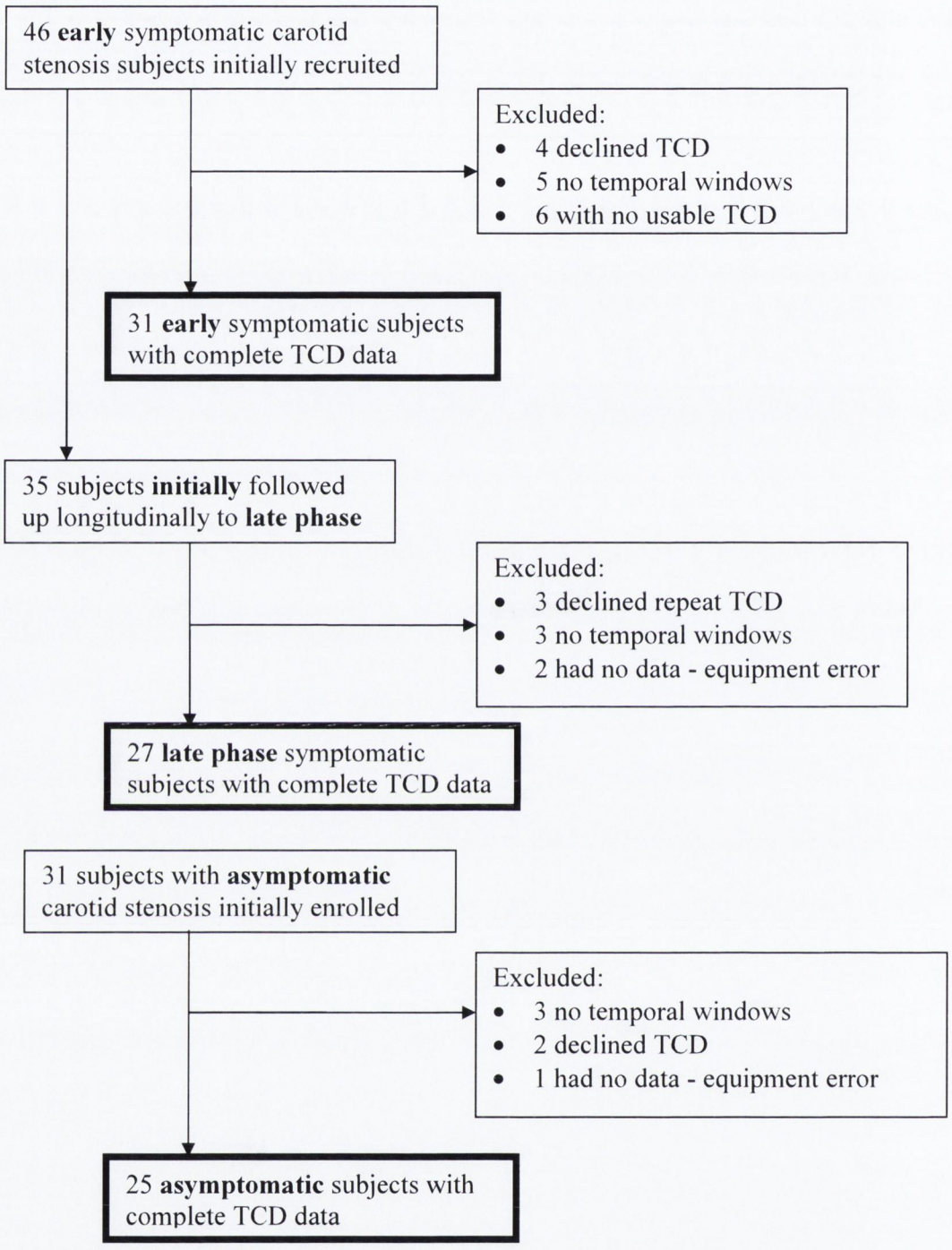


Figure 9.1: Algorithm of asymptomatic and symptomatic carotid stenosis patients included and excluded in the study.

Figure 9.2a: Example of a spectral waveform representing normal blood flow through the middle cerebral artery (MCA).

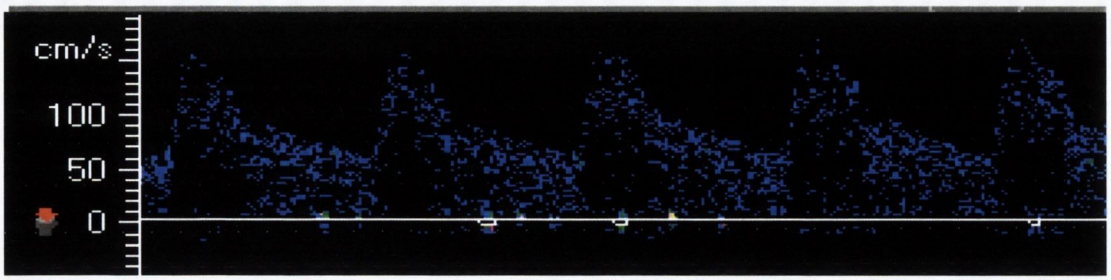


Figure 9.2b: Example of a microembolic signal (MES) within the visual spectrum (indicated by white arrow). The visual spectrum represents blood flow through the middle cerebral artery (MCA). The embolus is unidirectional and <300 ms in duration and accompanied by a characteristic high-pitched chirp or click. It is within the diastolic component of the cardiac cycle in this example. X-axis represents time (sec); y-axis represents velocity of flowing blood (cm/s).

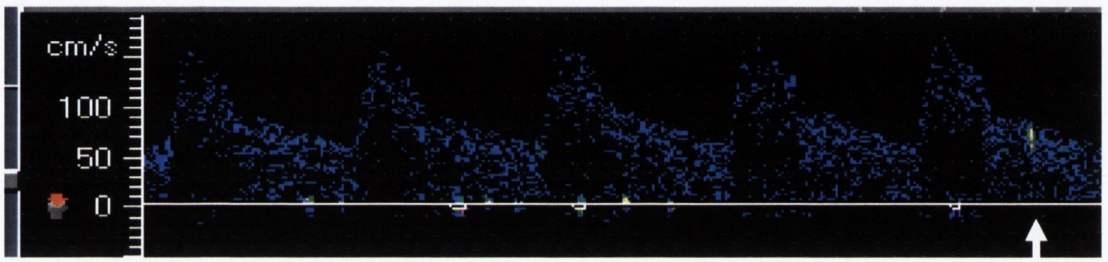


Figure 9.2c: Another example of a MES with accompanying time delay at different depths within the M (motion)-mode recording (white arrow). With multilevel recordings, it is possible to detect the subtle time delay as the MES travels from one sample volume (SV) to another at different depths (mm), increasing the likelihood of the signal representing a true MES rather than artefact.

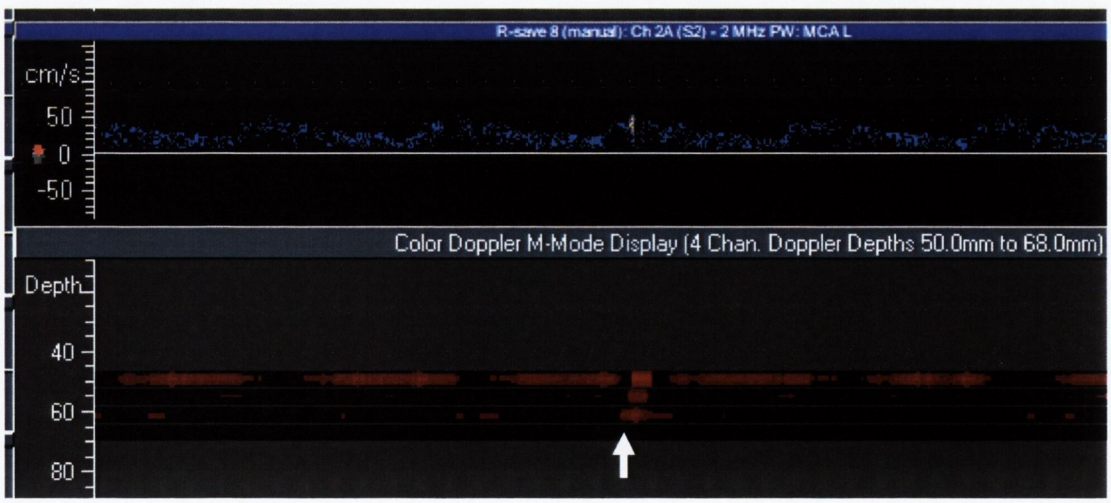


Figure 9.2d: Example of time delay as a result of a MES moving from a SV at one depth to another (54mm and 60mm). The soundtrack display on the right indicates the acoustic information from the MES, indicating the time delay (white arrows) at the 2 different depths indicating a true MES.

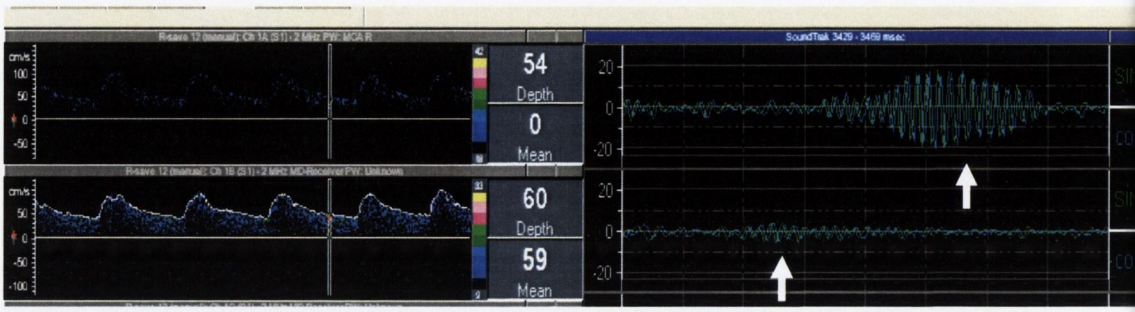
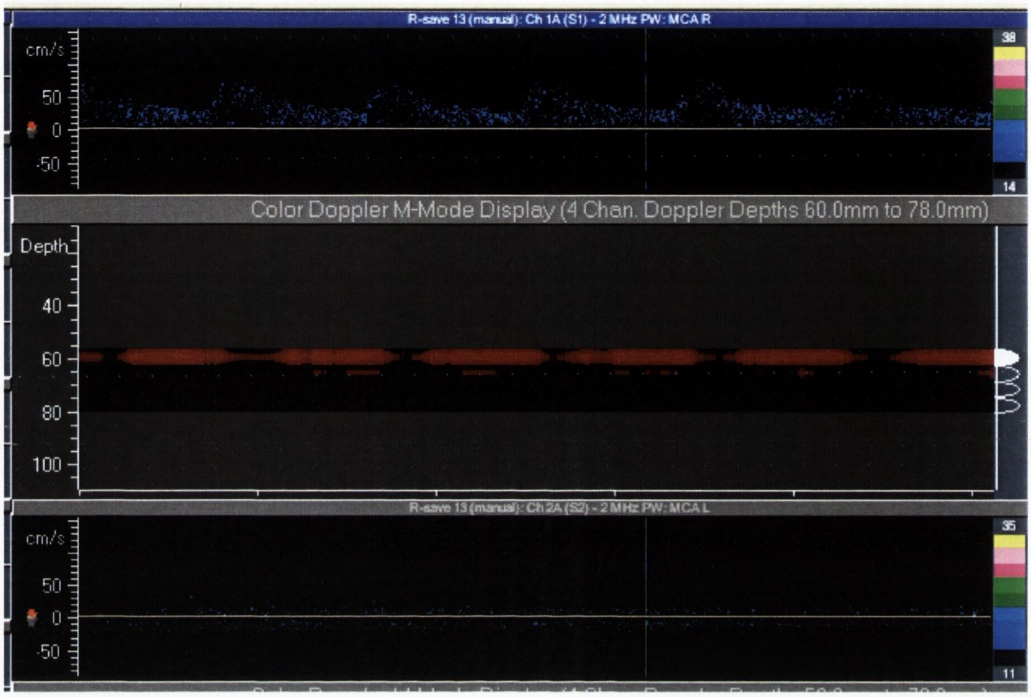


Figure 9.3: Example of a bidirectional signal extending outside the spectral waveform indicating artefact. It's presence on a bilateral recording increase the likelihood of this signal representing artefact rather than a MES (e.g. secondary to patient or transducer movement).



10. Assessment of the association between carotid plaque morphology and microembolic signals in asymptomatic and symptomatic carotid artery stenosis

10.1. Introduction

10.1.1 Colour Doppler ultrasound (CDUS) grading of the severity of carotid artery stenosis

As stated in Chapter 2, carotid endarterectomy may reduce the risk of subsequent stroke in patients with recently symptomatic ≥ 70% internal carotid artery stenosis, (North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991;Rothwell *et al*, 2003;The European Carotid Surgery Trialists Collaborative Group, 1995) and in some patients with ≥ 50% symptomatic (Rothwell *et al*, 2003) and ≥ 60% asymptomatic carotid artery stenosis.(Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995;Halliday *et al*, 2004) Colour Doppler ultrasound (CDUS) is widely used as a non-invasive, easily accessible, rapid method of evaluating the degree of internal carotid artery (ICA) stenosis (Figure 10.1)(Hallam *et al*, 1989). The degree of internal carotid artery stenosis is graded according to standardised measurements of the peak systolic velocity (PSV) and the end diastolic velocity (EDV) in the internal carotid artery, and the ratio of the PSV in the internal carotid artery (PSV_{ICA}) to that in the common carotid artery (PSV_{CCA}).(Sidhu & Allan, 1997;Grant *et al*, 2003)

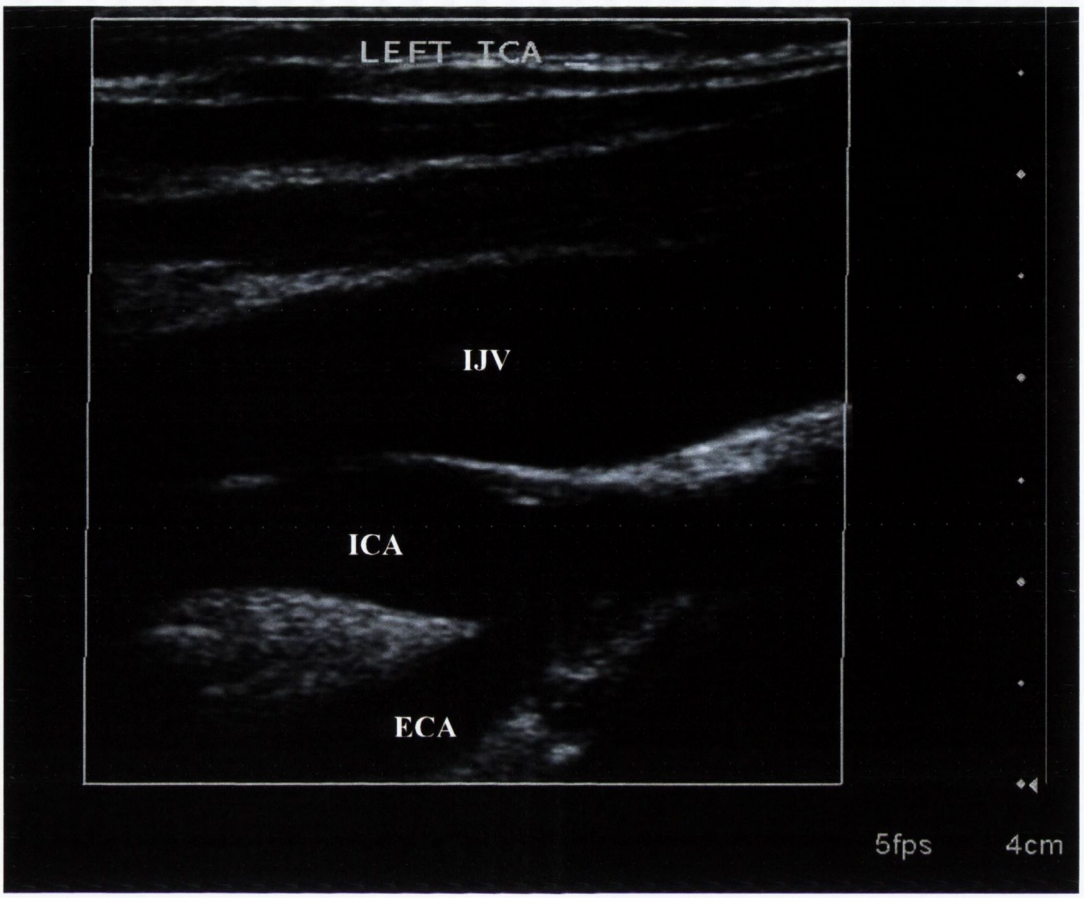


Figure 10.1: Ultrasound picture of a non-stenosed internal carotid artery (ICA) with the external carotid artery (ECA) and internal jugular vein (IJV) identified.

In 2003, an expert consensus committee proposed standardised criteria for grading ICA stenosis, which included a peak systolic velocity (PSV) of > 230 cm/s for identifying an ICA stenosis of ≥ 70%.(Grant *et al*, 2003) These data were based on appraisals from several prior studies comparing formal angiographic versus colour Doppler ultrasound classification of degrees of carotid stenosis. Huston *et al* previously demonstrated that a PSV ≥ 230 cm/s had a sensitivity of 86.4% and a specificity of 90.1% for detecting ≥ 70% ICA stenosis.(Huston, III *et al*, 2000) A PSV ≥ 130 cm/s had a sensitivity of 92.1 % and a specificity of 89.5% for detecting ≥ 50% ICA stenosis. Neale *et al* demonstrated that an end diastolic velocity (EDV) > 110 cm/sec provided a sensitivity of 96% and a specificity of 91% for detecting > 70% ICA stenosis.(Neale *et al*, 1994) A subsequent, independent, single centre study revealed that a PSV measurement > 230 cm/s had a sensitivity of 95.3% and specificity of 84.4% for identifying an ICA stenosis of ≥ 70% using the NASCET method of grading ICA stenosis on digital subtraction angiography.(Braun *et al*, 2008)

Accurate grading of the severity of carotid stenosis is therefore vitally important in determining whether patients should be treated with optimal medical management or surgical or endovascular treatment, but cannot identify all patients who actually require carotid intervention. For this reason, there is increasing interest in other plaque imaging characteristics that may help risk stratify patients with asymptomatic and symptomatic carotid stenosis. In this chapter, I will review the relevant literature on ultrasound-based plaque morphological assessment in patients with carotid stenosis, and then describe our novel, pilot study.

10.1.2 The role of B-mode ultrasound in the characterisation of symptomatic and asymptomatic carotid artery plaques

Duplex B-mode ultrasonography has been used to analyse the characteristics and morphology of carotid plaques *in vivo* in an attempt to identify potentially 'high-risk' plaques constituents which may be associated with a higher risk of ipsilateral ocular or hemispheric ischaemic events.(de Bray *et al*, 1998;Gronholdt *et al*, 2001;Gronholdt *et al*, 2002) There have been several scales used for the assessment and grading of plaque morphology, including the Gray-Weale score the Beletsky Score and the computer-assisted Gray Scale Median (GSM).

The Gray-Weale score categorises plaques into the following types based on visual inspection of the plaque by the operator: (1) dominantly echolucent, (2) substantially echolucent with small areas of echogenicity, (3) dominantly echogenic with small areas (< 25%) of echolucency, (4) uniformly echogenic, and (5) not visible because of heavy calcification.(Gray-Weale *et al*, 1988).

The Beletsky Score broadly describes the components of a carotid artery plaque visible on ultrasound based on appearances of organized thrombus; intraplaque hemorrhage/lipid deposition; fibrosis; and calcification.(Beletsky *et al*, 1996) The score has 3 plaque types: (1) soft plaque/organised thrombus, (2) intraplaque hemorrhage/fatty deposition, and (3) fibrosis/densely calcified plaque.

The Gray-Scale Median (GSM) is believed to be a more objective measure of carotid plaque echogenicity than the Gray-Weale classification. To calculate the GSM, B-Mode image normalisation and standardisation is performed, as previously described.(Elatrozy *et al*, 1998;Sabetai *et al*, 2000) As in previous studies,(Elatrozy *et al*, 1998;Iannuzzi *et al*, 1995) B-mode images were digitised and transferred to a personal computer, and Adobe

Photoshop CS2 software used to analyse the images. All images had colour eliminated and converted to gray-scale using the 'gray-scale' software function. The 'histogram facility' and the 'levels function' are employed to standardise the image for each plaque, and the GSM of 2 reference points is defined: (i) The GSM of the 'noiseless' blood in the centre of the carotid lumen is normalised to an 'input' value of 0 – 5, equivalent to 'maximal echolucency', and (ii) the GSM of the echogenic adventitia within the plane of imaging adjacent to the target plaque was normalised to an 'output' value of 185 – 195, equivalent to 'maximal echodensity'. This process normalises the gray-scale values of all pixels in the original B-mode image according to these input and output reference points (i.e. the vessel lumen and adventitia) (Figure 10.2). The distribution of the gray levels within the plaque is assessed, and the median of the normalised gray values is calculated to establish the 'gray scale median' of the plaque, and thus quantify plaque echogenicity (Figure 10.2). The lower the GSM value, the more echolucent the plaque.

Relationship between ultrasound plaque characteristics and symptoms in patients with carotid stenosis

Early studies attempted to determine the association between potentially 'unstable' plaques on ultrasound and the occurrence of cerebrovascular symptoms during follow up. One study assessed the value of duplex ultrasonography at predicting future cerebrovascular events in 242 asymptomatic carotid artery stenosis patients (49 patients with 0 – 15% stenosis, 99 with 16 – 49% stenosis, 61 with 50 – 79% stenosis, 13 with 80 – 99% stenosis and 20 with carotid occlusion).(Bock *et al*, 1993) The authors found that patients with echolucent, and presumably lipid-laden plaques had a higher annual incidence of TIA or stroke than patients with echogenic plaques (5.7% *vs.* 2.4% p = 0.03). Furthermore, carotid artery disease progression over time was highly correlated

with the occurrence of subsequent TIA and stroke during follow-up ($p < 0.0001$). Significant carotid stenosis progression occurred on serial ultrasound scans in 2 out of 15 patients before their stroke, and in 7 patients following their stroke. Of 20 TIA patients, carotid stenosis progression occurred before the event in 3 patients, but 11 showed progression on the post-stroke scan. Overall, 164 scans demonstrated progression, of which 18 (11%) were associated with stroke or TIA. These findings, and those from more recent studies,(Topakian *et al*, 2011) suggest that asymptomatic carotid stenosis patients with severe stenosis or echolucent plaque appearances on ultrasound are at higher risk of ipsilateral stroke risk than asymptomatic patients without these findings. A subsequent study assessed the relationship between colour Doppler carotid plaque appearances according to the Gray-Weale classification and subsequent symptoms in 285 symptomatic (ocular or hemispheric stroke or TIA) and 65 asymptomatic patients, all of whom had 60 - 99 % ICA stenosis on Doppler ultrasound.(Golledge *et al*, 1997) Information regarding prescribed antiplatelet therapy was not presented. Predominantly echolucent plaques were seen more frequently in symptomatic than asymptomatic patients (83 *vs*. 44 %; $p < 0.0001$), and plaque ulceration was also more common in symptomatic than asymptomatic patients (23 vs 14 %; $p = 0.04$). These early studies suggested an important association between echolucent carotid plaque appearances and the risk of subsequent cerebrovascular symptoms.

In one of the first studies to perform computerised GSM analysis of carotid plaque duplex ultrasound data, Biasi *et al*. retrospectively assessed plaque echogenicity in patients with asymptomatic and symptomatic > 50% carotid stenosis, and investigated its association with cerebrovascular symptoms and CT – documented cerebral infarction in the carotid territory of interest.(Biasi *et al*, 1998) Cerebrovascular symptoms were not significantly correlated with the appearance of cerebral infarction on CT in the relevant territory ($p =$

0.076). Mean GSM was 38 for plaques in patients who had infarction versus 56 for plaques in patients who did not have cerebral infarction on CT of brain ($p < 0.0001$). The prevalence of infarction on CT was 40% in patients with a plaque GSM < 50 *vs.* 9% in patients with a plaque GSM > 50 ($p < 0.001$). This early study demonstrated the potential usefulness of computerized GSM analysis of plaque echogenicity at predicting the risk of clinically evident or silent infarction on CT of brain.

A further study tested the hypothesis that the occurrence of ipsilateral stroke could be predicted by the GSM estimate of echolucency of carotid atherosclerotic plaques in previously symptomatic (n = 135) and asymptomatic (n = 111) patients with ≥50% carotid stenosis.(Gronholdt *et al*, 2001;Gronholdt *et al*, 2001) The group found that echolucent plaques predicted the risk of subsequent ipsilateral stroke in previously symptomatic but not asymptomatic patients. This study provided some evidence to suggest that measurement of plaque echogenicity with GSM analysis, as well as the degree of stenosis may facilitate improvements in the selection of patients for carotid intervention.

Relationship between ultrasound plaque characteristics and peri-procedural symptoms around the time of carotid intervention

Other studies that investigated the role of ultrasound in the identifying 'unstable' carotid plaques most likely to embolise around the time of carotid intervention, in particular stenting, have revealed conflicting results. Biasi *et al.* performed the first study to analyse the value of the GSM at predicting the risk of stroke during carotid stenting in patients enrolled in the Imaging in Carotid Angioplasty and Risk of Stroke (ICAROS) registry.(Biasi *et al*, 2004) 11 of 155 (7.1%) patients with a GSM ≤ 25 had stroke around the time of intervention *vs.* 4 of 263 (1.5%) with a GSM > 25 ($p = 0.005$). In addition,

patients with ≥ 85% stenosis had a higher incidence of stroke than those with less severe stenosis (p = 0.03). Multivariate analysis revealed that GSM (OR 7.11; p = 0.002) and degree of stenosis (OR 5.76; p = 0.010) were independent predictors of the risk of stroke in these patients. These data suggested that patients with echolucent carotid plaques with a pre-interventional GSM ≤25 were at an increased the risk of stroke around the time of stenting. A subsequent study by Reiter *et al.* that assessed the relationship between carotid plaque morphology on ultrasound and neurological complications in 698 consecutive patients who underwent elective carotid stenting for symptomatic or asymptomatic ≥ 70% ICA stenosis did not support the conclusions from the ICAROS registry.(Reiter *et al*, 2006) 70% of patients in this study were asymptomatic; 21% had had an ipsilateral TIA and 9% an ipsilateral stroke within the preceding 6 months. Pre-interventional plaque status was classified according to GSM, standardized Beletsky score, and Gray-Weale plaque scores. Neurological complications up to 30 days after intervention were recorded prospectively. 5.9% (41/698) of patients had a TIA, minor or major stroke during follow-up in this study. Median GSM, Beletsky and Gray-Weale scores in the entire cohort were 45, 3.0 and 2.0, respectively. However, none of these plaque measurements were significantly different between the symptomatic and asymptomatic carotid stenosis groups, even after adjusting for traditional risk factors, current medication, pre-interventional symptoms, degree of stenosis, contralateral occlusion and use of cerebral protection devices. This study suggested that plaque echolucency, as measured by different ultrasound grading systems, did not identify patients at increased risk of peri-interventional neurological events in patients undergoing carotid stenting.

Tegos *et al.* studied ultrasound plaque characteristics, including surface morphology, and degree of stenosis in patients with asymptomatic and symptomatic carotid

stenosis.(Tegos *et al*, 2001b) Echogenic characteristics as measured by GSM and the degree of stenosis on ultrasound were the strongest predictors of symptomatic carotid stenosis, with lower GSM ($p = 0.001$) and higher degrees of carotid stenosis ($p = 0.01$) being associated with symptomatic rather than asymptomatic stenosis. However, surface plaque characteristics on ultrasound were not significantly different between asymptomatic and symptomatic plaques.

Relationship between ultrasound plaque characteristics and microembolic signals (MES) on TCD, including around the time of carotid intervention

The relationship between microembolic signals (MES) on transcranial Doppler (TCD) with symptoms and plaque characteristics, including echodensity as measured by GSM and the degree of carotid stenosis was assessed in patients with 50 – 99% carotid stenosis ($n = 59$ patients, and 80 plaques).(Tegos *et al*, 2001a) As anticipated, MES were more frequent in symptomatic than asymptomatic hemispheres (Mann-Whitney U test, $p = 0.031$). There was a weak, but significant inverse correlation between GSM and emboli count, with increased MES counts associated with lower GSM values and thus more echolucent plaques (Spearman correlation $r = - 0.22$; $p = 0.045$). However, there was no significant correlation between increasing degrees of stenosis and the number of MES (Spearman correlation $r = 0.086$; $p = 0.44$). These data provide some support for the concept that echolucent plaques account for some of the emboli that occur in association with carotid stenosis, but the number of subjects included in this analysis was too small to make any definitive conclusions about the relationship between the degree of stenosis and the risk of MES in patients with carotid stenosis. A more recent study investigated the prevalence of MES in the MCA of patients with symptomatic high-grade internal carotid artery (ICA) stenosis to identify predisposing factors for MES.(Zuromskis *et al*, 2008)

Cerebral MES were present in 30% of patients with symptomatic carotid stenosis despite optimal anti-platelet therapy, but GSM ultrasound plaque characteristics were not predictive of the presence or absence of MES. A further study assessed the role of carotid plaque echolucency, as defined by GSM analysis, as a potential risk factor for cerebral embolism during carotid stenting in 27 patients with symptomatic severe carotid stenosis.(Rosenkranz *et al*, 2009). 13 carotid plaques were classified as echolucent (GSM < 50), and 14 plaques were classified as echogenic (GSM > 50). Solid microemboli were more common during stenting of patients with echolucent plaques than patients with echogenic plaques (85% vs. 42%; p = 0.031), but plaque echolucency alone could not reliably identify patients at high risk of periprocedural embolism.

Relationship between carotid plaque characteristics on ultrasound and histopathological plaque analysis

One study compared carotid plaque morphology on B-mode ultrasound with histopathological findings in patients who underwent carotid endarterectomy.(Tegos *et al*, 2000) Retinal symptoms were associated with echolucent plaques (median GSM = 0), hemispheric symptoms with intermediate plaque echolucency (median GSM = 16), and asymptomatic patients had hyperechoic/echodense plaques (median GSM = 34; p = 0.001 for comparison between the three groups using the non-parametric Kruskal-Wallis Test). Echolucent plaques were associated with the presence of plaque haemorrhage (median GSM for hemorrhagic plaques = 6, and for non-haemorrhagic plaques = 20; p = 0.04). These data suggested that distinct echomorphologic plaque characteristics were associated with retinal and hemispheric symptoms and asymptomatic status, but larger studies are needed to confirm these findings, especially in symptomatic patients. Goncalves *et al*. reported that plaque echogenicity on ultrasound is mainly determined by

elastin and calcium, but not collagen or lipid content, and that echolucent plaques have higher elastin content.(Goncalves *et al*, 2004)

More recent studies have used novel techniques in an attempt to further clarify the nature and stability of the carotid plaque, and in particular, the morphology of the overlying carotid plaque cap. These studies have incorporated colour mapping of plaques and contrast-enhanced US (Sztajzel *et al*, 2005;Shalhoub *et al*, 2010;Owen *et al*, 2010), or 3D ultrasound of carotid plaque ulcers to potentially identify high risk asymptomatic carotid stenosis patients who should be considered for carotid intervention.(Madani *et al*, 2011). Sztajzel et al. investigated whether stratified GSM analysis of carotid plaques combined with colour mapping correlated better with the presence of symptoms and intracranial microembolic signals on TCD than whole plaque measurement with B-mode ultrasound only.(Sztajzel *et al*, 2006;Sztajzel *et al*, 2005) Mean GSM was lower in symptomatic than asymptomatic plaques, and low mean GSM values with a predominance of red colour at the plaque surface (indicative of GSM < 50) correlated with symptomatic or MES-positive stenoses. The data suggested that stratified GSM measurements combined with colour mapping may improve our ability to identify plaques that are likely to become symptomatic or cause distal embolisation; these findings definitely warrant further study.

Previous studies have attempted to risk-stratify asymptomatic carotid stenosis patients who have ultrasonographic and other features of a potentially active plaque to identify those who may benefit from more urgent intervention.(Madani *et al*, 2011;Topakian *et al*, 2011) Other studies have also investigated the ability of advanced methods of carotid plaque ultrasound to identify patients likely to benefit from intervention.(Owen *et al*, 2010) Enhanced risk-stratification models could identify high-risk subgroups of asymptomatic and symptomatic patients who could derive most benefit from timely carotid endarterectomy or endovascular intervention, with a view to employing these

techniques in routine clinical practice.(Kwee *et al*, 2008) To our knowledge, prior to the commencement of this thesis, there were no data comparing platelet activation in asymptomatic versus symptomatic patients with concurrent reference to carotid plaque morphology and microembolic status.

Aims

The aims of this component of the PACS study were to determine whether:

(a) There were differences in atherosclerotic carotid plaque morphology on colour Doppler ultrasound between patients with asymptomatic and early symptomatic moderate or severe carotid stenosis;

(b) MES on transcranial Doppler ultrasound examination were more likely to be seen in association with echolucent than echodense plaques.

Hypotheses

We hypothesised that:

(a) Early symptomatic atherosclerotic plaques were likely to be more echolucent on colour Doppler ultrasound than asymptomatic plaques

(b) MES would be more common in symptomatic patients with echolucent than those with echodense plaques.

10.2. Methods

Consecutive eligible patients > 18 years old with asymptomatic or symptomatic moderate or severe carotid artery stenosis or carotid occlusion, identified on colour Doppler ultrasound using standardised velocity criteria (Sidhu & Allan, 1997;Grant *et al*, 2003) were recruited. Information on patient inclusion and exclusion criteria, ethical approval, clinical assessment and follow-up have been described in Chapter 3 and the patient population was the same as that in chapter 4.

10.2.1 Colour Doppler Ultrasound examination and grading of carotid artery stenosis and evaluation of plaque morphology on B-mode ultrasound

Equipment & Settings:

High-resolution colour Doppler and B-mode ultrasonography was performed by highly-experienced vascular technologists in our lab. The technologists followed a standardised protocol on a Siemens Acuson Antares Premium Edition device, and used a 7.5-MHz linear-array transducer with a maximum dynamic range of 60 dB. Images were digitally stored on the device hard drive, and transferred to digital compact discs before being saved onto a centralised research personal computer for later analysis. Gain settings during B-mode imaging were adjusted to allow for adequate visualisation of the carotid plaque and to minimise noise within the vessel lumen, so that the structural details of the far wall media-adventitia interface was clear. This ensured the availability of 2 reference structures for image normalization, i.e. the 'noiseless' blood in the vessel lumen, and the echo-dense area of adventitia in the vicinity of the plaque, as outlined above. Colour Doppler ultrasound assessment of the cervical arteries was performed within one week of

recruitment to the study in most early symptomatic patients recruited, with the remainder assessed up to one month from study entry. The asymptomatic cohort was assessed with CDUS of the cervical vessels within 3 months of recruitment in 91% of cases, but assessments occurred up to 6 months from recruitment in 2/23 (9%) patients.

10.2.2 Colour Doppler Ultrasound Imaging Protocol

Both common and internal carotid arteries were imaged with patients lying supine and comfortable. The carotid bifurcation was imaged in longitudinal (anterolateral, lateral, and posterolateral) and transverse projections. The transducer was kept parallel to the vessel, so that the near and far wall adventitia were imaged at right angles. The aim was to obtain a carotid plaque B-mode image with a relatively noiseless lumen, with an echo-dense area of adventitia as near to the most stenosing aspect of the plaque as possible, and with a clear image of the plaque that one could outline relatively easily. For this study, we defined a plaque as a localized protrusion of the vessel wall into the lumen that narrowed the lumen by $\geq$ 50% based on the velocity criteria outlined below.

The velocity criteria used in the grading of carotid stenosis were as follows: patients were classified as having 50-69% internal carotid artery stenosis on colour Doppler ultrasound if the peak systolic velocity in the internal carotid artery (PSV_{ICA}) was $\geq$ 130 cm/s but < 230 cm/s, the end diastolic velocity in the internal carotid artery (EDV_{ICA}) was < 110 cm/s, and the ratio of the PSV_{ICA} to the PSV in the common carotid artery (PSV_{CCA}) was < 4.0. The carotid stenosis severity was considered $\geq$ 70% if the PSV_{ICA} was $\geq$ 230 cm/s, the EDV_{ICA} was $\geq$ 110 cm/s, or the PSV_{ICA}: PSV_{CCA} ratio was > 4.0.(Sidhu & Allan, 1997) 37 out of 51 (73%) of the early symptomatic patient cohort had another concurrent, non-invasive angiographic imaging modality performed in addition to colour Doppler ultrasound (either extracranial MR or CT angiography) to assess for concordance with the

CDUS assessment of the degree of carotid stenosis. MRA or CTA was also performed in 9 of 31 (29%) asymptomatic stenosis patients. Plaque GSM analysis was performed in our lab using Adobe Photoshop CS2 software, as outlined above, where > 50% of the plaque area had analysable imaging information that was not obscured by acoustic shadowing due to calcification. The number of pixels in the area of interest had to constitute over half the total number of pixels in the area of the plaque, but only the clearly visualised area was subjected to analysis. Plaques for which we did not have adequate or any B-mode imaging information because of > 50% acoustic shadowing were excluded from this part of our study. The standardised images were analysed off-line by 2 experienced trained observers (JAK, CD) who calculated the GSM by consensus agreement when all data had been collected. Analysis was performed blinded to the symptomatic status of the patient, as well as the MES and platelet activation status of the patient. The two same trained observers classified the plaques according to the Gray-Weale score (Types 1-5).(Beletsky *et al*, 1996;Gray-Weale *et al*, 1988) Results were simultaneously recorded and entered onto a database at the time each plaque was scored, and formal statistical analysis was performed at a later date.

10.2.3 TCD recording for detection of microembolic signals

Bilateral simultaneous transcranial Doppler ultrasound monitoring of the middle cerebral arteries was performed for 1 hour to classify patients as MES positive or MES negative, as described in detail in Chapter 9.

10.2.4 Statistical Methods

The distribution of all variables was tested for normality with the Shapiro-Wilk test of normality. Normally distributed variables were expressed as mean ± 2 standard

deviations (SD). The median and interquartile range (IQR) were calculated for non-normally distributed variables. Paired or unpaired t-tests were used for comparison of paired and unpaired parametric variables, respectively. The Wilcoxon signed rank test and the Wilcoxon rank sum test were used for comparison of paired and unpaired non-parametric variables, respectively. Chi-squared or Fisher exact tests were used to compare proportions between groups. $P < 0.05$ was considered to be statistically significant. All calculations were performed with the online statistical package R version 2.9.1. (R Development Core Team, 2010)

10.3. Results

Twenty-three asymptomatic carotid stenosis patients had complete data available for analysis. Of the 31 asymptomatic patients originally included, 4 had heavily calcified and therefore 'unanalysable' plaques, and 4 had suboptimal imaging. Of the 46 symptomatic patients originally recruited in the early symptomatic phase, 6 had carotid occlusion and the plaque was not suitable for accurate analysis, 3 had heavy calcification which made interpretation of the carotid plaque suboptimal, and 9 had either suboptimal images or the plaque images were not available. Therefore, data were available in 28 patients with early symptomatic moderate or severe carotid stenosis. Seven symptomatic patients who were followed up to the late phase had ipsilateral >50% stenosis – 3 had developed carotid restenosis following carotid endarterectomy, and the remainder did not undergo carotid intervention (Table 10.1). There was a higher proportion of ex smokers in the asymptomatic group than in the early symptomatic group, but otherwise, there were no differences in demographic or vascular risk factors between groups (Table 10.1). In the asymptomatic carotid stenosis group, the median Gray-Weale score was 3, the median GSM score was 27. In the early symptomatic carotid stenosis group, the median Gray-Weale score was 2 and the median GSM score was 18.5. In the late symptomatic carotid stenosis group, the median Gray-Weale score was 3 and the median GSM was 23.

There was no significant differences between the asymptomatic and early symptomatic carotid stenosis groups in terms of plaque echogenicity measured by the Gray-Weale Score (median Gray-Weale score 3 vs. 2; $p = 0.16$) or by the GSM Score (median GSM 27.0 vs. 18.5; $p = 0.13$). Similarly, there was no difference between the asymptomatic and late symptomatic carotid stenosis groups in terms of plaque echogenicity measured by the Gray-Weale Score (median Gray-Weale score 3 vs. 3; $p = 0.5$) or by the GSM Score (median GSM 27.0 vs. 23.0; $p = 0.66$).

Plaque morphology and presence of MES:

In this substudy, 5 early symptomatic carotid stenosis patients (18%), and none of the asymptomatic or late symptomatic patients had MES detected on TCD. The presence of MES was non-significantly higher in early symptomatic versus asymptomatic patients (18 vs. 0%, $p = 0.056$). 2 of the 5 patients with MES had a Gray-Weale score <3, indicating predominantly echolucent plaques. However, one must emphasise that the number of subjects included in this analysis was very small.

10.4. Discussion

This pilot study simultaneously compared plaque morphology in asymptomatic versus symptomatic carotid stenosis patients, and examined the relationship between plaque morphology on colour Doppler ultrasound and MES on TCD in asymptomatic and early and late symptomatic carotid stenosis patients. We found a trend towards a lower median GSM score, thus indicating more echolucent plaques in early symptomatic versus asymptomatic cohorts, but the differences between the groups were **not** statistically significant. The lack of differences between the groups may represent a type II error, but these pilot data do not allow one to conclude that recently symptomatic patients have more echolucent and unstable carotid plaques than their asymptomatic counterparts. Larger studies are required to readdress this issue, especially in light of the findings outlined in the literature review at the beginning of this chapter which provided some evidence that symptomatic plaques are more echolucent.

Interestingly, we found that the presence of MES on TCD was nearly significantly higher in early symptomatic versus asymptomatic subjects, in keeping with our results in the larger patient population (Chapter 9), but again the number of subjects included in this pilot study was too small to comment on whether our results are subject to a type II error or not. Because only 5 patients in this component of the PACS study had MES, one cannot reliably comment on any potential association between MES status and plaque echogenicity on ultrasound.

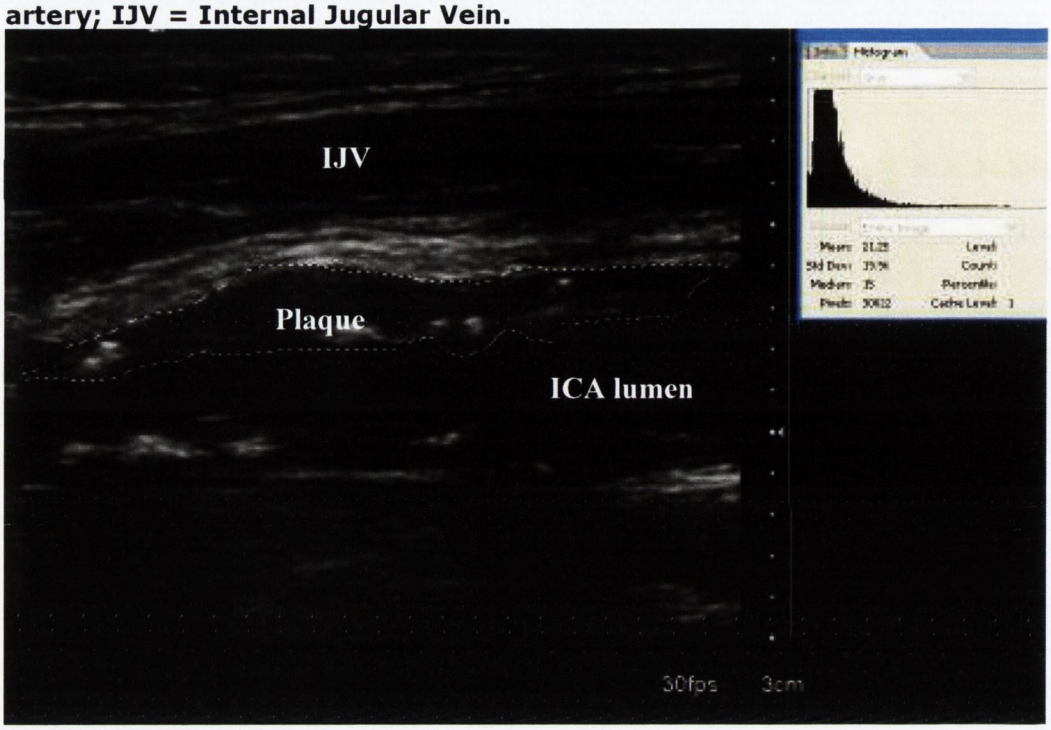
Based on these pilot data, we have designed and commenced a larger clinical follow-up study simultaneously assessing carotid plaque morphology, TCD assessment of cerebral MES, and biomarkers of platelet activation/hyper-reactivity to assess the potential predictive value of these measurements, alone and in combination, for risk-stratification of patients with asymptomatic and symptomatic carotid stenosis.

Table 10.1: Demographic data and risk factor profiles of patients assessing the relationship between Gray Scale Median (GSM) and Gray-Weale Score and platelet activation status in carotid stenosis. P values relate to chi-squared and Fisher exact testing between asymptomatic and other carotid stenosis patient groups. Values are Means (±SD), Medians (Range) or absolute counts. Significant P values are in bold.

Parameter	Asymptomatic Carotid Stenosis (n = 23)	Early Symptomatic Carotid Stenosis (n=28)	p	Late Symptomatic Carotid Stenosis (n=7)	p
Mean age (years)	68.3 (±8.4)	64.2 (±9.0)	**0.02**	63.9 (±11.7)	0.38
Gender (M; %)	11 (48%)	13 (46%)	0.92	3 (43%)	1.0
Median interval from symptom onset (days; range)	N/A	7 (0 - 27)		160 (97 – 250)	
Degree of Stenosis:					
Moderate:≥50 – 69%	8 (35%)	4 (14%)	0.1	5 (71%)	0.2
Severe: ≥ 70 – 99%	15 (65%)	24 (86%)	0.1	2 (29%)	0.2
Occlusion	0 (0%)	0 (0%)	1	0 (0%)	1
Plaque GSM entire cohort:					
Median GSM (range)	27 (6 – 81)	19 (2 – 84)	0.13	23 (8 – 64)	0.66
Median Gray-Weale Score (range)	3 (1 – 4)	2 (1 – 4)	0.16	3 (2 – 4)	0.50
Moderate stenosis group:					
Median GSM (range)	40 (14 – 81)	19 (2 – 84)	0.55	25 (8 – 64)	0.27
Severe stenosis group:					
Median GSM (range)	20 (6 – 64)	19 (2 – 49)	0.58	17 (15 – 19)	0.77
MES present	0	5 (18%)	0.056	0	1
Median GSM (severe stenosis)	N/A	19 (14 – 36)		N/A	
Antiplatelet therapy:					
Aspirin monotherapy	15 (65%)	19 (68%)	0.84	3 (43%)	0.4
Aspirin/Dipyridamole combination	2 (9%)	3 (11%)	1	1 (14%)	0.58
Clopidogrel monotherapy	5 (22%)	1 (4%)	0.08	2 (29%)	1
Aspirin/Clopidogrel combination	1 (4%)	2 (7%)	1	1 (14%)	0.4
Ischaemic heart disease	6 (26%)	6 (21%)	0.7	3 (43%)	0.64
Hypertension	20 (87%)	19 (68%)	0.2	4 (57%)	0.12
Diabetes Mellitus	3 (13%)	6 (21%)	0.49	2 (29%)	0.57
Prior TIA/Stroke before index event	6 (26%)	3 (11%)	0.27	1 (14%)	1
Prior venous thromboembolism	1 (4%)	0 (0%)	0.45	0 (0%)	1

Peripheral Vascular Disease	3 (13%)	5 (18%)	0.72	3 (43%)	0.12
Migraine (with or without aura)	6 (26%)	4 (14%)	0.32	2 (29%)	1
Family History Stroke	8 (35%)	10 (36%)	0.94	5 (71%)	0.19
Current smoker	5 (22%)	13 (46%)	0.08	4 (57%)	0.15
Ex-smoker	16 (70%)	10 (36%)	**0.02**	2 (29%)	0.08
Never smoker	2 (9%)	5 (18%)	0.44	1 (14%)	1
Statin therapy	21 (91%)	21 (75%)	0.16	7 (100%)	1

Figure 10.2 Example of Gray-Scale Median (GSM) analysis of a stenosed carotid artery plaque using Adobe CS2 software. ICA = Internal Carotid artery; IJV = Internal Jugular Vein.



11. Prevalence of *ex vivo* 'high on-treatment platelet reactivity' to commonly prescribed antiplatelet regimens on the PFA-100® and VerifyNow® following TIA or ischaemic stroke

11.1. Introduction

Antiplatelet agents play a key role in the secondary prevention of vascular events in patients with ischaemic heart disease (Antithrombotic Trialists' Collaboration., 2002;Mehta & Yusuf, 2003) or non-cardioembolic ischaemic stroke.(McCabe & Brown, 2000) Several groups have investigated the controversial topic of *ex vivo* 'non-responsiveness to antiplatelet therapy' in patients with ischaemic heart disease, including those undergoing percutaneous coronary intervention (PCI),(Dorsch *et al*, 2007;van Werkum *et al*, 2006;Krasopoulos *et al*, 2008;Lordkipanidze *et al*, 2007) in whom symptoms are usually believed to be caused by thrombotic subtotal or total occlusion of a coronary artery.(Gurbel *et al*, 2007) More recent studies have assessed the newly-termed concept of 'high on-treatment platelet reactivity' (HTPR) in patients with ischaemic heart disease.(Breet *et al*, 2010b;Bonello *et al*, 2010;Price *et al*, 2011) This term accounts for the fact that patients might have some degree of inhibition of platelet function with a particular antiplatelet regimen, but are still considered to have 'hyper-reactive platelets' compared with an established normal range; this information may still be clinically informative in ischaemic cerebrovascular disease (CVD) patients in whom 'longitudinal data' are not available from the same patients before and after starting a particular antiplatelet regimen.(Tobin *et al*, 2011) However, because of the heterogenous aetiology

of ischaemic CVD),(Adams, Jr. *et al*, 1993) one cannot assume that one may extrapolate data on *ex vivo* HTPR from ischaemic heart disease patients to those with TIA or stroke.

Aspirin is the most commonly prescribed antiplatelet drug for secondary prevention following TIA or ischaemic stroke, but the majority (82-87%) of patients are not protected from further vascular events with aspirin alone.(CAST (Chinese Acute Stroke Trial) Collaborative Group., 1997;The International Stroke Trial, 1997) This has led to clinical trials of aspirin and dipyridamole combination therapy versus aspirin monotherapy,(Diener *et al*, 1996;Halkes *et al*, 2006) aspirin versus clopidogrel monotherapy,(CAPRIE Steering Committee, 1996) and more recently aspirin and dipyridamole combination therapy versus clopidogrel monotherapy (Sacco *et al*, 2008) in ischaemic CVD. None of these landmark clinical trials routinely incorporated platelet function testing into the study paradigm.

The limited, available literature indicates that the prevalence of *ex vivo* antiplatelet 'non-responsiveness' in ischaemic CVD varies between 5-66% with aspirin monotherapy,(McCabe *et al*, 2005a;von Lewinski *et al*, 2009;Alberts *et al*, 2004;Gengo *et al*, 2008;Harrison *et al*, 2005;McCabe *et al*, 2005b) 5-44% with clopidogrel monotherapy,(von Lewinski *et al*, 2009;Serebruany *et al*, 2005) 0-73% on aspirin and clopidogrel combination therapy(von Lewinski *et al*, 2009), and 56-59% when dipyridamole is added to aspirin (Tobin *et al*, 2011) in the early, subacute (McCabe *et al*, 2005a;Harrison *et al*, 2005;Tobin *et al*, 2011) or late phases (McCabe *et al*, 2005a;Serebruany *et al*, 2005;Tobin *et al*, 2011) after symptom onset. Studies in ischaemic CVD patients have assessed inhibition of platelet function with platelet aggregometry in either platelet rich plasma (PRP) (Harrison *et al*, 2008) or whole blood,(Serebruany *et al*, 2007) with the whole blood Ultegra-Rapid Platelet Function Analyser (RPFA®) (Serebruany *et al*, 2007;Harrison *et al*, 2005;Serebruany *et al*, 2006)

or VerifyNow,(Harrison *et al*, 2008;Lee *et al*, 2009a;Lee *et al*, 2008) or the 'moderately high shear stress' whole blood platelet function analyser called the PFA-100®.(McCabe *et al*, 2005a;Harrison *et al*, 2007;Grau *et al*, 2003;Harrison *et al*, 2005;Harrison *et al*, 2008;Tobin *et al*, 2011) The reported prevalence of 'non-responsiveness' varied according to the definition employed.

Because aspirin and clopidogrel combination therapy is not routinely recommended for long-term secondary prevention in ischaemic CVD,(Bhatt *et al*, 2006;Diener *et al*, 2004) it needs to be established whether one can reliably detect inhibition of platelet function with commonly prescribed antiplatelet regimens (aspirin and dipyridamole combination therapy or clopidogrel monotherapy) in individuals following TIA or ischaemic stroke using established,(Grau *et al*, 2003;Serebruany *et al*, 2008) and relatively novel laboratory techniques.(Harrison *et al*, 2008) Furthermore, the controversy over whether one can reliably detect inhibition of platelet function on *long-term* clopidogrel with the PFA-100, and whether the collagen-ADP cartridge could serve to monitor platelet reactivity in these patients needs to be resolved.(Raman & Jilma, 2004;Grau *et al*, 2003)

We therefore assessed the ability of established and relatively novel point-of-care laboratory tests to simultaneously detect *ex vivo* inhibition of platelet function in whole blood in patients on aspirin and dipyridamole combination therapy or clopidogrel monotherapy in the late phase after TIA or ischaemic stroke. We hypothesised that there would be a substantial proportion of patients with *ex vivo* HTPR to their prescribed antiplatelet regimen, and that the prevalence of HTPR would be higher with the PFA-100 than VerifyNow.

11.2. Methods

This pilot cross-sectional, observational, translational platelet science study was performed at our secondary and tertiary referral university teaching hospital.

11.2.1. Clinical assessment

Eligible patients > 18 years of age, in the *late stable phase* (≥ 3 months) after TIA or ischaemic stroke, who had been prescribed aspirin and dipyridamole combination therapy or clopidogrel monotherapy by their treating physician were identified from our Vascular Neurology Research database. All patients had undergone thorough clinical and neurovascular workup by either an experienced consultant vascular neurologist or consultant stroke physician, as per European Stroke Organisation guidelines at the time of symptom onset, and were fully reassessed by a vascular neurology registrar (Dr Kinsella or Dr Tobin) at a special outpatient study visit at study entry.(Ringleb PA *et al*, 2008) Local Research Ethics Committee approval was secured, and all participants gave written informed consent. The treatment regimen was left to the discretion of the attending consultant vascular neurologist or stroke physician, and was not altered as part of this study. TIA or stroke subtyping was performed according to TOAST criteria.(Adams, Jr. *et al*, 1993) Exclusion criteria for patients included: active infection, inflammation or neoplasia; platelet count < 120 or > 450 x 10^9/L; recurrent TIA or stroke within the preceding 3 months; myocardial infarction, pulmonary embolism, deep vein thrombosis or major surgery within the preceding 3 months; ongoing unstable coronary or peripheral arterial disease; renal impairment (urea > 10 mmol/l); or other nonsteroidal anti-inflammatory drug (NSAID) intake (apart from aspirin in the patients on aspirin and dipyridamole) within the preceding 2 weeks.

The importance of antiplatelet compliance was re-enforced by phoning patients the day prior to their study visit to ensure they had remembered to take their medication over the preceding 10 days. Assessment was deferred for 10 days in patients in whom there was any initial concern about full adherence to their antiplatelet regimen at the time of recruitment.

We explored potential demographic and vascular risk factors which may have influenced non-responsiveness to either antiplatelet therapy regimen.

11.2.2. Laboratory Methods

As outlined in the methods in Chapter 3.10, blood was collected from a free-flowing vein via a 21G Butterfly® needle and a Vacutainer® system with a luer adaptor after resting for at least 20 minutes, as described previously.(McCabe *et al*, 2004a)

The VerifyNow is a cartridge-based platelet function analyser employing a modified light transmission aggregometry paradigm to assess inhibition of platelet function in response to stimulation with different platelet agonists, depending on the cartridge employed: arachidonic acid in the 'Aspirin cartridge' [Accumetrics Inc. VerifyNow-ASA (aspirin assay) package insert. San Diego, CA. 2004 (Pamphlet)]; adenosine diphosphate (ADP), iso-thrombin receptor activating peptide (iso-TRAP), and PAR-4 activating peptide in the '$P2Y_{12}$ cartridge' [Accumetrics Inc. VerifyNow-P2Y12 (P2Y12 assay) package insert. San Diego, CA. 2006, (Pamphlet)]; and iso-TRAP alone which activates the platelet PAR–1 receptor in the 'GpIIb/IIIa assay' [Accumetrics Inc. VerifyNow-GP IIb/IIIa (GP IIb/IIIa assay) package insert. San Diego, CA. 2006, (Pamphlet)]. We also analysed Platelet Aggregation Units (PAUs) obtained from the VerifyNow GpIIb/IIIa cartridge to assess whether the antiplatelet effects of aspirin and dipyridamole or clopidogrel could

influence the results obtained from this cartridge, and thus, to indirectly assess its specificity for detecting platelet inhibition with a GpIIb/IIIa inhibitor.

The PFA-100 activates platelets by exposure to moderately high shear stress (5000-6000 s^{-1}) and biochemical stimulation with collagen and epinephrine (C-EPI cartridge) or ADP (C-ADP cartridge).(Michelson, 2009;Kundu *et al*, 1995) The time taken for activated platelets to occlude an aperture in the cartridge is called the closure time (maximum closure time 300s), and we arbitrarily defined closure times above 300s as 301s.(McCabe *et al*, 2005a) Aspirin prolongs C-EPI closure times in 83-100%,(Harrison *et al*, 1999;Kundu *et al*, 1994;Mammen *et al*, 1995;Mammen *et al*, 1998) with prolongation of C-ADP closure times in 0-24% of healthy controls.(Harrison *et al*, 1999;Mammen *et al*, 1995;Mammen *et al*, 1998)

Blood samples were analysed on the VerifyNow GpIIb/IIIa cartridge within 15 minutes of venepuncture. PFA-100 and VerifyNow Aspirin and P2Y12 assays were performed simultaneously between 2–3 hours after venepuncture. Intra-assay coefficients of variation (CV) on the VerifyNow and PFA-100 were established in our own lab based on control data: [VerifyNow Aspirin (0.1%), $P2Y_{12}$ (5.5%), and GpIIb/IIIa (4.0%); PFA-100 C-ADP (7.0%), C-EPI (7.5%)], indicating a high level of reproducibility for all tests. Therefore, we did not perform analyses in duplicate. FBCs were performed between 2–4 hours after venepuncture on a Sysmex XE-2100 haematology analyser (Sysmex UK Ltd, Milton Keynes, UK).

Because all clopidogrel-treated patients reported being on treatment for at least 3 months, this study had the potential to resolve the uncertainty regarding the ability of the PFA-100 to reliably detect inhibition of platelet function with long-term clopidogrel in ischaemic CVD.(Raman & Jilma, 2004;Kotzailias *et al*, 2007)

11.2.3. Statistical methods

All statistical analyses were performed with R, version 2.9.1.(R Development Core Team, 2010) On the *VerifyNow*, aspirin HTPR was defined by Aspirin Reaction Units (ARU) ≥ 550 on the Aspirin cartridge, based on the manufacturer's definition of aspirin HTPR [Accumetrics Inc. VerifyNow-ASA (aspirin assay) package insert. San Diego, CA. 2004, (Pamphlet)]. Clopidogrel HTPR on the VerifyNow was defined as P2Y12 reaction units (PRU) ≥ 194, based on the manufacturer's definition of clopidogrel HTPR [Accumetrics Inc. VerifyNow-P2Y12 (P2Y12 assay) package insert. San Diego, CA. 2006, (Pamphlet)]. Antiplatelet HTPR on the GpIIb/IIIa cartridge was defined as PAU < 125 based on the manufacturer's definition of GpIIb/IIIa HTPR [Accumetrics Inc. VerifyNow-GP IIb/IIIa (GP IIb/IIIa assay) package insert. San Diego, CA. 2006 (Pamphlet)].

For the purpose of this study, we considered patients to have *ex vivo* antiplatelet HTPR on the PFA-100, if they had evidence of platelet 'hyper-reactivity' on the relevant cartridge despite stable antiplatelet therapy. Therefore, antiplatelet HTPR was defined as failure to prolong the closure time beyond the mean + 2 standard deviations of our control range for the CEPI and CADP cartridges i.e. failure to prolong (a) C-EPI closure times with aspirin and dipyridamole beyond 176s, and (b) C-ADP closure times with clopidogrel beyond 160s, as per usual 'cross sectional, case-control' definitions in the literature.(McCabe *et al*, 2005a;Grau *et al*, 2003)

Descriptive statistical calculations assessed the percentage of patients on aspirin and dipyridamole combination therapy or clopidogrel monotherapy who had HTPR on each device. Unweighted Cohen's kappa statistics (κ) were calculated to assess inter-device agreement in the definition of antiplatelet HTPR. A kappa value of 0–0.20 indicates poor agreement; 0.21–0.40 fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 good

agreement; and 0.81–1.00 very good/excellent agreement. Unpaired student's t-tests compared means, and the Wilcoxon rank sum test compared medians between antiplatelet non-responsive and responsive patients. Chi-squared or Fisher exact tests were used to compare changes in proportions between groups. $P < 0.05$ was considered statistically significant.

11.3. Results

Between August 2009 and February 2010, 76 eligible TIA or ischaemic stroke patients were recruited. Fifty-one patients were on combination therapy with a median dose of 75mg of aspirin daily and 200mg of modified release dipyridamole BD; 25 patients were on 75mg of clopidogrel daily (Table 11.1). None had recurrent vascular events on their prescribed antiplatelet regimen in the preceding 3 months at the time of recruitment. The median interval between index cerebrovascular event and study inclusion was 401 days (95 – 821 days) in patients on aspirin and dipyridamole, and 462 days (93 – 774 days) in patients on clopidogrel.

On the *VerifyNow*, 4/51 patients (8%) on aspirin and dipyridamole combination therapy had HTPR on the Aspirin cartridge as per the manufacturer's definition; two of these patients had experienced a stroke of undetermined aetiology, and two a lacunar stroke (Tables 11.2 and 11.3). Eleven of 25 (44%) patients on clopidogrel monotherapy had HTPR on the $P2Y_{12}$ cartridge (Table 11.3). No patients on aspirin and dipyridamole combination therapy, and only 1/25 (4%) patients on clopidogrel monotherapy had an antiplatelet effect detected on the GpIIb/IIIa cartridge, defined by PAU < 125.

Twenty-one of 51 patients (41%) on aspirin and dipyridamole combination therapy had HTPR on the PFA-100 C-EPI cartridge using our 'cross-sectional' definition (Table 11.3). The median C-ADP closure time was shorter in aspirin/dipyridamole patients with HTPR than in those without HTPR on the C-EPI cartridge (80s versus 106s, $p < 0.001$). Twenty-three of 25 patients (92%) on clopidogrel had HTPR on the PFA-100 C-ADP cartridge (Table 11.3).

The proportion of those with HTPR was lower on VerifyNow than PFA-100 on both aspirin and dipyridamole combination therapy ($p < 0.001$) and clopidogrel monotherapy ($p < 0.001$). Inter-device agreement in the definition of HTPR was fair for aspirin and

dipyridamole (κ = 0.33), and moderate for clopidogrel-treated patients (κ = 0.46). Three of four patients with HTPR on aspirin and dipyridamole combination therapy on the VerifyNow also had HTPR on the PFA-100 C-EPI cartridge. Interestingly, of the 11 patients with HTPR on clopidogrel on VerifyNow, 8 also had HTPR on PFA-100 C-ADP cartridge.

There were no significant differences in demographic or vascular risk factors between patients with and those without HTPR on either device. Of note, *post-hoc* analysis of our VerifyNow data did not reveal any differences in the use of proton pump inhibitors (2/11 [18%] *vs.* 6/14 [43%], p = 0.23) or calcium channel blockers (5/11 [45%] *vs.* 1/14 [7%], p=0.06) between patients with HTPR versus those without HTPR on clopidogrel.

In the aspirin and dipyridamole group, the median platelet count was higher in patients with HTPR than in those without HTPR on the VerifyNow Aspirin cartridge (p = 0.037). The median platelet distribution width [PDW] and mean platelet volume (MPV) was higher in patients with than in those without HTPR on the PFA-100 C-EPI cartridge (p = 0.001). There were no other differences in platelet parameters between patients with and those without HTPR on either antiplatelet regimen on PFA-100 or VerifyNow (Table 11.4).

11.4. Discussion

To our knowledge, this is the first cross-sectional study to employ a parallel testing paradigm to simultaneously assess *ex vivo* HTPR on aspirin and dipyridamole combination therapy or clopidogrel monotherapy in ischaemic CVD patients with the PFA-100 and this version of the VerifyNow.

The finding that 8% of ischaemic CVD patients on aspirin and dipyridamole, and 44% of patients on clopidogrel monotherapy exhibited *ex vivo* HTPR on the VerifyNow is slightly higher than the prevalence range for HTPR reported in chronic, stable ischaemic heart disease patients on aspirin monotherapy (0 - 6.7 %) (Lordkipanidze *et al*, 2007;Nielsen *et al*, 2008;Lordkipanidze *et al*, 2007) or aspirin and clopidogrel combination therapy (2.4 - 40%).(Pinto Slottow *et al*, 2009;Breet *et al*, 2010a;Lordkipanidze *et al*, 2009;Paniccia *et al*, 2007a) However, the prevalence of HTPR on the VerifyNow in patients on aspirin and dipyridamole in our study is lower than that reported by others who studied patients on aspirin monotherapy in either the subacute (Lee *et al*, 2009a) or late phases after TIA or stroke (10.1-12.8%).(Harrison *et al*, 2008;Lee *et al*, 2009a) One cannot conclude that dipyridamole enhances the antiplatelet effects of aspirin on the VerifyNow Aspirin cartridge because data from this and prior studies are not directly comparable: an earlier version of the VerifyNow Aspirin cartridge was utilised in one study;(Harrison *et al*, 2008) 31% of patients were studied within 3 months of symptom onset in another;(Lee *et al*, 2009a) and there are no longitudinal data comparing *ex vivo* ARUs in CVD patients on aspirin and subsequently on aspirin and dipyridamole. Furthermore, the limited available experimental evidence indicates that the addition of dipyridamole to aspirin *in vitro* does not affect the prevalence of HTPR to aspirin in patients in the subacute or late phases after ischaemic

stroke, as measured with the Aspirin cartridge on the Ultegra-RPFA®.(Serebruany *et al*, 2006)

The prevalence of HTPR to aspirin and dipyridamole on the PFA-100 C-EPI cartridge (41%) in this study is slightly higher than in IHD patients on aspirin (19 - 32.4%),(Paniccia *et al*, 2007b;Pamukcu *et al*, 2006) higher than that reported in patients on 75-150 mg of aspirin daily at least 11 months after TIA or ischaemic stroke (25%),(Harrison *et al*, 2008) and slightly lower than reported previously in patients in the late stable phase after TIA or stroke (43%) on aspirin monotherapy.(McCabe *et al*, 2005a) We did not measure simultaneous serum thromboxane B_2, salicylate, dipyridamole or urinary dehydro-thromboxane B_2 levels as additional measures of aspirin and/or dipyridamole adherence, absorption or HTPR, because this was not the purpose of this study.

The 92% prevalence of apparent clopidogrel HTPR on the C-ADP cartridge in our study is higher than reported in ischaemic heart disease (IHD) patients on clopidogrel (24.4%),(Paniccia *et al*, 2007a) but in keeping with reported figures for clopidogrel HTPR in pilot studies in patients with prior ischaemic CVD on 75 mg of clopidogrel monotherapy daily (75%-93.5%) (Kotzailias *et al*, 2007;Grau *et al*, 2003) Our data are in contrast to a small, longitudinal, pilot study in nine acute ischaemic stroke patients which suggested a time lag of at least 12 days before seeing an initial *ex vivo* antiplatelet effect with 75 mg of clopidogrel monotherapy daily, and that clopidogrel prolongs C-ADP closure times in the majority of patients.(Raman & Jilma, 2004) Our data indicate that the C-ADP cartridge is not a sensitive tool for detecting inhibition of collagen and ADP-induced platelet adhesion and aggregation with clopidogrel if one uses a 'cross-sectional definition' of clopidogrel HTPR on the PFA-100; we conclude that the C-ADP cartridge should **not** be used for this purpose. The recently licensed INNOVANCE® PFA P2Y

cartridge has been designed to address the limitations of the C-ADP cartridge in detecting the antiplatelet effects of clopidogrel (Breet *et al*, 2010a;Elsenberg *et al*, 2009;Linnemann *et al*, 2010). This cartridge was not available at the time of this study, but does deserve future study.

The prevalence of *ex vivo* aspirin HTPR in patients on aspirin and dipyridamole, and clopidogrel HTPR in patients on clopidogrel monotherapy, was significantly lower on the VerifyNow than on the moderately high-shear stress PFA-100 ($p < 0.001$). The discordance between the devices is in keeping with that reported previously by Harrison *et al*. in chronic stable ischaemic CVD patients, predominantly on aspirin monotherapy, who were tested with an earlier version of the VerifyNow Aspirin cartridge and the PFA-100 C-EPI cartridge.(Harrison *et al*, 2008) The lower prevalence of aspirin HTPR on the VerifyNow than PFA-100 is likely multifactorial. For example, platelets are exposed to biochemical stimulation with arachidonic acid on the VerifyNow, compared with moderately high shear stress and biochemical stimulation with both collagen and epinephrine on the PFA-100 that may potentially overcome inhibition of platelet cyclo-oxygenase 1 by aspirin.(McCabe *et al*, 2005a) In addition, the VerifyNow only assesses platelet aggregation compared with assessment of both adhesion and aggregation on the PFA-100. Because von Willebrand factor (VWF) plays an important role in high-shear stress-induced platelet adhesion and aggregation, the results of the PFA-100 in ischaemic CVD are influenced by VWF antigen levels.(McCabe *et al*, 2005a)

The finding of an elevated platelet count in the VerifyNow Aspirin group among the patients with versus those without HTPR indicates that those patients had elevated platelet production, potentially contributing to elevated platelet reactivity. Patients who had HTPR detected on the PFA-100 C-EPI cartridge had larger platelets as measured by the PDW and MPV versus those without HTPR. This may indicate elevated platelet

reactivity associated with larger, maybe younger platelets, which are potentially more reactive to stimuli and hence less responsive to the effects of antiplatelet agents, but again this is speculative.

We postulated that the VerifyNow $P2Y_{12}$ cartridge would be more reliable at detecting inhibition of ADP-induced platelet reactivity with clopidogrel than the PFA-100 C-ADP cartridge, and this was the case. Although platelets are exposed to ADP in both cartridges, the $P2Y_1$ ADP platelet receptor is blocked by prostaglandin E1 in the VerifyNow cartridge to allow one to specifically calculate the degree of $P2Y_{12}$ receptor inhibition with clopidogrel, [Accumetrics Inc. VerifyNow-P2Y12 (P2Y12 assay) package insert. San Diego, CA. 2006. (Pamphlet)] (Michelson *et al*, 2006b;2010) whereas ADP in the PFA-100 C-ADP cartridge may activate both $P2Y_1$ and $P2Y_{12}$ ADP platelet receptors.(Gachet, 2006) The lower prevalence of clopidogrel HTPR on the VerifyNow may also relate to other factors outlined above, including the influence of VWF antigen levels on C-ADP closure times, but systematic analysis of the effects of VWF antigen levels was not performed in this pilot, cross-sectional, observational study.

At present, the main indicator of 'presumed HTPR' to an antiplatelet regimen in patients with ischaemic CVD, in the absence of platelet function monitoring, is the occurrence of a recurrent vascular event, such as a further TIA, stroke or myocardial infarction. These patients have sometimes been referred to as "antiplatelet failures" despite the fact that apparent HTPR to the antiplatelet regimen in question may be related to several important factors. Although compliance with antiplatelet therapy was verbally confirmed in all our patients, if one uses VerifyNow data to assess whether patients had ingested the antiplatelet agents in question, we know that the vast majority of our patients were definitely taking their prescribed antiplatelet medication. In addition to the factors outlined above, medication dosage, body weight, the extent of intestinal drug absorption,

differences in platelet surface receptor expression, and genetic polymorphisms (e.g. mutations in polymorphisms involving the hepatic cytochrome P450 enzymatic system that convert clopidogrel to its active metabolite) may all play a role.(Lee *et al*, 2009b;Coma-Canella & Velasc, 2007)

11.5. Conclusions

These findings emphasise that clinicians treating patients with ischaemic CVD cannot compare data on *ex vivo* HTPR in one study with that in another without paying close attention to platelet function test employed and the shear stress that platelets are exposed to during the laboratory test in question. The availability of laboratory tests of platelet function that correlate with clinical effectiveness of antiplatelet agents, would enable physicians to tailor antiplatelet therapy and optimise secondary prevention regimens.

Table 11.1: Patient demographic and vascular risk factor profiles. *ASA/DP = Aspirin & Dipyridamole. CVE = Cerebrovascular Event; DVT – Deep Venous Thrombosis; PE = Pulmonary Embolus

	ASA/DP* (n=51)	Clopidogrel (n=25)
Mean age (years)	60	71
Gender (Male)	40 (78%)	15 (60%)
Mean days since CVE	458	461
Stroke as Qualifying Event	22 (43%)	14 (56%)
Prior history of TIA/Stroke before Qualifying Event	10 (20%)	9 (36%)
Diabetes Mellitus	4 (8%)	7 (28%)
Atrial Fibrillation	1 (0.5%)	2 (8%)
Hyperlipidaemia	29 (57%)	24 (96%)
Hypertension	23 (45%)	22 (88%)
Ischaemic Heart Disease	6 (12%)	9 (36%)
Previous DVT / PE	2 (4%)	2 (8%)
Migraine	13 (25%)	7 (28%)
Peripheral Arterial Disease	5 (10%)	3 (12%)
Family History Stroke / IHD	23 (45%)	16 (64%)
Smoker (Current or previous)	30 (59%)	13 (52%)
TOAST Subtypes:		
Large Artery Atherosclerotic	9	1
Small Vessel Occlusion (Lacunar)	13	4
Cardioembolic	8	5
Undetermined Aetiology	20	14
Other Determined Aetiology	1	1

Table 11.2: Comparison of demographic and vascular risk factors between patients with (HTPR) and without HTPR (no HTPR) on aspirin and dipyridamole combination therapy, and clopidogrel monotherapy; none of the differences between subgroups were statistically significant

	Aspirin / Dipyridamole				Clopidogrel			
	VerifyNow Aspirin		PFA-100 C-EPI		VerifyNow $P2Y_{12}$		PFA-100 C-ADP	
	No HTPR (n=47)	HTPR (n=4)	No HTPR (n=30)	HTPR (n=21)	No HTPR (n=14)	HTPR (n=11)	No HTPR (n=2)	HTPR (n=23)
Vascular Risk Factors:								
Previous Stroke/ TIA	10 (21%)	1(25%)	6 (20%)	5 (24%)	5 (36%)	4 (36%)	2 (100%)	7 (30%)
Diabetes Mellitus	4 (9%)	0	1 (3%)	3 (14%)	2 (14%)	5 (45%)	1 (50%)	6 (26%)
Atrial Fibrillation	1 (2%)	0	1 (3%)	0	2 (14%)	0	0	2 (9%)
Hyperlipidaemia	27 (57%)	2(50%)	14 (47%)	15 (71%)	13 (93%)	11 (100%	1 (50%)	23(100%)
Hypertension	21 (45%)	2(50%)	12 (40%)	11 (52%)	12 (86%)	10 (91%)	1 (50%)	21(91%)
Ischaemic Heart Disease	5 (11%)	1(25%)	4 (13%)	2 (10%)	6 (43%)	3 (27%)	0	9 (45%)
Current/ Ex Smoker	27 (57%)	3(75%)	18 (60%)	12 (57%)	10 (71%)	3 (27%)	1 (50%)	12 (52%)
Stroke as Qualifying Event	19 (40%)	3(75%)	13 (43%)	9 (43%)	6 (43%)	8 (73%)	0	14(61%)
TOAST Subtypes								
Large Artery Atherosclerotic	9 (19%)	0	7 (23%)	2 (10%)	1 (7%)	0	0	1 (4%)
Small Vessel Occlusion (Lacunar)	11(23%)	2(50%)	7 (23%)	6 (29%)	2 (14%)	2 (18%)	1 (50%)	3 (13%)
Cardioembolic	8 (17%)	0	4 (13%)	4 (19%)	3 (21%)	2 (18%)	0	5 (22%)
Undetermined Aetiology	18 (38%)	2(50%)	12 (40%)	9 (43%)	7 (50%)	7 (64%)	0	14(61%)
Other Determined Aetiology	1 (2%)	0	1 (3%)	0	1 (7%)	0	1 (50%)	0

Table 11.3: VerifyNow and PFA-100 data from study patients. Values are medians or means (range).

	ASA/DP* (n=51)	Clopidogrel (n=25)
VerifyNow cartridge		
Aspirin		
Mean ARU (range)	476 (350 – 660)	648 (546 – 674)
$P2Y_{12}$		
Mean PRU (range)	325 (233 – 428)	204 (35 – 372)
GpIIb/IIIa		
Mean PAU (range)	210 (126 – 288)	198 (113 – 318)
PFA-100 cartridge		
Median C-EPI CT (range in secs)	202 (81 - > 300)	120 (72 – 243)
Median C-ADP CT (range in secs)	95 (54 - >300)	102 (62 – 236)
*ASA/DP = Aspirin & Dipyridamole. CT = closure time. ARU = Aspirin Reaction Units. PRU = $P2Y_{12}$ Reaction Units. PAU = Platelet Aggregation Units		

Table 11.4: Comparison of platelet parameters on the FBC in aspirin and dipyridamole patients without HTPR (no HTPR) versus those with HTPR on the VerifyNow Aspirin and PFA-100 C-EPI cartridges, and in clopidogrel patients without HTPR versus those with HTPR on the VerifyNow $P2Y_{12}$ and PFA-100 C-ADP cartridges. Values are means (±SD) or medians (25th - 75th percentile).

Aspirin and Dipyridamole	**VerifyNow Aspirin**		**PFA-100 C-EPI**	
	No HTPR (n=47)	**HTPR** (n=4)	**No HTPR** (n=30)	**HTPR** (n=21)
Median Platelet Count (x 10^9/l)	220(197 – 268)	281(274 – 300; p=0.037)	244(191-286)	218(207-258; p=0.54)
Median PDW (fl)	12.3(11.4–13.6)	12.6(11.5-13.6; p=0.9)	11.7(11.1-12.5)	13.4(12-15; p=0.01)
Median MPV (fl)	10.6(10-11.3)	10.6(10.2-11.1; p=0.9)	10.3(9.8-10.8)	11.0(10.4-11.7; p=0.01)
Clopidogrel	**VerifyNow $P2Y_{12}$**		**PFA-100 C-ADP**	
	No HTPR (n=14)	**HTPR** (n=11)	**No HTPR** (n=2)	**HTPR** (n=23)
Mean Platelet Count (x 10^9/l)	207 (±56)	225 (±44; p=0.36)	238 (±81)	213 (±50; p=0.73)
Mean PDW (fl)	13.1 (±1.7)	13.2 (±1.8; p = 0.87)	11.5(±2.3)	13.3(±1.6; p=0.46)
Mean MPV (fl)	10.9 (±0.8)	10.8 (±0.8; p = 0.88)	10.1(±1.1)	10.9 (±0.7; p=0.5)
PDW = Platelet Distribution Width; MPV = Mean Platelet Volume				

12. Summary and Future Work

The studies described in this thesis investigated the levels of platelet activation and reactivity as well as thrombin generation potential and endothelial activation in asymptomatic compared with early and late symptomatic carotid stenosis patients. Our novel findings have raised new clinically important translational research questions. In this chapter, I will discuss proposals to investigate these outstanding research issues.

12.1. Platelet Activation in Carotid Artery Stenosis

In Chapter 4, I have described that the percentage of lymphocyte-platelet complexes on unstimulated whole blood flow cytometry was elevated in early symptomatic versus asymptomatic carotid stenosis subjects. Levels of the surface platelet activation markers and of the other leucocyte-platelet complexes were not different between asymptomatic and symptomatic patients. These data are consistent with enhanced platelet +/- lymphocyte activation early after TIA or stroke in association with symptomatic carotid stenosis, and improve our understanding of the potential mechanisms responsible for the high risk of recurrent cerebrovascular events in patients with recently symptomatic *vs.* asymptomatic carotid stenosis. Our data also suggest that measurement of the % circulating leucocyte-platelet complexes is a more sensitive method of assessing platelet activation status than measurement of % CD62P or CD63 expression in recently symptomatic carotid stenosis. Further longitudinal studies in asymptomatic carotid stenosis patients, with serial assessment of platelet activation markers and clinical outcome measures are warranted 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 are at

highest risk of experiencing cerebrovascular symptoms during follow-up, then perhaps one could target these patients with more potent antiplatelet regimens, or perhaps treat these individuals with earlier carotid endarterectomy to optimise TIA and stroke prevention.

12.2. Platelet Function in Carotid Artery Stenosis

The data described in Chapter 5 revealed a higher prevalence of 'high on-treatment platelet reactivity' (HTPR) on aspirin monotherapy in the subgroup of patients with asymptomatic versus symptomatic post-intervention carotid stenosis ($p = 0.03$) measured on the collagen-epinephrine cartridge of the PFA-100® analyser. In the longitudinal component of the study comparing 'matched' data from early symptomatic subjects followed up to the late stage following symptom onset or intervention, In the longitudinal component of our study comparing early symptomatic patient data with data from the same patients followed up to the late stage after symptoms or intervention, HTPR on the C-EPI cartridge was more prevalent in early symptomatic than late symptomatic carotid stenosis patients on aspirin monotherapy ($p = 0.016$), or on aspirin alone or in or combination with other antiplatelet agents ($p = 0.01$). The PFA-100® assesses platelet function under moderate to high shear stress conditions, and may be most informative in patients with TIA or stroke in which moderate to high shear stress plays a role, i.e. stroke of large-artery atherosclerotic aetiology, including carotid artery stenosis. However, as stated in Chapter 11, testing these patients with a low shear stress system ex vivo may actually be more clinically informative to avoid 'overexposure' to shear stress in the testing paradigm.

12.3. Circulating reticulated platelets in Carotid Artery Stenosis

This thesis assessed the percentage of circulating reticulated platelets (%RP) in asymptomatic and symptomatic carotid stenosis patients. In Chapter 6, we found no significant differences in %RP between asymptomatic and symptomatic patients at any stage after symptom onset ($p \geq 0.7$), or between early and late phase symptomatic patients who had data at each time point ($p \geq 0.82$). These data suggest that measurement of the %RP is unlikely to be a sensitive biomarker of increased platelet turnover in patients with recently symptomatic than asymptomatic carotid stenosis, but this study has not addressed the potential prognostic value of reticulated platelets in assessing the risk of long-term stroke recurrence because none of the patients in this study had a recurrent TIA or stroke during follow up.

12.4. Endothelial Activation in Carotid Artery Stenosis

In chapter 7 of my thesis, VWF:AgII levels were higher in early ($p < 0.001$), late ($p = 0.01$) and late post-intervention ($p = 0.038$) symptomatic versus asymptomatic patients. There were no significant differences in VWF:Ag levels or VWF:Ag/VWF:AgII ratio between symptomatic and asymptomatic patients ($p \geq 0.056$). In patients on aspirin monotherapy, VWF:Ag and VWF:AgII levels were elevated in early symptomatic versus asymptomatic patients. VWF:Ag levels decreased significantly in symptomatic patients followed up from the early to late phase after symptom onset ($p = 0.048$). These data indicate that endothelial activation is enhanced in symptomatic versus asymptomatic carotid stenosis, and decreases over time in patients with symptomatic carotid stenosis. They also indicate that VWF propeptide levels are potentially a more sensitive marker of endothelial activation than VWF antigen levels in carotid stenosis patients. Whether VWF:Ag II and / or VWF:Ag levels could act as 'endothelial biomarkers' in the

prediction of recurrent cerebrovascular events in early symptomatic carotid stenosis patients, or in the predication of index events in asymptomatic patients warrants further study in larger, longitudinal studies.

12.5. Coagulation System Potential in Carotid Artery Stenosis

In chapter 8, we demonstrated that peak thrombin and ETP were higher in early symptomatic versus asymptomatic patients, and in early severe symptomatic versus severe asymptomatic carotid stenosis patients. Peak thrombin production decreased in symptomatic patients followed up from the early to late phase after symptoms ($p=0.02$). In the subgroup of symptomatic carotid stenosis patients who did not undergo intervention, ETP was elevated in the early compared with late phase. These findings indicate that patients with recently symptomatic moderate or severe carotid stenosis have increased thrombin generation potential compared with their asymptomatic counterparts. Thrombin generation potential decreases over time following TIA or stroke associated with extracranial carotid stenosis. This increased thrombin generation potential in the early symptomatic patients may be as a consequence of the ischaemic event itself or could potentially be secondary to an acute phase response.

12.6. MES Detection in Carotid Artery Stenosis

In this novel aspect in chapter 9 of my thesis, we demonstrated that 32% ($p = 0.02$) of early phase and 19% ($p = 0.2$) of late phase symptomatic patients versus 12% of asymptomatic patients had MES detected on TCD ipsilateral to the carotid stenosis of interest. We also found that early symptomatic MES negative patients had elevated circulating lymphocyte-platelet complexes than asymptomatic MES negative patients ($p = 0.0085$), indicative of enhanced platelet activation status in this early symptomatic

subgroup. These data suggest that platelet activation data may further aid risk-stratification of patients with symptomatic carotid stenosis, who might be deemed to be at low risk of recurrent symptoms due to the absence of MSE on TCD monitoring.

12.7. Carotid plaque morphology characterisation in Carotid Artery Stenosis

This small, pilot substudy in chapter 10 of my thesis did not reveal any significant differences in measures of plaque echolucency between the asymptomatic and early symptomatic cohorts. The study was underpowered to make any definitive conclusions. However, based on these pilot data, we have designed and commenced a larger clinical follow-up study simultaneously assessing carotid plaque morphology, TCD assessment of cerebral MES, and biomarkers of platelet activation/hyper-reactivity to assess the potential predictive value of these measurements, alone and in combination, for risk-stratification of patients with asymptomatic and symptomatic carotid stenosis.

12.8. Ex vivo 'high on-treatment platelet reactivity' to commonly prescribed antiplatelet regimens on the PFA-100® and VerifyNow® following TIA or ischaemic stroke

This novel cross-sectional pilot study assessing *ex-vivo* platelet reactivity and HTPR on two different point-of-care devices in patients in the late phase after TIA or ischaemic stroke provided several interesting findings. On the PFA-100, 41% of the aspirin-dipyridamole group demonstrated HTPR on the C-EPI cartridge, with 92% in the clopidogrel group demonstrating HTPR on the C-ADP cartridge. The proportion of patients with HTPR was lower on the VerifyNow versus the PFA-100 ($p < 0.001$). Overall, our findings indicate that the prevalence of *ex-vivo* HTPR following TIA or

ischaemic stroke is markedly influenced by the method employed to assess platelet reactivity. Our data also clearly indicate that the PFA-100 C-ADP cartridge should not be used routinely to monitor the antiplatelet effects of clopidogrel *ex vivo*, when one classifies patients with a cross-sectional, case-control definition of HTPR.

12.9. Future Directions

In order to address many of the hypotheses generated by this thesis, future larger, longitudinal studies in patients with asymptomatic and symptomatic carotid stenosis are required. Such a study has been designed by me and other members of our neurovascular research group, and is currently ongoing at AMNCH, SJH and TCD under the supervision of Dr McCabe and his collaborators. This larger, longitudinal observational study, called the 'HaEmostasis In carotid STenosis (HEIST) Study' is assessing platelet activation status with fresh whole blood flow cytometry, platelet reactivity on the PFA-100 with C-EPI and C-ADP cartridges, as well as the novel, recently-licensed Innovance P2Y cartridge which has been designed to specifically to detect the antiplatelet effects of clopidogrel and P_2Y_{12} antagonists. Other devices that are being incorporated into the HEIST study include the relatively new Multiplate® analyser, and the VerifyNow® Aspirin and P2Y12 cartridges, as well as more novel tests of platelet function that are being developed with platelet and basic science collaborators. We are also simultaneously assessing ETP and VWF:Ag and VWF:Ag II levels in these patients to assess their ability to predict the risk of stroke recurrence in patients with recently symptomatic carotid stenosis, but also to assess whether levels of thrombin generation potential or endothelial activation are higher in asymptomatic patients who subsequently develop cerebrovascular symptoms during follow-up over time.

The data from the PACS study will be combined with data from the HEIST study to determine whether platelet biomarkers, in association with simultaneous data on carotid plaque morphology and MES detection can aid in the risk-stratification of patients with symptomatic and asymptomatic carotid stenosis. We will combine these data in a statistical model to attempt to risk-stratify asymptomatic and symptomatic carotid stenosis patients. It is hoped that these data will lead to important advances in primary and secondary stroke prevention.

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