Thromboembolic events after carotid surgery: causes and solutions

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The work on which this thesis is based is my own independent work except where acknowledged

P. D. HAYES

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This thesis is dedicated to my wife and family without whose constant support this work would not have been possible

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Abstract

Introduction:
Carotid endarterectomy (CEA) for patients with a significant (>70%) carotid stenosis reduces their risk of stroke over the subsequent years of follow up. This benefit is ameliorated by post-operative strokes and deaths. If the post-operative complication rate can be reduced then it is possible that patients with lesser stenoses may also benefit from CEA. This body of work has concentrated on identifying factors related to the development of strokes secondary to carotid thrombosis following CEA. The number of microembolic signals detected by transcranial Doppler ultrasound arising from the endarterectomised vessel wall has been shown in a number of publications to correlate with risk of post-operative carotid thrombosis.

Methods and Results:
1) Patients who undergo two carotid endarterectomies at separate time points have a significant correlation between the number of post-operative emboli seen at the first and second procedures (p=0.038).
2) A randomised controlled trial of 274 patients undergoing closure of the arteriotomy site with either a prosthetic Dacron patch or a vein patch was performed. This showed that whilst the vein patch had fewer emboli overall (median of 3 vs 5, p=0.028), the number of people having high levels of sustained embolisation or needing post-operative Dextran-40 therapy (to prevent carotid thrombosis) was not different between the 2 groups (p=0.62).
3) Laboratory platelet function studies (flow cytometry and aggregometry) showed that patients at increased risk of post-operative carotid thrombosis had platelets that were significantly more responsive to the physiological agonist, ADP (p<0.0001 for flow cytometry and p=0.0012 for aggregometry). The degree of inhibition by aspirin did not have a significant effect.
4) Dextran-40, an effective drug for the prevention of carotid thrombosis, was found to bind to the surface of platelets in a dose dependent manner (p=0.006). Unexpectedly, Dextran-40 increased the rate at which platelets aggregated together following stimulation with ADP (p=0.047). Activated platelets bound significantly more Dextran-40 than resting platelets (p<0.0002). Dextran-40 failed to cause disaggregation of previously aggregated platelets.

5) A platelet receptor polymorphism of the fibrinogen binding site (HPA-3) was found to be associated with both increased pre- and post-operative emboli (p=0.03 and p=0.04 respectively). This polymorphism was also associated with increased fibrinogen binding (p=0.024) and decreased post-operative blood loss (p<0.001). The met+ allele of the collagen receptor (HPA-2) was significantly associated with post-operative restenosis (p=0.01).

6) Studying patient risk factors for post-operative carotid thrombosis identified that women (who are at significantly increased risk of carotid thrombosis) had over 3 times as many emboli as men (p=0.004). Patients who embolised pre-operatively were more likely to do so in the post-operative period (p=0.027). No other risk factors were associated with emboli counts. Patients who had their operation in the morning were significantly more likely to develop high numbers of emboli relative to those performed in the afternoon (p=0.004).

Conclusion:
The findings of this body of work support the hypothesis that it is the patient who is prothrombotic rather than the procedure per se. It has also identified a number of potential targets and therapeutic strategies that may help to reduce the number of post-operative emboli seen after CEA, and thereby reduce patients’ risk of thrombotic stroke. The findings of this study may well have relevance to other areas of cardiovascular intervention.
PUBLICATIONS ARISING FROM THIS WORK

Papers:

Patients' thromboembolic potential between bilateral carotid endarterectomies remains stable over time.
Hayes PD, Lloyd AJ, Payne D, Bell PRF, Naylor AR

Randomized trial of vein versus Dacron patching during carotid endarterectomy: Influence of patch type on postoperative embolisation.
Hayes PD, Alroggen H, Steel S, Thompson MM, London NJL, Bell PRF, Naylor AR.
*J Vasc Surg.* 2001; 33; 994-1000

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The excess of strokes in female patients after CEA is due to their increased thromboembolic potential – analysis of 775 cases.
PD Hayes, DA Payne, NJ Evans, M Thompson, NJL London, PRF Bell, AR Naylor.
*Eur J Vasc Endovasc Surg* 2003 (accepted for publication).

Dextran-40 stimulates resting platelets and binds more avidly to activated platelets.
PD Hayes, H Box, NJ Hayes, AR Naylor, AH Goodall.
Submitted to *Eur J Vasc Endovasc Surg* 2003.

The platelet receptor HPA-3 (ile/ser) is a risk factor for pre- and post-operative thromboembolic events following CEA.
PD Hayes, C Hurd, W Ouwehand, AR Naylor, AH Goodall.
Abstracts:

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*Thromb Haemostasis 1999; 82: 162*

28) Hayes PD, Box H, Tull S, Bell PRF, Naylor AR, Goodall AH. Pre-operative platelet function predicts thromboembolic events after carotid endarterectomy. 
*Thromb Haemostasis 1999; 82: 449*

Thromboembolic events after carotid surgery are not prevented by aspirin, but are due to platelet response to adenosine 5-phosphate. 
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*Br J Surg 2000; 87: 503*

Dextran-40 stimulates resting platelets and binds more avidly to activated platelets. 
Hayes PD, Box H, Tull S, Bell PRF, Goodall AH, Naylor AR. 
ABBREVIATIONS

\(\alpha\) -alpha
\(aa\) Common homozygote
\(ab\) Heterozygote

ADP Adenosine diphosphate

AA Arachidonic acid

ACAS Asymptomatic Carotid Artery Surgery

Agg Aggregation

AHA American Heart Association

ANOVA Analysis of variance

APTT Activated partial thromboplastin time

bb Less common heterozygote

\(Ca^{2+}\) Calcium

CABG Coronary artery bypass graft

cAMP Cyclic adenosine monophosphate

CASANOVA Carotid Artery Stenosis with
Asymptomatic Narrowing: Operation
Versus Aspirin

CCA Common carotid artery

CD 62P Marker of P selectin expression

CEA Carotid endarterectomy

CHD Coronary heart disease

CI Confidence interval

COX Cyclo-oxygenase

CREST Carotid Revascularisation
Endarterectomy versus Stent Trial

CT Computed tomography

cm Centimetres

\(0^\circ C\) Degrees celsius

DAT Digital audio tape

DM Diabetes mellitus

DNA Deoxyribonucleic acid

ECM Extracellular matrix

ECST European Carotid Surgery Trial

ELISA Enzyme linked immunosorbent assay

FITC Fluorescein isothiocyanate

GAG Glucosamine glycan

GpIb Platelet collagen receptor

GpIb-IIIa Platelet fibrinogen receptor

GPRP L-glycyl-L-propyl-L-arginyl-L-proline peptide
<table>
<thead>
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<th>Full Form</th>
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<tr>
<td>HBS</td>
<td>Hepes buffered saline</td>
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<tr>
<td>HDL</td>
<td>High density lipoproteins</td>
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<td>HPA</td>
<td>Human platelet antigen</td>
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<td>hr</td>
<td>Hour</td>
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<td>ICA</td>
<td>Internal carotid artery</td>
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<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<td>IL-6</td>
<td>Interleukin –6</td>
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<td>ILE</td>
<td>Isoleucine</td>
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<td>IQR</td>
<td>Interquartile range</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<td>Litre</td>
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<td>LDL</td>
<td>Low-density lipoproteins</td>
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<td>M</td>
<td>Molar</td>
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<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
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<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
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<tr>
<td>MHz</td>
<td>Megahertz</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MRA</td>
<td>Magnetic resonance angiography</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>MWU</td>
<td>Mann Whitney U test</td>
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<tr>
<td>Na Cl</td>
<td>Sodium chloride</td>
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<tr>
<td>NASCET</td>
<td>North American Symptomatic Endarterectomy Trial</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>No</td>
<td>Number</td>
</tr>
<tr>
<td>OHS</td>
<td>Oxford handicap stroke score</td>
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<tr>
<td>Op</td>
<td>Operation</td>
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<tr>
<td>PC</td>
<td>Primary closure</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
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<tr>
<td>PG EP</td>
<td>Prostaglandin endoperoxides</td>
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<tr>
<td>POCT</td>
<td>Post operative carotid thrombosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
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<tr>
<td>PTFE</td>
<td>Poly-tetrafluoroethylene</td>
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<tr>
<td>Rargn-FITC</td>
<td>FITC conjugated polyclonal rabbit antibody to fibrinogen</td>
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<tr>
<td>$R^2$</td>
<td>Correlation</td>
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<tr>
<td>RPE</td>
<td>R-phycoerythrin</td>
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<tr>
<td>RTAT</td>
<td>Recombinant tick anticoagulant peptide</td>
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<tr>
<td>s</td>
<td>Second</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SER</td>
<td>Serine</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosis</td>
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<tr>
<td>st dev</td>
<td>Standard deviation</td>
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<tr>
<td>TCD</td>
<td>Transcranial Doppler</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>TIA</td>
<td>Transient ischaemic attack</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TX A$_2$</td>
<td>Thromboxane A$_2$</td>
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<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
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<td>US$</td>
<td>United States dollars</td>
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<td>µg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>µmol</td>
<td>Micromole</td>
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<tr>
<td>VCAM</td>
<td>Vascular cellular adhesion molecule</td>
</tr>
<tr>
<td>VPC</td>
<td>Vein patch closure</td>
</tr>
<tr>
<td>vWD</td>
<td>Von Willebrand disease</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>+ve</td>
<td>Positive</td>
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<tr>
<td>-ve</td>
<td>Negative</td>
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<td>vs</td>
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CAROTID ARTERY DISEASE AND ITS COMPLICATIONS
1.1 Introduction

Atherosclerosis currently accounts for more death and serious morbidity than any other cause in the Western world. Although any artery may be affected, it is primarily a disease of the elastic arteries, with the aorta, the coronaries and the carotid vessels being most commonly affected. Disease within the carotid arteries is a major cause of stroke.

The basic atheromatous lesion consists of a raised focal plaque, or atheroma, within the intima of the vessel which has a lipid core comprised mainly of cholesterol and cholesterol esters, and a covering fibrous cap. As plaques increase in size, they progressively encroach on the lumen of the affected vessel and into the subjacent media. Clinical disease manifests itself via two distinct mechanisms: occlusion of the vessel by an expanding atheroma thereby compromising blood flow to the distal organs, or plaque rupture, causing distal embolisation and potential acute thrombosis of the vessel. Both of these sequelae can cause acute cerebral ischaemia and subsequent stroke.
1.2 Classification of atherosclerosis

The American Heart Association committee on Vascular Lesions has proposed that the progression of the atherosclerotic plaque is divided into five phases with each phase defined by a distinct set of morphological characteristics (1). Phase 1 consists of small lesions that progress over a number of years and these are sub-divided into three types. Type I denotes lesions comprised of macrophage derived foam cells that contain lipid droplets; Type II lesions contain both macrophages and smooth muscle cells with extracellular deposits; and Type III lesions contain smooth muscle cells surrounded by extracellular connective tissue, fibrils and lipid deposits. Phase 2 lesions are plaques that may not yet be stenotic in nature, but have a high lipid content and therefore exhibit an increased tendency to rupture. Type IV plaques comprise confluent, cellular lesions with a significant lipid content. Finally, Type V lesions possess a lipid core covered only by a thin fibrous cap.

Phase 3 refers to disruption of one of the preceding types of plaque and Phase 4 denotes complete occlusion of the affected vessel by thrombus formation generated in response to exposure of the plaque contents. Phase 5 refers to the final stage whereby the thrombus has become partially or completely lysed, and the lesion is reorganised. This is referred to as a Type Vb plaque.
1.3 Aetiology of atherosclerotic plaques

The Type III lesion, or 'fatty streak', is the earliest macroscopically detectable atherosclerotic lesion of atherosclerosis and is present in most individuals by the age of 25 (2). Chronic minimal injury to the endothelium is often physiological in its nature, resulting from a disturbance in the pattern of blood flow in certain parts of the arterial tree, such as bending points and bifurcations including the common carotid bifurcation. The effects of these local shear forces may be exacerbated by the presence of several other factors which then potentiate chronic endothelial injury, leading to the accumulation of lipids and macrophages at this site. These factors include hypertension (3), hypercholesterolaemia, advanced glycation products in diabetes, chemical irritants in tobacco smoke, circulating vasoactive amines, immune complexes and infection (4-7). The progression of the plaque beyond the type III stage is related to lipid metabolism and monocyte-macrophage function and can be categorised into five stages:

- Firstly, circulating lipid derived from plasma low density lipoproteins (LDL) enters the vessel wall through endothelium damaged by the processes outlined above (8).
- The LDL is mildly oxidised by all cell types in vessel wall, but primarily the endothelial cell (9).
- A combination of the oxidised LDL, shear forces and turbulent flow are instrumental in attracting circulating monocytes into the vessel wall, by increasing the expression of the adhesion factors ICAM-1 and VCAM-1 on the endothelial cells (9). Further oxidation of the LDL by the macrophage now occurs, and the subsequent product is engulfed by the active cell, thus converting it into a foam cell.
CHAPTER ONE: Carotid Artery Disease and its Complications

• High density lipoproteins exhibit a protective mechanism against lipid accumulation in the vessel wall by inhibiting the LDL oxidation and by removing lipid from the wall and macrophages.

• Macrophages damaged by the excessive accumulation of lipid within them begin to release a number of other deleterious products, including oxidised cholesterol, macrophage colony stimulating factor (M-CSF) and platelet derived growth factor (PDGF) (10). The combination of these factors leads to a shift in the balance of vasoactive metabolite production, from predominantly vasodilating factors such as nitric oxide (NO) or prostacyclin, to vasoconstrictive agents (11)

1.4 Plaque disruption

As further lipid accumulation occurs, the Type III lesion is converted to a Type IV or Va lesion, both of which are more prone to rupture than the relatively stable Type III plaques. Two types of disruption have been described: “passive” and “active” plaque disruption. Passive disruption denoted plaque disruption secondary to a collection of physical factors acting upon a thinned portion of the fibrous cap. This is often at the junction of the plaque and adjacent vessel wall (12) and the vulnerability of the plaque to rupture is determined by three factors.

• The circumferential wall stress, which relates to the thickness and collagen content of the cap,

• Blood pressure

• Lumen radius (13).
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Chronic repetitive stresses of this nature progressively weaken the wall, giving rise to sudden plaque rupture. There is also an ‘active’ component to plaque rupture and this is influenced by the cellular content of the plaque. Activated macrophages are able to degrade extra-cellular matrix components directly by phagocytosis, or by secreting proteolytic enzymes including plasminogen activators or matrix metalloproteinases (14). These enzymes have previously been identified in human carotid plaques and their presence shown to correlate with an elevated risk of rupture (15). The action of MMPs is directly inhibited by naturally occurring tissue inhibitors of matrix metalloproteinases, or TIMPs. In the physiological state, careful regulation of MMP and TIMP synthesis ensures extracellular matrix homeostasis; a disruption of this balance can have direct implications for plaque stability, with an increase in MMP activity or a decrease in TIMPs favouring ECM breakdown and therefore placing the plaque at risk of rupture.

Plaque rupture initiates a cascade of thrombotic events in response to the release of prothrombotic material from its core. The extent of this response is governed by three major determinants:

(a) *The extent and thrombogenicity of the exposed plaque tissues.*

Significantly more platelet deposition occurs on lipid rich plaques than on those with a collagen rich, sclerotic matrix (16), and it has been suggested that it is the amount of tissue factor protein found in the lipid rich plaques that gives them their enhanced thrombotic potential (17). Tissue factor is one of the many products released by activated macrophages but contrary to this view is the fact that thrombin generation in patients with ruptured
plagues appears to be more dependant on the extrinsic coagulation pathway (contact activation mediated), rather than the intrinsic (tissue factor initiated) pathway (18;19).

(b) The nature of flow disturbances around the disrupted plaque surface

Local blood flow patterns directly influence the thrombotic response; the tighter the stenosis (20) and the rougher the surface (16), then the greater the number of platelets that are deposited. This is likely to be a reflection of the fact that von Willebrand factor, a key protein for platelet receptor binding via Gp Ib, is activated by the high shear flow rates seen at these sites.

(c) Thrombogenic factors

Platelet hyperaggregability, hypercoagulability, and impaired fibrinolysis are all known to be associated with an increased risk of thrombus mediated coronary events (1). This is illustrated by the fact that a transient, systemic hypercoaguable state can be identified in patients with acute coronary syndromes (18), mediated partly by activated macrophages in the circulation (21). The importance of the fibrinogenic-fibrinolytic equilibrium at the time of plaque disruption is clearly demonstrated by the protective effect exerted by antiplatelet agents and anticoagulation in stroke and myocardial infarction (1); if fibrinolysis is prevalent then the accumulated thrombus is rapidly dispersed, preventing vessel occlusion and end organ infarction.
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1.5 The consequences of carotid plaque disruption

Disruption of the plaque surface results in one of three clinical scenarios; TIA, stroke, or a clinically insignificant event. TIAs occur as a result of small emboli passing along the internal carotid artery (ICA) to the small end arteries that supply the cerebral tissue. This results in short lived periods of ischaemia causing alterations in cerebral function, which by definition last less than 24 hours. The causative emboli are usually derived from within the lumen of the plaque, the loosely adherent cellular and lipid content being washed piecemeal out of the plaque. A further source of emboli causing TIAs is microthrombi of platelets and fibrin that are dislodged from the surface of the plaque itself.

The second consequence of carotid plaque disruption is the syndrome of stroke. This clinical term refers to the sudden development of a neurological deficit, caused by an unspecified abnormality of the blood supply to the cerebral tissues. The anatomical location of the lesion determines the nature of the symptoms and signs exhibited by the patient, and encompasses a wide spectrum, including motor, sensory, and speech deficits. In turn, the size, location and extent of tissue damage are all determined by the size of the vascular bed affected, and the adequacy of any collateral circulation to the area in question. The major source of collateral supply is the contralateral internal carotid artery, via the circle of Willis, if this is intact, and a further collateral route exists via the external carotid - ophthalmic artery pathway. Partial and inconstant reinforcement is available over the surface of the brain from the distal branches of the anterior, middle and posterior cerebral arteries via the
cortical - leptomeningeal anastomoses. However, there is negligible collateral supply to the deeper structures of the brain.

The disruption of a carotid plaque may cause cerebral infarction in one of two ways; namely secondary to embolic occlusion from either plaque or thrombus, or due to thrombotic occlusion of the ICA with a failure of the collateral supply to support the metabolic needs of the cerebral tissue. Complete internal carotid artery occlusion results in a stroke in between 38 and 52 percent of cases (22;23). This figure is not as high as one might expect, but it has been postulated that many fatal infarctions may have been missed from these studies (24). Currently, the most common cause of stroke is embolic in nature, with microthrombi being dislodged from the surface of the disrupted causative plaque. However, the mechanisms that underlie the development of an occlusive lesion in some patients and an embolic event in others remain unclear at present.
CHAPTER ONE: Carotid Artery Disease and its Complications

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CHAPTER TWO

TREATMENT OF CAROTID ARTERY DISEASE
CHAPTER TWO: Treatment of Carotid Artery Disease

2.1 Introduction

First time stroke currently affects 0.2 percent of the population of England and Wales per annum, amounting to 120,000 cases per year, with another 40,000 suffering a recurrence (1). There are a further 500,000 people living with the long term consequences of a stroke at the present time and the cost of treating, rehabilitating and caring for a single stroke has been estimated at £16,868 (2), £29,351 (3) and £27501 (4) per patient. This costs the National Health Service (NHS) and Social Services approximately £3.9 billion a year and as age is a significant risk factor for stroke and our population continues to live longer, stroke care will continue to present an increasing financial burden to the NHS.

Just as there is a spectrum of carotid artery disease, there are number of therapies available to treat the problem, although none of the available therapies alone is suitable to treat all stages of the disease. The question of which treatment to apply to a particular scenario has been the subject of a number of significant trials over the last two decades (5-12).
2.2 Clinical trials in symptomatic disease

(a) *The European Carotid Surgery Trial (ECST)* (12)

The European Carotid Surgery Trial (ECST) enrolled 3024 patients in a multi-centre randomised controlled trial. All patients had proven stenosis of varying severity and had experienced an ischaemic vascular event within the last six months. Between 1981 and 1994, 1811 (60 percent) patients were allocated to surgical management and the remainder to the best medical therapy available at the time, with surgery to be avoided for as long as possible. The mean follow up was 6.1 years, and the main analyses were by intention to treat.

The overall outcome, major stroke or death, occurred in 37 percent of the surgery group and 36.5 percent of the medical group. The risk of major stroke or death was 7.0 percent, and this did not vary with the severity of the stenosis operated upon. However, the risk of ischaemic stroke during medical follow up did vary with the degree of carotid stenosis, being greatest for those with stenoses greater than 70-80 percent. This effect was present for the first three years of medical follow up and then the risk of stroke appeared to decline in this population. The Kaplan-Meier estimate of stroke risk at three years was 26.5 percent for the control group with significant stenoses, against 14.9 percent for the equivalent surgical group.
This trial demonstrates a definite benefit for surgery in those patients with a stenosis greater than 80 percent. However, there are a number of factors that may influence this result.

Patients allocated to the study were operated on within one year of the onset of symptoms, whereas the risk of a further neurological event rapidly declines within two to three months of the event (12). Thus, earlier surgery may have shown a greater net benefit. Patients were only entered into the study when the clinician was "substantially uncertain" of whether to recommend surgery or not, therefore patients with multiple ischaemic neurological events who would benefit most from surgery were not entered into the trial.

Also of note is the fact that patients who were allocated to the control group but underwent surgery because of further symptoms (3.5 percent) were analysed within the control group. A greater proportion of the control group were taking aspirin at randomisation (58.7 percent versus 54.7 percent, p<0.05) and the control group were also treated more aggressively with other anti-platelet agents, anticoagulants and lipid lowering drugs (p=0.003).

Using a Kaplan-Meier survival curve, for patients in the 80 to 99 percent stenosis group, 139 events were prevented over 3 years by surgery, per 1000 operations performed. The risk-benefit assessment for patients with 70 to 79 percent stenoses appeared to be equal for surgery and medical therapy at three years. The authors of the report felt that the cut-off point at which surgery was indicated fell between 70 and 80 percent, but with more aggressive medical therapy for the surgical group and a reduction in the 7 percent perioperative major stroke/death complication rate, surgery may well be indicated for lesser degrees of stenosis.
(b) The North American Symptomatic Carotid Endarterectomy Trial (NASCET) (13)

This randomised trial was performed in 50 centres across the USA and Canada from 1988. Patients were eligible if they exhibited symptoms of cerebral ischaemia within the previous six month period and demonstrated an ipsilateral 30 to 99 percent stenosis of their carotid artery. This cohort of 659 patients was divided into 2 according to severity of stenosis; 30 to 69 percent, and 70 to 99 percent stenosis. The method of calculating the degree of stenosis differed from that used in ECST as follows.

NASCET:  Percentage stenosis = \( \frac{A-B}{A} \)

ECST:  Percentage stenosis = \( \frac{C-B}{C} \)

Where  
\( A = \) diameter of the unaffected ICA distal to the stenosis  
\( B = \) the minimum diameter of the ICA due to the stenosis  
\( C = \) the estimated width of the carotid bulb at it's widest point.

All measurements were performed on pre-randomisation angiograms and these formulae generated the equivalent values given in table 2.1 (14;15):
Within the first 30 days after surgery, the risk of major stroke or death was found to be 2.7 percent, with a total stroke rate of 5.5 percent. Long-term follow up was continued for two years, after which time an interim analysis demonstrated a significant benefit for in surgery in the 70 to 99 percent stenosis group. This arm of the trial was halted at this point. At 2 years, the major stroke and death rate was 8.0 percent for surgery, versus 18.1 percent for medical management. The comparable figures for any stroke event were 12.6 percent and 27.6 percent respectively. In this trial, no difference existed between the medical therapy received by each group and sub-division of the 70 to 99 percent stenosis group into deciles revealed an increasing benefit from surgery with increasing degree of stenosis.
To date the results of the 30 to 69 percent stenosis group have not yet been published, but they have been presented (14;15) and this communication indicated that the NASCET study now suggests a significant benefit for those patients with a stenosis of 50 percent. Comparison of the ECST and NASCET results using the preceding table would support this conclusion, with a 50 percent according to the NASCET formula equating to 75 percent under ESCT criteria.

(c) The Veterans Affairs Co-operative Studies Program 309 Trialist Group (16)

This trial randomised 193 male patients to receive either surgery plus best medical therapy, or best medical therapy alone. All patients had been symptomatic within the previous four month period and had angiographic evidence of a greater than 50 percent stenosis in the ipsilateral carotid artery using the NASCET criteria. At a mean follow up of 11.9 months the surgically managed group had suffered significantly fewer strokes than the medically treated group; 7.7 percent against 19.4 percent respectively, giving an absolute risk reduction of 11.7 percent. There was an absolute risk reduction for surgery of 17.7 percent, when the stenosis reached 70 percent, but this trial was terminated early in the advent of the publication of the NASCET study.
2.3 Clinical trials in asymptomatic disease

(a) The Asymptomatic Carotid Atherosclerosis Study (ACAS) (17)

The Asymptomatic Carotid Atherosclerosis Study (ACAS) was commenced in 1987 as a prospective randomised trial of 1662 patients with a 60 percent or greater stenosis of a carotid artery, and recruited patients from 39 clinical centres across the USA. It’s objective was to determine whether surgery plus best medical therapy could reduce the incidence of cerebral infarction. After a mean follow up of 2.7 years, with 4657 patient-years of observation the aggregate risk over five years for ipsilateral stroke and any peri-operative stroke or death was 5.1 percent for surgical patients and 11.0 percent for those treated with best medical therapy alone (absolute risk reduction 5.9 percent, aggregate risk reduction 53 percent). The degree of carotid stenosis did not alter the magnitude of benefit seen by the surgical arm of the trial.

The absolute risk reduction of 5.9 percent indicates that 19 patients must undergo CEA for the prevention of one stroke. The risk reduction stated here is an aggregate over the course of five years, and for the first 10 months of follow up is worse in the surgical group. Most strokes occurring in the surgical group occurred within 30 days of surgery, and these exerted a residual effect for the remaining five years, whilst the risk seen in the medical group was distributed over the five years. When the patients were divided according to gender, the benefit of surgery appeared to persist in men (aggregate risk reduction 66
percent (95 percent CI, 36 percent to 82 percent)), whilst for women this benefit apparently disappeared (aggregate risk reduction 18 percent (95 percent CI, 50 percent to 87 percent)). This was felt to be due to an excess of peri-operative complications in the female group, 3.6 percent versus 1.8 percent for the male group.

(b) The Veterans Affairs Asymptomatic Carotid Artery Trial (18)

This randomised study enrolled 444 asymptomatic men, with a stenosis of greater than a 50 percent stenosis from 11 Veteran’s Medical Centres across the US. The mean follow up was 47.9 months. A significant reduction in the number of ipsilateral strokes was demonstrated in the surgical group (4.7 percent) when compared to the group receiving best medical therapy alone (9.4 percent). However, when all strokes and death were used as end points, no significant difference existed between the two groups. Despite this finding, many of the deaths during the long-term follow up period were due to coronary atherosclerosis, and the authors felt that a modest long-term benefit from surgery could not be excluded in view of this.

(c) Other studies

The Carotid Artery Stenosis with Asymptomatic Narrowing: Operation Versus Aspirin (CASANOVA) (19) trial involved 410 patients, excluding those with stenosis greater than 90. The peri-operative complication rate was relatively higher in this trial at 6.9 percent when compared to the two trials discussed previously, although no significant difference was found between the groups in terms of either stroke or death. A further study conducted
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at the Mayo Clinic in the US (9) compared surgery with no aspirin with best medical therapy but was terminated early due to an excessive number of deaths in the surgery group, most of which were cardiac in origin. The European Asymptomatic Carotid Artery Surgical trial continues to enrol patients at the present time.

(d) Summary

It is currently accepted that patients with a significant carotid stenosis (50 percent in NASCET and 75 percent in ECST) and recent symptoms of cerebral ischaemia who are otherwise fit for surgery, should undergo a CEA. In addition, all patients should be treated with best medical therapy, alongside their surgical management. However, some areas of uncertainty remain, including the optimum management of patients with a contralateral occlusion and lesser degrees of stenosis. The publication of the final results of the NASCET data combined with the ECST findings should allow meaningful subgroup analysis in order to address these outstanding issues (12). A reduction of the peri-operative mortality rate will exert a significant effect on the overall risk reduction seen for surgery and the potential impact of new and more potent anti-platelet therapies on the success of best medical therapy alone remains a further avenue of investigation.

The American Heart Association (AHA) has published guidelines for the use of CEA in patients with asymptomatic carotid stenosis (20). For patients exhibiting a low surgical risk of less than 3 percent, they state that it is appropriate to perform CEA for asymptomatic unilateral stenoses of greater than 60 percent. Patients with a moderate surgical risk of less than 5 percent should undergo CEA of an asymptomatic stenosis if greater than 75 percent
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if significant contralateral stenosis also of greater than 75 percent coexists. Furthermore, CEA should only be performed on patients carrying higher surgical risks than this within the auspices of a randomised trial. The trial data (17,19) available to date would seem to indicate there whilst there is a small benefit of surgery in men with asymptomatic stenosis, the data is less certain for women. However, the AHA guidelines make no distinction between gender groups at present.

2.4 Best medical therapy

Best medical therapy refers to a combination of treatments designed to lower patients’ overall risk of mortality, and reduce or limit risk factors directly implicated in stroke pathogenesis. Consequently, it comprises both lifestyle modification and specific drug therapy, although few studies have prospectively assessed the role of risk factor control following CEA. However, there exists a wealth of data to illustrate the relationships between risk factor control in stroke incidence, and this is outlined below.
2.4.1 Risk factors and lifestyle

(a) Circulating lipids

Mortality from stroke and coronary heart disease has long been thought to be related to elevated cholesterol and low-density lipoprotein (LDL) plasma concentrations, in addition to decreased levels of high density lipoproteins (HDL) (21). Some meta-analyses have cast doubt on the ability of a reduction in serum lipids to truly reduce overall mortality, in light of a relative increase in traumatic and cancer related deaths (22). However, three large studies have recently confirmed a benefit in reduction of serum cholesterol by using hydroxymethylglutaryl coenzyme A reductase inhibitors, or 'statins' (23-25). The primary end points of these studies were not originally the reduction of stroke risk, but two meta-analyses have recently focused on this issue. Herbert et al (26) studied 16 trials involving 29,000 patients and found that the use of statins reduced serum cholesterol and LDL by 22 percent and 30 percent, respectively; this was associated with a substantial reduction in trials on 105,000 patients. Conversely, diet modification and the use of fibrates to lower cholesterol exerted no effect on the stroke rate seen. However, statins reduced the risk of major and minor strokes by 24 percent. Two studies have examined recurrence of carotid disease following CEA and found a positive association between hypercholesterolaemia and restenosis (27;28). Indeed, other studies have noted a slowing in carotid artery disease progression when statins have been used to lower cholesterol (29;30), although there has been no prospective study to examine reduction of lipids and it's effect on restenosis rate to date.
(b) Hypertension

Hypertension represents the most powerful, prevalent and treatable risk factor for stroke identified to date (31). Both the systolic and diastolic blood pressure are implicated in stroke incidence, with a reduction in systolic blood pressure by 6mmHg resulting in a 42 percent decrease in stroke risk (31). One study has demonstrated that hypertension following CEA is also a risk factor for recurrent stenosis (27) although this finding has not yet been confirmed elsewhere. However, the control of hypertension in the immediate postoperative period warrants careful attention, as it is recognised as a significant risk factor for the development of the hyperperfusion syndrome, a complication of CEA discussed later.

(c) Cigarette smoking

Cigarette smoking increases the risk of stroke 1.5 to 2.2 fold (32;33), with the risk encountered being dependent upon the number of cigarettes smoked. Cessation reduces the risk to that of a non-smoker within two to three years (32;33), and of all of the risk factors for stroke, smoking exhibits the most significant association with restenosis post CEA (34;35).

(d) Other risk factors

Several other risk factors have been postulated for stroke, although conflicting data has cast doubt over their role in stroke aetiology. The consumption of alcohol in relation to stroke risk exhibits a J-shaped curve, with higher risk of stroke at either end (36). The post-menopausal use of oestrogen has been reported both as a benefit (37) and a risk for stroke (38), and although diabetes mellitus is a risk factor for carotid artery related stroke (39), its relationship with recurrent carotid disease post-CEA remains unproven at present.
2.4.2 Medical therapy

The role of anti-platelet agents in the peri-operative period for CEA has not yet been comprehensively studied, although numerous studies have sought to address the post-operative use of these medications. In the first randomised trial of aspirin versus placebo, patients were commenced on 1300mg aspirin or placebo five days after surgery (40). Six months post-CEA, there were fewer deaths or strokes in the aspirin group but the small number of patients involved in the study casts doubt over the validity of these results. A further randomised trial conducted by Kretschmer et al, found that the mortality rate following CEA was reduced by the administration of 1000mg of aspirin post-operatively (41). The number of strokes in this study was not reported but a Danish trial that administered low dose aspirin at 50 to 100 mg post-operatively failed to find any significant benefit in terms of stroke reduction (42). However, the aspirin was not initiated until between one and twelve weeks after the CEA and so it is difficult to ascertain the true role of aspirin in the immediate post-operative setting from this trial alone. The use of aspirin prior to the CEA versus placebo was studied during a Swedish trial (43), and there was a significant decrease in the number of intra- and peri-operative strokes seen in the cohort commenced on aspirin. However, a further trial used aspirin at 325mg alongside dipyridamole at 225mg in an effort to prevent restenosis following CEA, but failed to demonstrate any significant benefit from this regime (44).
In contrast, The Anti-platelet Trialists Collaboration (45) found a 23 percent reduction in risk of non-fatal stroke compared with placebo in patients with a history of TIAs. There was also a 22 percent reduction of events in the cluster "non-fatal stroke, non-fatal myocardial infarction and vascular death). This benefit was independent of age, sex, diabetes or hypertension.

NASCET have retrospectively examined their data with regard to peri-operative stroke in those patients with 70 to 99 percent stenoses undergoing CEA. The ipsilateral stroke rate at 30 days was 2.1 percent, 1.1 percent, 6.5 percent, and 7.8 percent in patients receiving 1300mg, 650mg, 325 mg or no aspirin, respectively. This non-randomised data has formed the basis for a double-blinded trial that is underway at present (46).

As illustrated the issue of the timing of admission and optimum dose of aspirin for prevention of stroke in patients undergoing CEA remains unresolved. However, it would seem prudent in the light of data from the Antiplatelet Trialist (45) and the Mayo Clinic study (9) to administer low dose aspirin in order to decrease the peri-operative mortality from cardiac events during CEA. The use of aspirin to prevent stroke in a non-surgical setting will be discussed later in Chapter 4 and the mode of action and use of other anti-platelet drugs will also be considered.
2.5 Carotid Angioplasty

Angioplasty has an established role in the treatment of lower limb peripheral arterial disease but its place in the management of carotid artery disease is currently under evaluation. The first report detailing the dilatation of an atherosclerotic carotid plaque appeared in 1980 (47). Since then there have been many reports of ad hoc case series purporting to demonstrate outcomes similar to CEA, but at present only one randomised trial has reported its results. CAVATAS was sponsored by the UK Medical Research Council and randomised 504 patients who were fit for CEA between 1992 and 1997 (48). The 30-day death and any stroke rate was nor different between the two groups (9.9% for surgery and 10.0% for angioplasty), but this study has been criticised for its high operative complication rate which may have compromised the analysis of the outcomes.

The proponents of angioplasty often quote cranial nerve injury rates as a reason to avoid a neck incision, but these injuries are usually transient and of minor consequence (49). In addition, arguments that angioplasty is cheaper and results in less hospitalisation has also been questioned (50). The major hazard of angioplasty is that of distal embolisation of the soft material within the plaque during the compression phase of the procedure. Very high rates of embolisation can be detected by the use of TCD monitoring during angioplasty and this probably accounts for the high rate of TIAs associated with the procedure. A number of “cerebral protection” devices exist that aim to trap the embolic material before it reaches the brain but at present none of them are proven to reduce stroke rates. In addition, angioplasty cannot prevent the most common cause of post-operative stroke – carotid thrombosis – as the disrupted plaque is at least as thrombogenic as the subendothelium. The rate of
restenosis of the angioplasty site is significantly higher than that of an endarterectomy zone (51), but as the morphology of these restenoses differs from that of the original lesion the impact on long term clinical outcomes remains unclear.

Intuitively, it would seem that angioplasty may have a role in the management of carotid disease, especially in patients where surgery is associated with greater degrees of morbidity than a straightforward CEA. These areas of unproven benefit include the treatment of significant carotid restenosis, very proximal CCA lesions or very distal ICA disease and those patients who are not fit to withstand surgical intervention. At the present time, two large-scale trials are underway to assess angioplasty versus standard CEA, CAVATAS II (a UK based study) and the north American Carotid Revascularisation Endarterectomy versus Stent Trial (CREST). A degree of controversy already exists around these studies before a single result has been published for their decision to use unproven carotid stenting as opposed to routine angioplasty.

2.6 Carotid Endarterectomy

Carotid endarterectomy was initially developed as a procedure for the prevention of ischaemic stroke in 1954 (52), and rapidly disseminated into clinical practice. The popularity of the procedure, particularly in the USA lead to a dramatic rise in number of cases, from 15,000 in 1971 to a peak of 107,000 in 1985 (53). However, the publication of two randomised trials in the late 1980’s that failed to confirm a significant benefit for CEA on unselected patients (54;55) alongside a high mortality and post-operative stroke rate
resulted in a decline in this popularity. The number of carotid endarterectomies performed fell with each subsequent year thereafter until 1991 the publication of the ECST (5) and NASCET (13) data, which revealed a significant benefit for patients with severe stenosis of the carotid artery. Advances in surgical technique and ongoing research intended to reduce mortality rates and stroke incidence have helped to re-establish the role of CEA for stroke prevention.

Since the publication of ECST and NASCET, the patients groups for whom CEA is recommended are more clearly defined, although not yet fully decided. Just as there is a wide spectrum of clinical disease that directly affects the outcomes of this surgical procedure, so variation in surgical technique for CEA and the optimum approach remains open to debate at present. However, it is clear that opportunities exist for minimising stroke risk to patients of all groups through modification of imaging modalities, surgical technique, and pre-operative and post-operative management, and a review of the literature regarding these factors will be presented next.

(a) Imaging

Although not strictly part of the procedure of CEA, imaging of the ipsilateral and contralateral arteries is fundamental in selecting the correct patients for surgery. The major trials have all used angiography as the gold standard for imaging, although this technique is not without its own risks. During the Asymptomatic Carotid Artery Surgical study (ACAS), angiography resulted in four non-fatal and one fatal cerebral infarctions of a total of 414 angiograms performed (1.2 percent). This study was performed on patients deemed to have
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A low stroke risk group, and when the angiogram related stroke rate of 1.2 percent is compared to the peri-operative stroke rate of 2.3 percent, it is apparent that this was a significant complication. A review of eight prospective and 7 retrospective trials suggested a minor stroke or TIA rate of 4 percent with angiography, and a major stroke rate of 1 percent. A study of 1000 angiographic procedures produced a minor stroke rate of 0.5 percent and a major stroke rate of 0.5 percent. Angiography was also associated with other complications including distal embolisation, puncture site haematoma, allergic reactions and renal impairment.

A prospective study of angiography compared with duplex scanning in 99 patients prior to CEA, revealed that Duplex had a positive predictive value of 91.6 percent when compared to angiographic imaging (56). This study also aimed to identify which duplex criteria correlated most closely with angiography, and found that a peak systolic velocity greater than 125 cm/sec plus an end diastolic velocity greater than 135 cm/sec were the most accurate parameters. A further study compared duplex and magnetic resonance angiography (MRA) with standard angiography (57), and in determining a significant degree of stenosis, duplex demonstrated a sensitivity of 89 percent and specificity of 93 percent compared with figures of 85 percent and 70 percent for MRA. When the MRA and duplex results were concordant, the sensitivity was 100 percent and the specificity 95 percent, and as a result, the authors felt that these findings justified the use of duplex as definitive diagnostic tool. However, this should be the case only when the local vascular laboratory has audited and validated its results in this way (58;59).
Despite its apparent benefits for identification of stenoses, duplex has been criticised for its inability to detect anatomical variants that may render the CEA procedure technically difficult. Wain et al addressed this issue in a recent study that examined the ability of duplex to predict a high bifurcation, the distal extent of carotid disease, and identification of a coiled or kinked artery (60). The specificity for these factors was 100 percent, 92 percent and 100 percent, respectively and the corresponding sensitivity figures were all 100 percent. Two separate studies (13;61) have addressed the diagnosis of intra-cranial or proximal carotid disease and intra-cerebral aneurysms with duplex, and both concluded that this is not reliably possible. However, the actual incidence of these lesions in the study population was less than 1 percent and the role of CEA in the presence of concurrent disease remains inconclusively elucidated.

(b) Arteriotomy closure

The options for closure of the arteriotomy following the endarterectomy include primary closure or patch angioplasty with natural or artificial materials. Advocates of patch closure claim a reduced incidence of peri-operative stroke and later re-stenosis, compared to primary closure, and this issue will be addressed by a review of the current relevant literature. A systematic review of the literature by Counsell et al (62) identified and analysed data from well conducted controlled trials that addressed the role of primary closure and different patch types. Six randomised trials were considered suitable to be included in the analysis and using meta-analysis of odds ratios, routine patching with Dacron, PTFE or vein revealed a significantly lower number of ipsilateral peri-operative strokes and ipsilateral strokes during long-term follow up. However, if a worse case scenario approach is used to analyse the data on the 53 patients missing from follow up, then both results become
non-significant. However, it seems unlikely that all the patch closure patients lost to follow up did indeed suffer an ipsilateral stroke, whilst none of the lost primary closure patients did. On reviewing the data correlating different types of patches with outcome, the numbers concerned are significantly smaller and no difference in outcomes was demonstrated.

A prospective randomised trial of 399 patients with severe carotid artery stenosis comparing primary closure (PC) with PTFE patch or vein patch closure (VPC) has been reported recently (63). The results are summarised in table 2.2:

<table>
<thead>
<tr>
<th>Outcome</th>
<th>PC (percent)</th>
<th>PTFE (percent)</th>
<th>VPC (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peri-operative stroke</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4 year stroke rate</td>
<td>18</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Recurrent stenosis</td>
<td>34</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Redo CEA</td>
<td>11</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2.2
Comparison of outcomes of carotid endarterectomy in severe stenosis classified according to method of arterotomy.
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The PTFE and VPC groups fair significantly better (p<0.05) than the PC cohort in respect to outcomes considered. There were fewer recurrent stenoses in the PTFE arm compared to the VPC arm but interestingly, no difference in the number of these that became symptomatic requiring further surgical input.

Reasonably good evidence that patching improves outcomes in terms of restenosis and possibly, ipsilateral stroke. However, there are a number of complications associated with the procedure, including vein harvest site infections, seromas, and haematoma formation. Operative time is prolonged with PTFE patches is due to the increased time required for haemostasis (64;65), and infection rates of 2.3 percent have been reported for artificial (66) patch closure and a 0.3 percent rate for vein closure (67). Post-operative vein patch rupture is a serious but infrequent complication, arising in between 0.5 and 1.0 percent of cases, but which carries high mortality and morbidity rates (68;69). It is seen almost exclusively when the saphenous vein is harvested from the ankle, rather than the thigh or groin (69), and the use of the internal jugular vein for preservation of the long saphenous vein for coronary artery bypass graft or peripheral bypass has been suggested as a viable alternative (70).

Perkins et al have described a carotid artery bifurcation technique that uses the ECA to angioplasty the ICA with some success in an attempt to avoid the complications of vein patch rupture or pseudoaneurysm formation (71)
(c) Prevention of intra-operative stroke

Intra-operative strokes are primarily attributable to one of three sources: embolic events prior to clamping of the internal carotid artery, cerebral hypoperfusion post clamping of the internal carotid artery, and residual perfusion defects during flow restoration post-procedure. Embolic events occurring prior to clamping of the ICA may be detected by the use of intra-operative trans-cranial Doppler ultrasound (TCD) monitoring of the ipsilateral middle cerebral artery (72,73). This allows visualisation of circulatory flow through the middle cerebral artery and the identification of potentially embolic material, thus enabling the operator to clamp the ICA early to prevent further distal embolisation, or to modify the dissection technique.

Numerous methods have been employed to assist in detecting inadequate cerebral perfusion during the clamping of the ipsilateral carotid artery. A lengthy review of this area is not relevant to the main topic of this thesis, but a short overview is presented. Advocates of regional anaesthesia for CEA suggest that having the patient conscious and responsive during clamping allows inadequate perfusion to become apparent by the failure of the patient to respond to given commands (74,75). If this occurs then the surgeon can opt to insert a shunt to restore sufficient cerebral flow for the remainder of the procedure. In patients undergoing CEA under general anaesthesia, measurement of physiological variables must be undertaken instead. Methods currently used include:
• Electroencephalographic techniques which examine changes in brain wave patterns as an indication of ischaemia (76);
• Measurement of stump pressure in the ICA to determine the extent of cerebral perfusion from other sources (77)
• Blood flow velocity in the middle cerebral artery before and after ICA clamping (77)

However, the problem with all of the above is clarification of the level at which a change in a particular physiological variable, in an individual patient, reliably indicates inadequate perfusion, and therefore the need to insert a shunt. Some groups perform all carotid endarterectomies without a shunt, including those with contralateral occlusions, and claim good results (78). The argument for not using a shunt is that it may increase the number of intra-operative complications by damaging the intima of the ICA and CCA (79). However, the incidence of definite shunt related complications in the published literature is rare (79). At the other end of the spectrum are groups that always use a shunt (80), and who claim that familiarity with the procedure of shunting is safer than episodic use in tense situations.

Imaging of the endarterectomy zone prior to the restoration of flow can detect lesions that may give rise to intra-operative stroke, such as retained luminal thrombus or intimal flaps (80;81). Lingenfelter et al (81) examined the reliability of intra-operative arteriography, continuous-wave Doppler with audible interpretation of signals, and colour-flow duplex, in detection of intra-operative abnormalities. The sensitivity and specificity for each procedure was 66 and 96 percent, 16 and 98 percent, and 100 and 100 percent respectively, and they concluded that of the three methods assessed, only the colour-flow Duplex was sufficiently reliable for use in the clinical setting. Gaunt et al (80) examined angioscopy, B-mode
ultrasound, continuous-wave Doppler, in this setting and reported that angioscopy was technically the easiest and most reliable method of detecting intra-operative defects.

(d) Prevention of post-operative stroke

Thromboembolic ischaemic stroke remains the major complication of CEA, occurring most frequently in the first few hours post-operatively (82). Studies using TCD to detect cerebral embolisation during CEA have provided important insights into the pathogenesis of peri-operative cerebral ischaemia (72;73;83;84) and work aimed at post-operative detection of emboli has identified an association between frequent embolic signals and the development of post-operative stroke (73;85), (86).

Lennard et al have demonstrated that the administration of Dextran-40 to patients who exhibit more than 25 embolic signals in any 10 minute post-operative period, is effective in preventing post-operative carotid thrombosis and therefore, the associated stroke (85). All of the patients in this group had undergone intra-operative monitoring, and none of the patients that later embolised had a previously identified and uncorrected technical defect. The reason for the propensity of some patients to embolisation and subsequent stroke remains unknown at the present time.

Although the blanket use of post-operative monitoring can reduce the number of thrombotic post-operative strokes seen, it is labour intensive and relatively costly. It requires a TCD machine and a vascular technician to monitor each patient for three hours post-operatively. A further disadvantage with this system is that it only signals a warning when the thrombus has already begun to form in the carotid artery and is rapidly embolising. Patients found to
have carotid thrombus formation may suffer many hundreds of emboli before administered Dextran-40 is effective, and this obviously places them at risk of ischaemic, embolic stroke. Between 60 and 90 minutes are usually required before the Dextran-40 significantly reduces the embolus rate from a forming thrombus. A previous solution to this problem was thought to lie in routine administration of Dextran-40 to all patients undergoing CEA. Unfortunately, this was associated with a significant excess of peri-operative bleeding problems which negated any benefits achieved in terms of stroke risk reduction.

A potential solution to the first two problems outlined above is to identify those patients at risk of carotid thrombosis prior to surgery. This may enable the surgeon to administer anti-platelet or anti-coagulant therapy in a directed manner, to those patients at high risk of carotid thrombosis only. Thus, the identification of patients with pro-thrombotic tendencies or who demonstrate hypercoaguable states may not only be of relevance in the treatment of carotid disease, but may be applicable in the management of peripheral vascular disease, vascular access for dialysis and coronary artery disease.
References

Ref Type: Report


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CHAPTER THREE

THE COAGULATION SYSTEM
Haemostasis is a stringently regulated mechanism that under resting physiological conditions maintains normal blood flow and enables rapid coagulation in the event of localised tissue damage. This requires the delicate balance of four interconnected systems, namely the vascular endothelium, platelets, the coagulation system and fibrinolysis.

This chapter will provide a brief overview of normal coagulation and discuss the known defects within the system that can cause it to become overactive. Many of the effects of excessive coagulation are predominantly evident in the venous system, but evidence for the roles of these defects in arterial disease will be examined, which has direct relevance for carotid thrombosis.

3.1 Normal coagulation

The localised formation of a fibrin clot at the site of blood vessel injury is critical for the maintenance of vascular integrity. The conversion of soluble fibrinogen into stable, insoluble fibrin necessitates a series of complex and interrelated enzymatic reactions, involving plasma serine proteases and their co-factors.
Under normal conditions, the endothelium prevents thrombus formation by acting as a physical barrier between haemostatic and reactive subendothelial components, and by repelling platelets through its negative surface charge (figure 3.1). However, tissue damage leads to exposure of the endothelial basement membrane and the extracellular matrix that initiates a cascade of interlinked events in order to repair the defect. Collagen, von Willebrand factor and fibronectin encourage platelet adhesion to the injury site, and the area becomes prothrombotic in response to stimulation by circulating inflammatory cytokines including interleukin-1, tumour necrosis factor and interferon-γ. Tissue factor is up-regulated, which stimulates the coagulation pathway, but this is in turn regulated by the production of plasminogen activating factor which initiates fibrinolysis in an attempt to prevent excessive thrombotic activity. The complex nature by which these numerous factors and their inhibitors interact is obviously finely balanced in the physiological setting, but if disrupted in disease states, the potential exists for either over or under coagulation with significant repercussions.

Earlier this century, Schmidt and Morawtz proposed a system of coagulation involving four factors: fibrinogen, prothrombin, and calcium from the intra-vascular compartment, and tissue thromboplastin (also called tissue factor) from the perivascular tissues (1). Upon the disruption of a blood vessel, the exposure of the first three factors to the latter lead to the formation of solid clot. This is known as the extrinsic pathway.
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Inhibition of platelet aggregation

Nitric oxide and prostaglandin

Thrombomodulin Heparan sulphate

Anticoagulant surface

Negative charge

Endothelial damage

Exposure of collagen and von Willebrand factor

Platelet adhesion

Tissue factor

Coagulation pathway

Fibrin generation

↓ Thrombomodulin

Plasminogen activator inhibitor
It was also noted that blood placed into a container would also clot in the absence of tissue factor (TF), a process that appeared to be "intrinsic" to the blood. Initially, it was thought that the extrinsic system was by far the most important mechanism for initiating coagulation, but the discovery of defects in the intrinsic pathway referred to as haemophilia A and B altered this perception; tissue factor coagulation, as measured by the prothrombin time, is normal in haemophiliacs patients, whilst intrinsic clotting is grossly abnormal.

Approximately 30 years ago, the classical cascade of sequential activation of the clotting mechanism was first proposed (2). In this model, the intrinsic and extrinsic pathways converge at step activating factor X. Factor VII from plasma interacts with TF to directly activate factor X. The intrinsic pathway is activated when factor XII comes into contact with a negatively charged surface. This is referred to as contact activation and under normal circumstances, requires the presence of two additional plasma components, namely the serine protease prekallikrein and the non-enzymatic cofactor, high molecular weight kininogen. Activated factor XII activates factor XI, which in turn then activates factor IX. The combination of factor VIII and activated factor IX then activate factor X (Figure 3.2).
CHAPTER THREE: The Coagulation System

VII Tissue factor XI

Tissue factor pathway inhibitor

VIIa.TF

IXa + VIIa

Activated protein C

Protein s

Prothrombin

Phospholipid + Ca2+

IXa + VIIIa

Protein s

Thrombomodulin

XIIa

XIII

Prothrombin X IIIa

Antithrombin

Fibrinogen

Fibrin

Cross-linked fibrin

Vitamin K-dependent serine proteases

Anticoagulant system

Cofactors
Although this is a useful model it is somewhat simplistic and therefore not entirely representative of in vivo coagulation. Interaction must exist between the intrinsic and extrinsic pathways otherwise a deficiency of factor XII or its co-factors would lead to a severe bleeding diathesis and in reality it does not. Factor VIIa and TF are able to activate factor IX, and therefore the intrinsic system (3). However, a paradox is evident in that if the extrinsic system were truly able to stimulate both arms of the coagulation system, by what mechanism do haemophiliacs bleed? The discovery of the endogenous factor, tissue factor pathway inhibitor (TFPI), has altered the general interpretation of the coagulation system (4). Based on the properties of TFPI, it has been proposed that VIIa-TF initiates the coagulation, but that factors VIII and IX are absolutely required to sustain haemostasis.

3.2 Hypercoaguable states

The prothrombotic reactions involved in the clotting cascade are regulated by a number of inhibitory enzymes and cofactors under normal physiological conditions. Deficiencies of these regulators can result in inappropriate or excessive coagulation, and similarly, overproduction could, at least in theory, result in an increased tendency to bleed. It is possible that these hypercoaguable states resulting from a pathological defect in clotting regulation may have a role to play in the aetiology of carotid thrombosis, and the mechanism by which they arise are discussed below.
(a) Protein C

Under normal circumstances, the combination of thrombin and thrombomodulin activates the Vitamin K dependant plasma zymogen, protein C, which then binds to its co-factor, protein S (5). This complex assembles on membrane surfaces in a calcium dependent reaction, which renders it stable. Once this has occurred, it proceeds to inactivate factors Va and VIIIa (5) and this has been proven to be a critical negative regulatory mechanism, since total deficiency of protein C leads to neonatal death (6). Furthermore, patients who exhibit the heterogeneous phenotype for thrombomodulin, have a notable predisposition to venous thrombotic events (7).

Deficiencies in the protein C pathway are present as a genetic defect in about one in 200 unselected patients (8) although protein C deficiency can also be acquired as a result of liver disease, disseminated intra-vascular coagulation, carcinomatosis and surgery (9). The evidence for protein C as a defensive mechanism against venous thrombosis is clear, although conflicting reports exist regarding its role in arterial disease. (7;10-12). Evidence to suggest a protective role often involve thrombosis in the post-surgical setting, whereas communications purporting the absence of any protective role for protein C are largely concerned with spontaneous events.
(b) Activated Protein C resistance

One of the fundamental reactions in coagulation is the conversion of prothrombin into thrombin by the complex of factors Va, Xa and calcium ions. Factor V activation increases the rate of prothrombin activation by 1000-fold (13), and it stabilises factor Xa (14). Thus, failure to inactivate Va will lead to a prothrombotic state. As stated above, Va is normally inactivated by the protein C complex, but a mutation in the DNA sequence for factor V can lead the rate at which it is inactivated being greatly reduced. The most common mutation is the conversion of Arginine $506$ to a Glutamine mutation, referred to as Factor V Leiden. This site is the principle cleavage site whereby protein C inactivates Va, and the presence of a mutation here inhibits the cleavage process (15). Factor V Leiden is therefore able to exert its normal pro-coagulant activity, and this is known as activated protein C resistance.

This mutation is very common in the Caucasian population, with a 1 to 15 percent prevalence (16) and those patients who are heterozygous for it have a six-fold increase in their risk for venous thromboembolism (17), whilst homozygotes have a 30- to 140- fold increase in risk (17;18). Since this is a common defect it is often seen in conjunction with other anti-coagulant defects, and these patients have a significantly increased propensity for thromboembolic disorders (16). The evidence to suggest a role for Factor V Leiden in the pathogenesis of arterial thrombosis is extremely limited, with most large studies demonstrating no association (19). However, one study does deviate markedly from the others in reporting a 15-fold increase in the rate of TIAs seen in patients who are heterozygote for Factor V Leiden when compared to a control group (19).
However, this study did not demonstrably prove that the cause of TIA was arterial in nature, rather than from cardiac or venous sources, such as a patent foramen ovale for example.

(c) Protein S

Protein S is a vitamin K dependent zymogen produced primarily by hepatocytes, but which is also found in endothelial cells and platelets (20). The inactivation of Va is a biphasic reaction with rapid cleavage at Arginine_{506} followed by a further slower cleavage at Arginine_{306} (21). Full inactivation occurs only after both steps are completed. Protein S is known to increase the rate at which the second cleavage occurs by 20-fold (22), and is also involved in the inactivation of VIIIa by means of a similar mechanism (23). There is also evidence that protein S is able to bind to and therefore reduce the rate of activation of factors VIII and X (24). A deficiency in protein S production or activity therefore has direct repercussions for the normal haemostatic equilibrium and favours a prothrombotic state.

The prevalence of PS deficiency appears to lie between those of protein C and Factor V Leiden at between 1 and 5 percent (25-28) in European populations. In heterozygote individuals, the risk of a thrombotic event before age 45 is 50 percent (29) but unlike protein C and Factor V Leiden, protein S deficiency appears to have a clear role in the pathogenesis of arterial thrombosis (30;31). In a French series, 13.5 percent of patients heterozygote for defective or deficient protein S experienced either stroke, myocardial infarction, or peripheral arterial thrombosis before the age of 46 years (32;33), and in
two further studies, the rate of arterial thrombosis in such patients was between 5 and 10 percent (29;34). However, the data from two further cohort studies failed to confirm this relationship (19), rendering the definitive implications of protein S deficiency somewhat unclear.

(d) Tissue Factor Pathway Inhibitor

Tissue Factor Pathway Inhibitor (TFPI) directly inhibits Xa and is a potent inhibitor of TF-VIIa complexes on either cell surfaces or in the subendothelial tissues (14). There is some evidence from animal models that it may exert a protective effect against venous thrombosis (35) and prevent re-thrombosis after successful thrombolysis of arterial thromboses (36). The anti-coagulants molecules discussed previously all circulate as free intravascular proteins, allowing measurement of their relative plasma concentrations to be taken as a reflection of activity. In contrast, most of the body's TFPI is bound to the vessel wall, with some bound to LDL, with only a small fraction in the region of ten percent circulating as free protein (37)(14). This has direct implications for measuring TFPI activity on a large and reproducible scale, and therefore, little success has been achieved to date in assessing the contribution of TFPI deficiency to the pathogenesis of hypercoaguabable states.

(e) Antithrombin III

The major physiological role performed by antithrombin III is the inhibition of thrombin activity, although it is also able to inhibit Xa, IXa and XIa to a lesser extent. In addition to this, it is capable of promoting dissociation of the TF-VIIa complex and of inhibiting its re-association (38). The mechanism of thrombin inhibition is a two-step
process. Firstly, it binds to the active site on the antithrombin III molecule and in doing so, initiates a conformational change in the antithrombin structure, trapping and inhibiting the action of thrombin (39). Heparin promotes thrombin inactivation by providing a binding site for the antithrombin III and thrombin, and this results in an acceleration in binding rates of 2000-fold (40).

Antithrombin deficiency is relatively uncommon, being reported in only 0.5 to 1.1 percent of European populations (41;42). Although it is less common than other anti-coagulant deficiencies, it’s effects seem to be more severe in terms of the number of venous thrombotic episodes seen (43) and the young age of first onset (44). Despite this, only occasional reports have emerged to suggest an association between antithrombin deficiency and arterial thrombosis, and most cohort studies do not support a role for antithrombin III in this setting (19).

(f) Antiphospholipid antibodies

This term refers to a heterogeneous group of antibody-mediated disorders in which both allo- and autoimmune immunoglobulins have been implicated. The autoimmune category of disorders can be idiopathic or in nature, or arise secondary to systemic lupus erythematosus and other connective tissue disorders. Disorders resulting from alloimmune antibodies are often only transient and are mediated by a variety of infectious agents and a number of malignancies which usually haematological in origin (45).
The first member of the anti-phospholipid antibody class to be described was the anti-cardiolipin antibody, which is detected in blood by a standard ELISA technique (46). Lupus anti-coagulant was later identified by its ability to interfere with one or more of the phospholipids-dependent in vitro coagulation tests such as the APTT ratio (47). However, the term lupus anticoagulant is a misnomer since many of the patients with this antibody have neither SLE nor a clinical state of anti-coagulation. Although all classes of antibody are associated with APA, it is the IgG isotype that is most commonly seen in prothrombotic states (48). In particular, a polymorphism of an IgG$_2$ subtype has been associated with platelet and endothelial cell activation (49).

The spectrum of antigenic targets against which these antibodies are directed is diverse. Although referred to as anti-phospholipid antibodies, the antibody is targeted against a protein molecule bound to a phospholipid rich surface in most cases. Anti-cardiolipin antibody targets include $\beta_2$-glycoprotein I (50) which has a number of anti-coagulant functions including inhibition of the contact phase of coagulation and a time-dependent inhibition of the prothrombinase complex (51). Other candidate proteins that bind to phospholipid are protein C and S, factor X and Va, high molecular weight kininogen. Approximately, 70 percent of the thrombotic events occurring in conjunction with the presence of antiphospholipid antibodies are venous, with the remainder arising in the arterial tree, and recurrent thromboses usually occurring in the same side of the circulation as previously (45). Patients with antiphospholipid antibodies have increased levels of coagulation system activation markers including fibrinopeptide A and prothrombin fragments 1 and 2 in their blood when compared to control groups (52) and the 5% of the population with the highest levels of antiphospholipid antibodies have a 5.3-fold increase in
their risk of deep venous thrombosis or pulmonary embolism (53). Similarly, the top quartile of this group carry a two-fold increase in their risk of myocardial infarction. In a recent study, 44 percent of young patients with cerebral ischaemia of undetermined origin were found to be positive for one or more antiphospholipid antibodies (54) and although the prevalence in the general population is approximately 5 percent, the wide variation in the sensitivities of different assay techniques used to identify these antibodies makes an accurate assessment of their true prevalence difficult.

3.3 Fibrinogen and fibrinolysis

The primary platelet clot formed in response to endothelial injury is strengthened by a series of reactions culminating in the formation of fibrin and the cross-linking of its polymers. This is also mediated by factor XIIIa and signals the final stages of the clotting pathway. This process is counterbalanced by fibrinolysis, which is a prerequisite for normal haemostasis, as revealed by the prothrombotic tendency of patients exhibiting a plasminogen deficiency. Tissue plasminogen activator (tPA) is released from endothelial cells and converts plasminogen into plasmin, which in turn hydrolyses arginine and lysine bonds. This results in the proteolysis of various substrates including fibrinogen, fibrin, and factors V, VIII, and XII. Fibrin and fibrinogen cleavage generates several fragments which inhibit both thrombin and fibrin polymerisation (figure 3.3). Evidently, careful regulation of the factors and cofactors implicated in this process is again required in order to prevent either excessive fibrinolysis and a structurally ineffective clot, or over-production of fibrin and the creation of a prothrombotic state.
Cytokines, adrenaline, thrombin, venous occlusion, exercise, vasodilators

<table>
<thead>
<tr>
<th>Tissue plasminogen activator</th>
<th>Tissue plasminogen activator-fibrin</th>
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<tbody>
<tr>
<td>Urokinase</td>
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<tr>
<td>Plasminogen</td>
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<tr>
<td>Activated protein C</td>
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Plasmin

Fibrin

Fragment X

Fragments Y + D

Fragments E + D
The degradation of cross-linked fibrin generates the production of several specific and measurable marker fragment, one of the best characterised being the *D-dimer* fragment.

Elevated levels of this protein fragment are indicative of active coagulation and the formation and degradation of intra-vascular thrombus. Two studies to date have indicated a link between elevated D-dimer levels and increased risk of future coronary artery thrombosis (55,56).

Fibrinogen degradation products may illustrate the rate of ongoing fibrin turnover, but they do not indicate the potential of the fibrinolytic system to overcome the coagulation pathway. Clearly, a reduced ratio of thrombus degradation to clot formation increases thrombotic potential in any given individual and impaired fibrinolysis has already been demonstrated to increase the future risk of stroke events and myocardial infarctions in male patients (57,58). As illustrated, fibrinolysis represents a stringent balance between tissue plasminogen activator activity and that of its primary inhibitor, plasminogen activator inhibitor-1 (59). Tissue plasminogen activator levels appear to correlate more significantly with the risk of arterial thrombosis than plasminogen activator inhibitor-1 (60), with elevated levels of tPA showing a significant associated with an increased risk of subsequent stroke in the recent US physicians health study (58).
Data from a number of large studies including the Gothenberg Heart study (61), the Northwick Park (55) and the Framingham Heart study (56) have all indicated that elevated fibrinogen levels are a positive risk factor for coronary artery thrombosis, and fibrinogen is also thought to be implicated in the risk factor profile for peripheral artery disease (62). Although these studies identify fibrinogen as a risk factor only, and not a cause of arterial thrombosis, the association of fibrinogen with arterial thrombosis is plausible. Increasing levels of fibrinogen may increase platelet aggregation, and the volume and rigidity of fibrin thrombi, as well as resulting in a reduction in the susceptibility of thrombi to lysis (63;64). Furthermore, fibrinogen also plays a major role in determining the plasma viscosity, which has additional implications for thrombotic potential.

3.4 Summary

A fully functioning coagulation system is essential for the maintenance of haemostasis under normal physiological conditions. Hypercoaguable states are positively associated with increased venous thrombosis rates, although the evidence to suggest a role for these conditions in arterial disease is less convincing. However, the diverse family of antiphospholipid antibodies may contain subgroups that predispose to arterial complications, and this remains an area of investigation that is open to further debate at present.
In terms of thrombosis after carotid endarterectomy, either a deficiency in, or abnormal form of any of the anticoagulant factors discussed may be involved. Proteins C and S, and anti-thrombin III all require binding to the endothelium in order to exert their optimum effect and as the endothelium is removed during CEA, the assumption is that they cannot be implicated in this setting. Excessive expression of tissue factor in the newly exposed sub-endothelium, or lack of tissue factor pathway inhibitor, could pose additional risk factors for the development of post-CEA thrombosis, and as indicated previously, alterations in the fine balance of fibrin production and degradation may feasibly play a vital role in predicting the occurrence of post-operative carotid thrombosis. It is possible that a relative failure of the fibrinolytic system to overcome the prothrombotic state created by surgery may be present in those who suffer this complication.
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References


CHAPTER FOUR

PLATELETS
4.1 Platelet physiology

Large cells known as megakaryocytes are the precursors of platelets and are found primarily in the bone marrow, where they constitute around 0.5 percent of the cells. Mature megakaryocytes have a typical morphology, combining large size and abundant cytoplasm with numerous granules and a distinctive demarcation membrane system (1). A characteristic of megakaryocytes is their polyploidy and their ability to undergo endomitosis and they can split into 64 portions, although 16 or 32 segments are more commonly seen (2). Megakaryocytopoiesis is regulated by multiple cytokines, most notably thrombopoietin (3), although the precise mechanism underlying this remains open to debate. It has been suggested that the entire megakaryocyte is shed into the circulation after production and reaches the pulmonary circulation where it fragments into platelets. Alternatively, a second theory proposes that platelets are shed gradually into the blood stream from megakaryocytes lying adjacent to the endothelium.

Platelets comprise an integral component of haemostasis and under normal circumstances circulate in close proximity to the endothelial cell wall. Adhesion to it is prevented by high local concentrations of prostaglandin and nitric oxide, although adherence is rapid in the presence of endothelial disruption. Following this, von Willebrand factor previously secreted into the subendothelium binds to extracellular collagen via the A3 domain and platelet adherence occurs when platelet glycoprotein GpIb-IX-V complex binds to the A1 domain of von Willebrand factor. The
CHAPTER FOUR: Platelets

The multimeric nature of this factor and the increased local concentration of A1 binding sites potentiates adhesion. During this phase, platelets are activated and the GpIIb-IIIa receptor subsequently undergoes conformational change that allows it to bind to both vWF and fibrinogen, resulting in irreversible platelet adhesion and aggregation.

Platelet activation is caused by the binding to specific receptors of a variety of agonists including thrombin, thromboxane A2, ADP, collagen, and arachidonic acid. Signal transduction is mediated by G-proteins and intracellular cAMP, increased concentrations of which inhibit platelet adhesion, aggregation, and release. Following platelet activation, adenylate cyclase activity is reduced and cAMP levels therefore fall. Platelet activation also results in structural changes; the smooth biconcave disc morphology is replaced by a spherical form with protuberant pseudopodia. Granules migrate centrally and contraction of the cytoskeletal contractile apparatus results in clot contraction and promotion of platelet plug formation.

4.2 Platelets and atherosclerosis

The understanding of the aetiology and pathogenesis of atherosclerosis has undergone radical change during recent years. The mainstay of the theory of pathogenesis was based upon the Rokitansky-Duguid theory, which implicates thrombus formation as the primary event, with subsequent organisation and overgrowth of endothelium (4). First introduced by Rokitansky in 1852, this “encrustation theory” was
dismissed by Virchow, who held that atherosclerosis was caused by proliferation of intimal connective tissue proliferation, or inflammation (5). Although these pioneers had yet to establish the definitive pathophysiology that underlies arterial disease and results in such devastating sequelae as myocardial infarction, stroke, and limb amputation, Virchow secured his place in the literature with his ‘triad’ concept. This cites changes in the vessel wall, changes in blood flow, and changes in blood composition as possible candidates in the initiation of atherosclerosis. Although these factors are accepted as playing a fundamental role in the pathogenesis of this condition, further variables are now known to be implicated and include local shear forces, clotting factors, fibrinolysis, platelets, and vessel wall constituents.

The presence of high local shear forces results in platelet aggregation (6) and alterations to both the biochemical and physical environment, including the initiation of radical conformational and functional changes to proteins within the vicinity (7). Shear forces are considered to represent a true risk factor for atherosclerosis when found in the presence of hypertension, and are important determinants for the localisation of the atherosclerotic process, and for sites of thrombosis.
4.3 Pathogenesis of arterial thrombosis

In the course of atherogenesis, endothelial cells undergo degenerative changes resulting in endothelial gaps that inevitably expose the subendothelium to the circulation or alternatively, can detach, paving the way for adhesion of platelets and white cells. Primarily, this involves the attachment of von Willebrand factor (vWF), to the endothelial defect, which then undergoes a series of conformational changes to become an attractive ligand for the platelet glycoprotein (GP) Ib-IX and GP IIb-IIIa complexes. This results in platelet adhesion and aggregation (8). The process of adherence activates platelets and they then undergo a morphological change, synthesise thromboxane A2, secrete ADP from dense granules, and release fibrinogen, vWF, and platelet derived growth factor from alpha-granules as a result. These events generate also an attractive phospholipid surface for assembling clotting factors at the site of the endothelial defect. This is achieved primarily by displacing phosphatidlyserine from the inside to the outside of the platelet membrane (9) and is described as 'flip-flopping' of the platelet membrane. Its net result is to induce a series of internal conformational changes within the platelet, in addition to increasing the adhesive properties of the external platelet membrane.
The primary arterial thrombus subsequently proceeds into a secondary phase by the formation of thrombin and a fibrin network within it. Initiation of the coagulation cascade occurs by either tissue factor-VIIa or the factor XI-IX pathway and occurs as a consequence of the tissue factor produced by incorporated, activated monocytes and platelets being presented on the flip-flopped platelet membrane. The platelet-fibrin network that is subsequently formed entraps red blood cells and is capable of obstructing the vessel lumen, resulting in distal tissue infarction. An increased consumption of platelets and evidence of activated coagulation in atherothrombotic individuals (10-12) are the natural consequences of these events, and the coagulation process progressing within the thrombus can continue for several hours. This progression cannot be halted by indirect anticoagulants such as heparin but importantly, thrombin activity may be quenched by direct-acting inhibitors including hirudin. If such inhibitors are administered, particularly within the first hour, the thrombus dissolves rapidly due to shear forces, fibrinolysis, or both. However, without such intervention, dissolution may take several hours, or alternatively, the thrombus becomes permanent (13). This would imply that in this setting, the effects of the natural anticoagulant systems are insufficient.

Remaining thrombi are involved in atherogenesis either by the effect of a variety of white cell or platelet derived cytokines or mitogens including IL-6, PDGF, and thrombin. These agents induce chemotaxis and proliferation of smooth muscle cells by becoming incorporated into the vessel wall in the Rokitansky-Duguid sense. The smooth muscle cells subsequently synthesise tissue factor, collagens, elastin, glucosaminoglycans (GAGs), and other connective tissue components. Monocytes
that are either incorporated with the thrombus, or to it attracted by various chemokines develop into macrophages that also express tissue factors, engulf lipids and ultimately become foam cells (5;14).

4.4 The role of thrombin in primary thrombus formation

Arterial thrombosis and haemostasis mirror each other in several respects, notably in that both processes occur under high shear stress. It is therefore reasonable to assume that the mechanisms by which haemostasis occur may also be relevant in arterial thrombus formation. By the late 1950s, research into the process of haemostasis had revealed that thrombin formation is not necessary for the primary arrest of bleeding and is apparent in that primary bleeding time remains within normal limits under the following circumstances:

- Severe deficiencies of fibrinogen or factor IX, VIII, V or VII.
- The Scott syndrome, whereby platelets lack the ability to undergo the flip-flop mechanism and therefore, are unable to provide an acceptable surface for thrombin formation (15;16).
- After infusion of active site inactivated factor VIIai into baboons, which quenches factor VIIa in subnanomolar concentrations (17;18).
- With the administration of r-hirudin, a direct thrombin inhibitor, at therapeutic concentrations (19).
As a result of these observations and work of their own, Luscher and Weber (20) proposed that the ability to achieve haemostasis in the absence of thrombin is due to the fact that so many platelets accumulate at the wound margins in a matter of milliseconds that there is no significant time available for thrombin formation.

There is also credible evidence to suggest that thrombin and fibrin formation is not a prerequisite in the process of primary arterial thrombosis. Weiss et al. (21) used thrombopenic blood and subendothelial arterial tissue to demonstrate that platelet adhesion is necessary for fibrin formation. They also revealed that Scott syndrome platelets, despite normal adhesion but deficient in the ability to support coagulation, were inactive in this scenario, and furthermore, that platelet thrombus formation was normal in afibrinogenemia. Ex vivo perfusion experiments at different shear rates with large concentrations of heparin and with direct thrombin inhibitors have shown that complete inhibition of fibrin deposition had no impact on platelet thrombus formation at high shear (22), although some inhibition was observed at low arterial shear (23). Further studies appear to support this conclusion: recombinant tick anticoagulant peptide (rTAT), a selective FXa inhibitor, exerted no effect on collagen-driven thrombogenesis at high arterial shear (24), and specific thrombin inhibitor had no effect on thrombogenesis on rabbit subendothelium at arterial shear (22).

Investigation of thrombogenesis using human fibrillar collagen and applying active site inactivated F IXa (F IXai) and F VIIai as inhibitors, revealed that whereas the former inhibited both fibrin deposition and fibrinopeptide A formation without affecting platelet deposition, the latter had no effect (25). This study was performed at venous shear rate but indicates that thrombin may form independently of the TF-
FVIIa pathway. The authors even showed that fibrin fibres emanated from single platelets or small aggregates. The observation of arterial thrombosis in an afibrinogenenic patient (26) would also indicate that the coagulation system has only a minor role to play in primary arterial haemostasis and thrombosis.

Despite this, recent in vivo studies of thrombogenesis in porcine carotid arteries demonstrate that minute amounts of thrombin are formed well before fibrin formation and irreversible platelet aggregation can be recognised. If this thrombin is quenched within 1 or 2 hours, by the use of direct acting anticoagulants, it will resolve (13), presumably due to the effects of local shear forces. These results may relate to studies of wound bleeding times (27), which showed that early thrombin formation occurs independently of platelets and appears to be tissue factor-factor VIIa driven. Whether or not this is also the case in the porcine studies discussed is unknown, but could be solved by the use of factor VIIa or tissue factor monoclonal antibodies. However, these very specific and highly active inhibitors do not prolong the bleeding time in baboons (17), which indicates that thrombin from this source is not involved in primary haemostasis. The contradictory nature of these findings suggests that further work is required to provide a definitive answer to this question.
4.5 The role of thrombin in secondary thrombus formation

Thrombin-fibrin formation is in most instances required for the progression of a primary arterial thrombus. This is evident, not only from the histological evidence of the presence of a red thrombus in the majority of infarcts, but also from the preventive effect of anticoagulants. However, thrombin formation is probably equally important in the recruitment and activation of platelets for thrombus growth in its role as a vital platelet agonist. Although it may enter the stage after the platelets in the process of thrombus formation, its importance is undisputed as a determinant of clinical outcome.

After the first molecules of thrombin have formed, either via the intrinsic or the extrinsic system, sustained production depends upon two factors:

(a) Access to a suitable surface for steric arrangement of the clotting factors.

(b) Tissue factor-synthesising cells that are themselves provided with such a surface.

In the first instance activated platelets, either as a result of the adhesion process or by thrombin, provide this surface through flip-flopping of the membrane. In the second instance, white cells, particularly activated monocytes, produce tissue factor, and are entrapped in between the platelets and provide igniting islands for thrombin formation. It is clear therefore that thrombin formation is dependent upon the
presence of both platelets and white cells, which again testifies to the significance of the role of platelets in this process. Patients with platelets lacking the ability to provide such a surface have an increased tendency to bleed, and should theoretically exhibit resistance to fatal thrombosis, although their platelets adhere and aggregate normally and they have a normal bleeding time (15;16). The fact that induction of intravascular coagulation is possible by an infusion of factor Xa requires a concomitant infusion of phospholipids, such as a 3:1 molar mixture of phosphatidylcholine and phosphatidylserine provides further proof that such a surface is not normally available in the circulation.

Inherent mechanisms exist to temper the activity of thrombin in order to avoid catastrophic clotting. One is the site restriction inherent in the immobilisation of platelets and white cells at the site of vessel damage, and further examples include specific inhibitor systems such as the Antithrombin III-GAG system, the Protein-Protein S system, the PCa-FV-inactivation mechanism, and a number of direct feedback reactions (28). Similarly, the effects of abnormal platelet regulation could be equally catastrophic and physiological control is achieved by vessel wall constituents and inhibitory agents including nitrogen oxide (NO), prostacyclin (PGI2), ADP-ase and Annexin V (29-32). At present, the relative importance of these factors in the pathogenesis of arterial and venous thrombosis remains unknown.
It should also be remembered that fibrin formation is not entirely necessary for normal haemostasis, as illustrated by the fact that patients with complete afibrinogenemia may not only develop arterial thrombosis, but are also able to sustain a reasonable life for decades (26), whereas the chance of survival for an individual lacking platelets is zero.

4.6 Extravascular coagulation

The fact that fibrin and fibrin-derived components are evident in plaques does not prove the role of thrombus encrustation in atherogenesis. It is known that all major plasma proteins are present in the intima and subintima, and that fibrinogen, fibrin, fibrinolytic enzymes, fibrin degradation products, prothrombin, and antithrombin are all present in plaques (33;34). As discussed, thrombus inhibition is increased in areas with damaged or detached endothelium (35), and also, free thrombin is found (33), in this scenario, having been either formed in loco or freed from clots by lysis, indicating that binding by AT III or other thrombin-neutralising compounds is insufficient. As the plaques also contain a number of white cells, including activated macrophages with expressed TF and a series of enzymes that may activate the FXII pathway, the stage is set for extravascular fibrin formation and degradation. It is possible that this is a further source of fibrin, equal to that of thrombus encrustation, but at present the impact of this source of fibrin in atherogenesis remains to be established.
4.7 The encrustation theory

During early studies in this area, the presence of fibrin in plaques was assumed to indicate encrustation of a clot and investigators failed to recognise that the presence of platelets, or their remnants is necessary for such a conclusion to be drawn. However, later studies using platelet markers have in fact verified that encrustation does indeed occur and is actually rather common (36). One investigation revealed that encrustation occurs in the majority of raised aortic plaques and in approximately one third of coronary plaques (37). Consequently, the Rokitansky-Duguid theory remains valid, but whether thrombus incorporation has implications for plaque development alone, or is also implicated in the progression of atherosclerosis in general remains to be determined. As with the fibrin derived from extravascular coagulation, it is unclear what role encrusted thrombi play in atherothrombogenesis in comparison to non-hemostatic risk factors. Despite this, it is now clear that encrustation occurs secondary to the factors that cause the initial endothelial damage; with undisturbed endothelium, no significant arterial thrombi and therefore no encrustation will occur.
The platelet-von Willebrand factor axis in atherothrombosis

It is well known that the coagulation cascade, von Willebrand factor (vWF), and platelets are all pivotal in thrombogenesis, as demonstrated by a host of sources (8). In contrast, a full understanding of their role as risk factors in atherosclerosis remains incomplete. Several studies conducted on patients with angina post infarction revealed increased levels of vWF, fibrinogen, and platelet activation in these individuals. However, reliable data regarding the implications of this in terms of risk assessment can be generated in prospective studies only. To date, existing evidence does not support a risk factor role for vWF. Until recently there has been no direct clinical evidence for altered platelet function as an atherothrombogenic risk factor. It had been assumed that platelets played a crucial role in atherothrombosis, which is a commonly used term to indicate the close connection between atherosclerosis and arterial thrombosis in terms of pathogenesis. This is due to a number of factors:

- The abundant presence of platelets in arterial thrombus.
- The established role of platelet-secreted products in smooth muscle proliferation.
- The presence of platelet remnants in plaques.

It would appear that vWF and platelets are entirely mutually dependent in atherothrombogenesis; where high shear prevails, vWF represents the necessary glue for platelet attachment to the vessel wall and for platelet cohesion (8).
However, that these components also exert independent effects is clearly demonstrated by the fact that the vessel wall is normal in even severe von Willebrand disease (vWD), whereas it begins to leak when platelet numbers approach one-fifth of normal. This would indicate that endothelial nurturing is taken care of by the platelets alone. However, in spite of its importance for repair, and possibly also in atherogenesis, this function of platelets remains poorly understood. A further example of individual activity of platelets and vWFF has emerged and suggests that platelet function and platelet mass are indeed true atherothrombotic risk factors. In a prospective study (38) designed primarily to evaluate the development of coronary heart disease (CHD), 2014 healthy male patients aged between 40 and 59 were recruited between 1973 and 1975. A broad selection of investigations related to CHD were performed as baseline evaluation and among these, platelet counts were performed in 487 patients, and in 150 of these aggregation studies were conducted in platelet-rich plasma. ADP, adrenalin and collagen were administered as platelet agonists. Data was collected after 13.5 years for CHD and total mortality, and showed that CHD mortality was five times higher in the 50 percent fastest aggregation rate when compared to the 50 percent slowest rate cohort and similar results were observed when the lag time was the parameter. When the platelet count was the parameter, cardiac mortality was almost three times higher in the upper quartile than in the lowest. This could indicate that platelet function is the dominant factor, rather than mass. The impact of platelets becomes even more evident when compared to that of the most common risk factors: platelet count was associated with the highest relative risk with the lowest p-value. Therefore, alterations in platelet function with or
without changes in platelet mass are of clearly notable importance in clinical atherothrombogenesis.

Interestingly, neither adrenalin nor collagen induced aggregation were related to CHD or total mortality. This could indicate that ADP plays a greater role, although the choice of agonist concentrations, or other technical reasons could be explanatory. The lack of effect of these other agonists may also indicate that the influence of variation in fibrinogen level and platelet number was insignificant for the ADP results. With the low shear forces in the applied method (Born method) vWF has no influence on aggregation and the outcome is therefore wholly representative of the reactivity of platelets to ADP.

4.9 Platelets and ADP

Adenosine diphosphate (ADP) has a number of important roles in platelet function. A study of a platelet storage pool deficiency indicated that this was due to a failure of ADP signalling (39) and ADP is also known to be important in platelet aggregation induced by high shear flow rates (40). This recently recognised type of aggregation is probably implicated in the development of atherosclerosis, where very high shear flow rates are often detected. This mode of aggregation is not affected by aspirin, but is affected by ADP-removing enzymes. ADP plays a crucial role in maintaining the active flip-flopped platelet membrane by sustaining intra-cellular calcium at elevated levels (41). It is also pivotal in the expression and release of vWF, thus further
promoting platelet adhesion (42). Fortunately, ADP also has some anti-thrombotic properties and these are induced by the release of nitric oxide and prostaglandin $I_2$.

### 4.10 Platelets and arterial surgery

Since platelets adhere to sites of endothelial damage it is unsurprising that $^{111}$-In labelled platelets can be found adhering to angioplasty and endarterectomy sites, and also to vascular grafts inserted into the arterial circulation (43). Indeed, even the small amount of suture that is exposed to the lumen of an arterial anastomosis acts as a stimulus to platelet adherence (44). As well as platelets adhering to the endarterectomy zone of a CEA they adhere to the patch angioplasty, regardless of whether the patch is made of autologous vein, Dacron or PTFE (45). Application of arterial clamps also leads to platelet deposition very shortly after flow restoration (46). Laboratory analysis of vascular grafts that had subsequently failed demonstrated the presence of a platelet rich thrombus as the cause of the failure in the majority of cases (47). It is clear from this evidence that platelets often play a major role in the development of complications following arterial surgery. The prevention of these complications will be discussed in the following chapter.
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CHAPTER FOUR: Platelets


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CHAPTER FIVE

ANTI-PLATELET THERAPY
5.1 Aspirin

Acetylsalicylic acid, or aspirin, is the acetic acid ester of salicylic acid and was synthesised by Felix Hoffmann at the Bayer Company in 1897. At that time and during the following 50 years, aspirin was solely used as an anti-inflammatory, antipyretic, and analgesic agent. An increased bleeding tendency with the administration of aspirin has been recognised for more than 40 years and had previously been considered an unwanted side effect (1) but this opinion has changed considerably. Today, it is the inhibition of platelet function by aspirin that qualifies the compound for prophylaxis and treatment of thrombotic vessel occlusions, specifically stroke, angina pectoris and myocardial infarction, and aspirin has become the "gold standard" medication for these indications.

5.1.1 Prostaglandin synthesis

In 1967 Quick (2) reported that aspirin but not salicylate prolonged bleeding times. However, due to the fact that only marginal effects on prothrombin time were seen with aspirin administration, it was proposed that nonplasmatic factors may have contributed to, or even caused the prolongation of bleeding times. At about the same time, several reports were published that suggested a role for aspirin as a potent inhibitor of platelet aggregation after oral administration. This anti-platelet effect was detectable within 2 hours of drug ingestion and could be demonstrated for between 5 and 6 days post drug withdrawal (3;4). In addition to this, it was also demonstrated that the inhibition of platelet function required an intact aspirin molecule, and was independent of the plasma level of the substance.
Moreover, "subclinical" oral doses of 150 mg aspirin but not salicylate were sufficient to achieve complete inhibition of platelet aggregation (4).

In 1971, Vane detected that aspirin inhibits prostaglandin biosynthesis and proposed the concept that this mechanism explains the analgesic and anti-inflammatory action of the compound (5). Another paper from the same laboratory by Smith and Willis implicates the inhibition of prostaglandin synthesis in platelets in the mechanism of the anti-platelet action of aspirin (6). In 1975, it was shown that aspirin inhibits the fatty acid cyclo-oxygenase (COX) by acetylation of a functionally important group ("active site") within the COX molecule, and furthermore that this action was irreversible in platelets (7). Later work suggested that this functionally important group was the amino acid serine at position 520 or 530 (8).

Aspirin and indomethacin, but not salicylate, cause the inhibition of ADP-induced platelet aggregation and thromboxane formation. Aspirin does not completely prevent platelet aggregation, and this is evident in the fact that initial platelet aggregation subsequent to activation of the platelet fibrinogen GPIIb/IIIa receptor is not directly modified by aspirin. Prevention of thromboxane generation by aspirin only eliminates an amplification mechanism of activation of the fibrinogen receptor, which eventually results in an impairment of platelet-platelet interactions. Under physiological concentrations of calcium (Ca^{2+}), there is markedly reduced thromboxane biosynthesis subsequent to stimulation by ADP, and no irreversible aggregation subsequent to ADP (9). Therefore, it is evident that aspirin is not a direct inhibitor of platelet aggregation, but solely an inhibitor of the thromboxane-dependent amplification mechanism. This is clearly demonstrable in Ca^{2+}-
depleted medium or citrated plasma with concentrations of between 40 and 60 µM, when compared to the physiological Ca\textsuperscript{2+} levels of between 2 and 3 mM in circulating blood. The pharmacological consequence of these findings was the design and development of new compounds that acted more specifically on platelet-dependent thromboxane formation, and at the same time did not inhibit the endothelial cyclooxygenase activity that would prevent vascular prostacyclin formation. Selective inhibitors of thromboxane biosynthesis (dazoxiben), thromboxane receptor blockers (daltroban), and combined mode agents (ridogrel) were synthesised and tested in clinical trials. However, the results were not encouraging and none of the compounds was found to be significantly superior to aspirin in the prevention of acute thromboembolic events. In contrast, the clinical consequence was the design of numerous trials that compared healthy subjects to patients suffering from atherosclerotic vessel disease, the objective being to determine whether low-dose aspirin of 100 mg per day was as effective as high-dose aspirin treatment in the prevention of thromboembolic events. This culminated in the report of the Antiplatelet Trialists’ Collaboration (10), which suggested that any kind of antiplatelet therapy reduced the risk of acute vascular thromboembolic events by between 25 and 30 percent, with aspirin emerging as the most intensively studied compound.

Fresh insights into the molecular mechanisms of the action of aspirin came with the introduction of novel molecular biology techniques. Recent work by William Smith and colleagues demonstrated that the concept of “active site” acetylation at Ser530 was incomplete and failed to explain the inhibition of the catalytic function of the COX enzyme by aspirin. It was found that acetylation of Ser530 did indeed result in inactivation of the
enzyme, but that this serine position was not essential for its catalytic function. Replacement of Ser530 by Ala530, that is, an amino acid without an acetylation site, did not modify the catalytic activity of the COX enzyme, but prevented the inhibitory action of aspirin (11) and related compounds. This resulted in the concept of a steric hindrance of COX activity by aspirin, whereby aspirin prevents the enzyme (COX)-substrate (arachidonic acid) interaction after binding to Ser530, which is not in fact the active centre of the enzyme. The group of catalytic importance was identified as tyrosine 385 (12), and after elucidation of the crystal structure of COX by Garavito's group (13), a steric model of the molecular action of aspirin was proposed (14). Tyr385 is located at the end of a tunnel in the COX molecule through which the substrate, arachidonic acid, has to pass to reach the catalytic centre. Covalent binding of aspirin to Ser530 located in a narrow part of this channel inhibits the access of arachidonic acid to the catalytic site, eventually resulting in an irreversible inhibition of enzyme activity (14).

5.1.2 Cyclooxygenase isoforms

There are two isoforms of the COX enzyme, namely COX-1 and COX-2, with the genes for these isoforms located on different chromosomes. The human COX-1 gene spans about 22 Kb, contains 11 exons, and is located on chromosome 9. Conversely, the COX-2 gene spans only 8 Kb, contains 10 exons, and is located on chromosome 1 (15;16). Both COX-1 and COX-2 genes code for COX-1 and COX-2 proteins that only slightly differ in their length and amino acid composition, and the prostaglandin endoperoxide products (PG G2 or PG H2) which are the immediate precursors of prostaglandins and thromboxane A2, are the same. However, the promoter sites and therefore the regulation of the genes, is completely
different. For example, the COX-2 gene contains a TATA box and a number of cis-regulatory elements in the 5'-upstream regulatory region that are involved in the transcriptional activation of the gene and are not found in the COX-1 gene (15). This different promoter structure is probably related to different roles of these encoded proteins in different physiological and pathophysiological settings. The constitutive enzyme (COX-1) is expressed in most if not all cells and determines the physiological functions of prostaglandins, including control of local tissue perfusion, haemostasis, and protection of mucosa in the gastrointestinal tract. In contrast, the expression of the inducible isoform of the enzyme (COX-2) is only evident after exposure to cytokines, immunological stimuli, or growth factors, and probably serves as a defence mechanism of the organism (15).

Aspirin is a relatively selective inhibitor of the constitutive isoform COX-1 (17). This would explain the different doses required for anti-platelet and anti-inflammatory activity on the different enzyme targets, namely COX-1 and COX-2. The anti-thrombotic action of aspirin is due to the inhibition of platelet COX-1, with subsequent prevention of thromboxane biosynthesis. This mechanism is particularly effective in platelets. Nonacetylated salicylates have no effect on platelets at antithrombotic doses comparable to those of aspirin (18), and also do not appear to impair the anti-aggregatory capacity of aspirin in man (19).

Aspirin inhibits the cyclooxygenase-catalysed conversion of arachidonic acid (AA) to the prostaglandin endoperoxides (PG EP). This results in inhibition of the generation of the downstream metabolites, namely thromboxane A2 (TX A2) in platelets, and prostacyclin (PG I2) in vascular cells. TX A2 and PG I2 have opposite effects on platelet function and vessel tone. In healthy volunteers with functionally intact endothelium, the action of aspirin
on platelets is dominant at low doses. In patients with atherosclerotic vessel disease and endothelial dysfunction, platelets adhering to the subendothelium may transfer prostaglandin precursors (PGEP) to vascular components such as smooth muscle cells, eventually resulting in enhanced PG12 biosynthesis. Under these conditions, all doses of aspirin that block the platelet enzyme (COX-1) will also reduce vascular prostacyclin production. Conversely, it is possible that induction of COX-2 has also occurred in vascular cells under the influence of inflammatory cytokines or growth factors (15). Because of the low potency of aspirin against COX-2, a retrograde transfer of prostaglandin precursors (PGEP) may occur towards platelets, eventually resulting in enhanced platelet-dependent thromboxane biosynthesis.

5.1.3 Optimum anti-platelet dosage in healthy subjects

Numerous studies in healthy volunteers have indicated that the regular uptake of approximately 30 mg aspirin per day is sufficient to inhibit platelet COX without simultaneous suppression of vascular prostacyclin generation (20;21). A significant inhibition of platelet function and thromboxane generation is obtained even doses of aspirin as low as 10-20 mg per day for 3 weeks, and this has been found to be accompanied by a 61 percent inhibition of serum thromboxane formation (22;23). Although this inhibition of thromboxane formation was statistically significant, it is of doubtful clinical significance as a prolongation of bleeding time was not observed at this dose but was seen with the regular administration of 30 mg per day. Overall, available data are compatible with the perception that inhibition of thromboxane forming capacity of platelets in excess of 90 percent is required to obtain clinically significant inhibition of platelet function in man in vivo. Regular
daily doses of 40 mg may be sufficient to obtain this effect in healthy volunteers, assuming that they are free of significant atherosclerotic vessel disease and endothelial dysfunction (24;25). However, even in healthy subjects who demonstrate no overt vascular risk, there are differences in aspirin efficacy between different vascular regions. For example, the number of myocardial infarctions recorded was reduced, whereas the number of strokes tended to be increased even at very low doses of aspirin (26), and this observation was also made in later trials.

5.1.4 Aspirin in patients with vascular disease

In healthy volunteers, a functionally intact endothelium exists, and there is no platelet adhesion to the vessel wall. Conversely, in patients suffering from atherosclerotic vessel disease, there is demonstrable platelet activation and adhesion to the subendothelium, plaques, and other injured areas of the vessel wall. It has been shown that patients in advanced stages of atherosclerotic vessel disease exhibit enhanced urinary excretion, not only of thromboxane but also of prostacyclin metabolites (27). This suggests that platelets may provide a significant source of prostaglandin precursors for vascular prostacyclin formation. Consequently, limitation of endothelial prostacyclin formation with low-dose aspirin administration may not be possible in such patients due to the precursor exchange mechanism from platelets to the vessel wall (27-29). If this is true, inhibition of platelet COX-1 will also result in reduced precursor transfer to the vessel wall, and subsequently in a reduced level of prostacyclin generation at any anti-platelet dose of aspirin.
Alternatively, the possibility of induction of COX-2 in endothelial and vascular smooth muscle cells within the vessel wall should be considered (30). These cells are able to alter their phenotype under the transforming stimuli of cytokines and growth factors (31). This is associated with the expression of the COX-2 gene, and can result in increased stimulation of prostaglandin formation in excess of 100-fold in well-controlled cell culture studies (32). It is possible that this also allows for "retrograde" precursor transfer from the vessel wall to adhering platelets, which eventually results in enhanced thromboxane production not sensitive to aspirin at anti-platelet doses. This may be an important factor in the mechanism of aspirin resistance, which is discussed later in this chapter.

In light of these complex alterations in platelet and vascular prostaglandin and thromboxane formation, establishing an optimal antithrombotic dosage for aspirin based solely on theoretical considerations is likely to pose difficulties. Clinical experience suggests that regular daily aspirin doses of 100 mg are sufficient to afford optimum protection in the secondary prevention of stroke and myocardial infarction (10). It is also evident that in comparison with other drugs such as heparin, aspirin is not the treatment of choice for prevention of venous thrombosis. However, the role of aspirin is less clear in the setting of peripheral arterial occlusive disease (33). The CAPRIE trial (34) compared the antithrombotic effects of aspirin at 325 mg with those of clopidogrel at 75 mg, and demonstrated that patients with peripheral arterial occlusive disease are less well protected from thromboembolic events by aspirin than are patients with cerebrovascular disease or myocardial infarction. The debate surrounding the designation of low dose aspirin at 100 mg per day as the optimum dosage for cerebral vascular disease remains a contentious issue (35-37). Evidence exists to suggest that higher doses of aspirin may be more efficient in
inhibiting the elevated thromboxane production and platelet activity in patients with cerebrovascular disease (38,39). This is illustrated in table 5.1.

<table>
<thead>
<tr>
<th>Platelet activity</th>
<th>Aspirin Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No aspirin</td>
</tr>
<tr>
<td>% collagen aggregation</td>
<td>73</td>
</tr>
<tr>
<td>% ADP aggregation</td>
<td>72</td>
</tr>
<tr>
<td>Serum TX B2</td>
<td>49</td>
</tr>
<tr>
<td>Urinary TX B2</td>
<td>994</td>
</tr>
</tbody>
</table>

Table 5.1

Platelet activity according to dose of aspirin

It is also possible that inflammatory events independent of or alongside thromboxane-independent mechanisms of platelet activation may contribute to ischaemic stroke. However, it should be noted that according to a recent meta-analysis of controlled trials of aspirin administration in patients suffering from transient ischaemic attacks or minor stroke, any dose of aspirin above 30 mg is able to prevent only 13 percent of vascular events (39). This clearly highlights the need for a more effective anti-platelet strategy, and these conclusions are also supported by the data of the TASS trial.
5.1.5 Aspirin resistance

Several controlled studies have demonstrated a remarkable inter-individual variability in the inhibition of platelet function by aspirin and it has been proposed that the failure of aspirin to sufficiently protect against thromboembolic vessel occlusion can be explained by either pharmacokinetic or pharmacodynamic mechanisms, a situation referred to as aspirin resistance. In truth, it is likely that both are involved (40). Possible pharmacodynamic explanations for an insufficient anti-platelet effect in response to aspirin include the induction of COX-2, which exhibits aspirin resistance, and a reduced sensitivity of hyperreactive platelets to aspirin for example, which could occur subsequent to activation by thromboxane-independent pathways. Additionally, differences in aspirin metabolism between individuals may also exist, especially at low dose administration.

As the anti-platelet action of aspirin is attributable to inhibition of COX-1, with COX-2 remaining essentially unaffected at anti-thrombotic doses (<300 mg), it is to be expected that the anti-platelet action of aspirin will be less effective if COX-2 becomes involved in platelet-dependent thromboxane production. As previously discussed, expression of COX-2 may occur in the vessel wall in atherosclerotic conditions (30) and therefore, could contribute to the overall increase in thromboxane biosynthesis seen in cardiovascular disease (41) associated with endothelial dysfunction. Vejar and colleagues (42) have reported that 17 percent of patients with unstable angina pectoris who were treated with a low-dose aspirin regime of 60 mg on the first day of symptoms, and 20 mg daily thereafter exhibited higher thromboxane excretion in urine than untreated patients despite inhibition of platelet COX in excess of 95 percent. Interestingly, the success rate of aspirin in most of the
controlled studies on unstable angina pectoris was approximately 50 percent (10;43), and although this can be interpreted as either a 50 percent success or 50 percent failure rate, in either case it suggests that a significant percentage of aspirin-treated patients were not protected from platelet-dependent vascular events. In contrast, the TASS trial (44) has demonstrated a significant and comparable therapeutic efficacy for aspirin and ticlopidine in patients with cerebrovascular disease. Because the mechanism of action of the two compounds is completely different, this data suggests that anti-platelet drugs offering different antiplatelet activity may confer similar therapeutic benefits, and that the combined use of such compounds may be more effective than either treatment used in isolation. The question surrounding the role of aspirin resistance in this scenario remains open to debate (36;45-48). Conversely, some data exists to suggest that in patients with cerebrovascular disease, high-dose aspirin is indeed more efficient that low-dose treatment in this group of patients.

Several studies suggest that the mechanism of platelet activation may explain the low efficacy of aspirin in protecting patients against thromboembolic events. For example, platelet activation by shear stress does not result in greater stimulation of platelet-dependent thromboxane formation (46;49) and therefore, is largely resistant to aspirin (50). Similarly, aspirin does not block the potentiation of proaggregatory factors by circulating catecholamines (51). In addition, blood flow may be affected differently in response to vasoconstrictors that are released during platelet secretion, including TXA2 and serotonin. Serotonin release from platelets has been found to be only partially inhibited by low-dose aspirin administration at 40 mg per day, despite inhibition of platelet-dependent thromboxane biosynthesis in excess of 95 percent (52).
The possibility exists that the thromboxane-independent action of aspirin may be implicated in its anti-thrombotic effect. It has been demonstrated that high doses of aspirin up to and above 500 mg inhibit thrombin generation in whole blood (53), although the potency was low and no evidence exists to date to suggest that thromboxane-independent mechanisms are indeed involved in the anti-thrombotic efficacy of aspirin.

Finally, it is possible that the pharmacokinetics of aspirin may differ between subjects, eventually resulting in insufficient acetylation of COX, and clinical trials have shown that this may occur at low doses of aspirin (54,55). Genetic polymorphism may also exist, specifically for expression of COX and thromboxane synthase (40) and could account for differences in response to aspirin between individuals although there are no systematic studies designed to address this issue at the present time.

5.2 Dipyridamole

Dipyridamole is a compound that has been available for a number of years, yet only a limited amount of information is known about its mode of action. A number of theories regarding its mechanism of activity have been postulated, including:

a) Increased stimulation of prostacyclin release by the vessel wall.

b) Elevation of platelet cAMP levels by inhibition of phosphodiesterase.

c) Local vasodilatory effects.
At present, dipyridamole alone would appear to offer no benefit over aspirin alone in preventing major vascular complications. However, the recent European Stroke Prevention Study-2, has demonstrated that a combination of these two treatments is more effective than aspirin alone in this setting (56). This study enrolled 6600 patients over 2 years, the and found that a combination of aspirin and dipyridamole reduced the recurrence of stroke by 37 percent when compared to reductions of 18 percent and 16 percent respectively when these agents were used alone.

5.3 Ticlopidine and Clopidogrel

Ticlopidine and its more recently developed analogue clopidogrel, are thienopyridine derivatives that are known to inhibit platelet function. Both agents can be administered orally and require post-digestion modification to the active metabolite form (4). Ticlopidine and clopidogrel act by inhibiting the binding of ADP to its platelet receptor (9;10) and this ADP receptor blockade leads to direct inhibition of fibrinogen binding to its Gp IIb-IIIa receptor (16). There is also evidence that ticlopidine can interfere with the platelet-vWF interaction, resulting in a decreased tendency to thrombosis (17).

Two types of ADP receptor exist on platelets surface, with one exhibiting low affinity and one possessing high affinity for ADP (15). The low affinity site is G-protein coupled and causes the release of intra-cellular calcium stores when activated. This results in a conformational change and subsequent activation of the glycoprotein Gp IIb-IIIa receptor. Both ticlopidine and clopidogrel inhibit this site, which leads to a reduction in thrombus

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formation (15). Neither affect platelet morphology, nor the influx of calcium into the platelet (19). These drugs require between 3 and 4 days to reach maximal effect, and appear to irreversibly inhibit platelet function over the subsequent 7 days.

Three major trials have demonstrated a small but significant benefit in the reduction of vascular events after the administration of ticlopidine and clopidogrel (42;44) (51). The largest of these was the CAPRIE study (51), which assigned over 19,000 patients to treatment with either clopidogrel or 325mg of aspirin. The mean follow up period was 1.9 years, and an overall relative reduction in major vascular events of 8.7 percent was revealed. Sub-group analysis demonstrated that clopidogrel conferred no benefit over aspirin in the prevention of either stroke or myocardial infarction. The sole group to benefit from clopidogrel administration were those patients with severe peripheral vascular disease. Incidentally, two smaller studies have shown a small reduction in the risk of cerebral ischaemia following ticlopidine administration (42;44).

5.4 Inhibition of platelet-fibrinogen binding

Following initial platelet activation, through binding to subendothelial vWF via Gp Ib, there is a rapid up-regulation in the number of fibrinogen receptors (Gp IIb-IIIa) expressed on the platelet surface, to a figure of approximately 50,000 per platelet. Fibrinogen has active sites for platelet binding at both ends of its structure, which enables it to cross-link platelets and therefore form a large thrombus. This is the final common pathway by which most forms of stimulation cause platelet aggregation. The active sites within the fibrinogen molecule
contain a specific arginine-glycine-aspartic acid sequence, referred to as the \textit{RGD sequence}. The RGD sequence is also found on other adhesive proteins including vWF, and therefore, inhibition of binding to this sequence should exert a profound effect upon platelet ability to form thrombus (57).

The first group of compounds found to act at this site were peptides retrieved from monoclonal antibodies targeted against the active site and include 7E3, or Reopro (58). These agents have a proven role in the prevention of coronary thrombosis and restenosis following angioplasty, although there currently exists no published evidence of their role in stroke prevention. However, the coronary angioplasty provides a similar environment to carotid endarterectomy, in that there is significant stripping of the endothelium and high flow shear rates and so it may be possible to draw a parallel between these procedures and suggest that Reopro may have antithrombotic potential in patients post carotid endarterectomy.

\subsection*{5.5 Dextran polymers}

The dextran molecule consists of a long chain of polysaccharide moieties produced by the organism \textit{Leuconostoc mesenteroides}. It was first used in 1944 as a plasma expander and the first reports of its ability to induce a bleeding tendency in patients did not emerge until the 1960's (59). Many of the earlier studies used dextran molecules with a weight of 65,000-80,000 but more recently, researchers have tended to concentrate on the shorter chain polymers such as dextran -40.
Although dextrans have been used clinically for many years, the mode of action in terms of bleeding time and platelet function has not yet been fully established. The infusion of between 500 and 1000ml of dextran into healthy volunteers and patients undergoing minor surgical procedures induced a significant reduction in platelet aggregating ability in response to a collagen stimulus in the in vitro setting (60). However, the effect of dextran on bleeding time in these subjects was variable. Dextran sulphate binds factor VIII, and the loss of this factor from circulating plasma was also associated with a decrease in aggregation in response to ristocetin administration (61). Dextran has also been shown to inhibit the function of platelet factor-3, with the use of dextran in patients undergoing minor surgical procedures demonstrating that it reduced the plasma concentration of the anti-coagulant anti-thrombin III in the post-operative period (62). This may indicate that dextran assists the stabilisation of the thrombin-antithrombin III complex. In a rabbit model of thrombosis, the administration of a dextran infusion significantly reduced the weight of the retrieved thrombus produced in response to trauma to the blood vessels (63). This approach was further developed in a later paper using the same model, where it was demonstrated that the dextran infusion also led to an increase in thrombus fragility.

Most of the clinical studies using dextran have focused on its ability to reduce the incidence of post-operative deep venous thrombosis. The first clinical report of this effect was by Koekenberg et al, who found that the incidence of post-operative DVT was reduced from 21 percent to 4 percent in patients who received dextran. This conclusion has been reproduced in 28 other studies (64).
CHAPTER FIVE: Anti-platelet therapy

The findings of studies that have assessed the prevention of arterial occlusions have been less convincing. An animal model that investigated PTFE interposition grafts in sheep carotid arteries demonstrated that dextran-70 reduced the rate of platelet and fibrinogen deposition in the grafts (65), although this was only true when the blood flow through the grafts was restricted to a low flow state. A recent clinical study of autogenous infrainguinal grafting in patients failed to confer a benefit to dextran administration terms of graft patency in the short-term or in the long-term (66).

A number of studies have demonstrated that small volumes of dextran-40 can reduce the number of post-operative carotid thromboses seen following CEA, and indeed a decrease in embolisation rates from the endarterectomised vessel can be seen after a peripheral infusion of only 20-30ml of Dextran-40 (67 - 69). However, the exact mechanisms that underlie this dramatic effect have yet to be elucidated. The ability of transcranial doppler to monitor the development of thrombosis within the carotid artery in the in vivo setting provides an excellent model for examination of the effects of Dextran-40 on thrombosis formation. It also presents an excellent opportunity to assess the effect of other markers of haemostatic function that may place individuals at increased risk of carotid thrombosis after CEA. Obviously, these findings may have wider implications in other clinical scenarios whereby endothelium is damaged and the subendothelium exposed, such coronary or lower limb angioplasty. The interaction of the endarterectomised carotid artery, haemostatic risk factors and platelet function, as measured by TCD monitoring will provide the basis for the research reported in this thesis.
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PART TWO

ORIGINAL WORK
CHAPTER SIX

PATIENTS’ THROMBOEMBOLIC POTENTIAL BETWEEN BILATERAL CAROTID ENDARTERECTOMIES REMAINS STABLE OVER TIME
Abstract

Objectives: There is limited understanding of the reasons underlying post-CEA carotid thrombosis. Clinicians have often implicated operative technique, such as patch type or shunting, however the evidence for this is limited. We have studied whether it is the patients themselves who are prothrombotic, by studying the rates of emboli detection in patients undergoing bilateral CEAs at separate time points.

Materials and methods: Sixteen patients (3 women) underwent CEA during the study period, all of whom were taking aspirin. CEA was performed in a standardised manner throughout the study. All patients were monitored for 3 hours post-operatively using a 2MHz fixed head probe.

Results: Those patients who had no emboli detected on TCD after the first operation, had a mean of 2.5 emboli after the second operation. Patients with emboli after the first op had a mean of 41.3 emboli after the second CEA (MWU test, p=0.02). The dose of aspirin administered did not affect emboli rates. Correlation of the number of emboli detected after the first CEA with the second CEA gave a significant correlation (p=0.038).

Conclusions: There appear to be factors relating to the patient that places some individuals at an increased risk of thrombotic stroke. Further elucidation of these factors may enable more effective, targeted therapy to be applied in the prevention of arterial thrombosis.
CHAPTER SIX

Introduction

The clinical effectiveness of carotid endarterectomy (CEA), is limited by the peri-operative stroke rate. Previous work has shown that the intra-operative stroke rate can be reduced by a programme of intra-operative transcranial Doppler (TCD) monitoring and completion angioscopy. A significant number of post-operative strokes are due to carotid thrombosis, but the incidence of these post-operative strokes can be reduced by a programme of TCD directed Dextran-40 therapy.

Although effective therapy for this complication is now available, there is limited understanding of the underlying causes of this problem. The routine use of completion angioscopy and intra-operative TCD limit the scope for technical error to be the cause of post-operative thrombosis. Secondly, in patients who suffer a post-operative thrombosis and are returned to theatre, the thrombus is found to be adherent to the endarterectomy zone, not at the site of a technical error.

We hypothesised that patients' potential to form thrombus represents a spectrum from hypocoaguable to thrombophillic and that their tendency to generate microemboli after CEA would be conserved between operations at different time points. We were not attempting to identify a specific factor that caused patients to be relatively thrombophillic. The post-operative TCD detected emboli counts of patients who had undergone two separate CEAs were studied, and the correlation of emboli counts between the first and second operation was noted.
Patients and methods

All patients were taking aspirin (75 - 300mg) at the time of CEA. No patient was using any other systemic anti-platelet or anti-coagulant therapy, or suffered from a known hypo- or hyper-coaguable state. Antiplatelet therapy was not stopped prior to surgery and the patient’s usual medication administered on the morning of surgery. Carotid endarterectomy was performed by a consultant vascular surgeon or supervised trainee with normotensive, normocarbic general anaesthesia using loupe magnification, systemic heparinisation (5000u), routine shunting (Pruitt-Inahara, Ideas for Medicine, Fla) and routine tacking sutures to the distal intimal step to prevent distal intimal dissection. Patients had a patch angioplasty with either a segment of long saphenous vein harvested from the groin or a Dacron Hemashield Finesse patch (Boston Scientific Ltd.). Both were anastomosed with a 6:0 prolene suture (Ethicon Ltd). The vein patch was simply used at the width which it was when harvested (ie the circumference of the vein), it was not trimmed to size. The Dacron patch was a standard 7mm in width.

Blood flow velocity in the middle cerebral artery (MCA) was monitored throughout the procedure using 2MHz pulsed wave TCD ultrasound, a SciMed PC2-64B, with a fixed head probe system (Scimed UK Ltd, Bristol UK) protected by a semi-circular metal headguard with recording onto digital audiotape (DAT) for off-line analysis of emboli. A 5mm segment of the anastomosis (adjacent to the orifice of the external carotid artery) was left open. The shunt was removed and the lumen vented and irrigated with heparinised saline. Prior to final closure and restoration of flow, the lumen was inspected with a flexible hysteroscope (Olympus 1070-48) to exclude residual luminal thrombus and intimal flaps.
After the procedure, the patient was transferred through to the high dependency unit for further TCD monitoring. All emboli detected in the first three post-operative hours were recorded onto DAT tape and quantified off-line. Previous studies from this unit have shown that post-operative emboli are exclusively particulate. Studies have shown that sustained post-operative embolisation is highly predictive of progression onto thrombosis and stroke and that this phase can be abolished by an incremental infusion of Dextran. Other centres have noted a link between clinical events and radiological findings and embolisation rates. Accordingly, any patient who had ≥25 emboli in any 10-minute period or whose emboli distorted the MCA waveform (suggesting they were large) were commenced on an incremental infusion of Dextran 40. An initial bolus of 20ml 10% Dextran-40 was administered, followed by an infusion at rate commencing at 20ml/hr. If the embolisation rate failed to settle, then the infusion rate was increased by 10ml/hr until embolisation was controlled. The number and rate of embolisation were quantified for every patient using TCD.

Results

None of the 17 patients suffered a stroke or major complication as a result of the CEA. One patient out of the 16 had no temporal window to allow TCD monitoring to take place and could not be included in the study. There was an average of 13 months between the first and second procedure (range 2 - 50 months). The use of aspirin, or the dose of aspirin taken, did not influence the post-operative emboli counts. The patch type, Dacron or vein, had no influence on embolisation rates. Only 1 patient needed Dextran-40 therapy in the
study, and this patient also had high numbers of emboli at the second operation, but they did not reach levels requiring dextran therapy.

Those patients who had no emboli after the first procedure (n=8), had a mean of 2.5 emboli per patient after the second operation (range 0-7). Those patients who embolised after the first CEA (n=8), had a mean of 41.3 emboli after the second CEA (range 0-264). Analysis of this non-parametric data using the Mann-Whitney U test demonstrated significantly higher numbers of emboli in the second group (Z = -2.27, p=0.02). Correlation of the number of emboli seen after the first CEA compared to the second, gave a correlation coefficient of r=0.52 (p=0.038). The numbers of emboli seen after each CEA were also ranked in numerical order and compared.

Discussion

This study indicated that the number of emboli detected by TCD monitoring after a CEA correlates with the number of emboli to be seen after a second CEA. These operations are often separated by a significant period of time. This may indicate that this trait is at least partly inherent. The strong correlation between emboli counts after first and second CEAs would argue against technical error being a significant cause of post-operative embolisation since the same technical error is unlikely to be repeated twice in the same patient. No technical errors were noted at completion angioscopy in any of these patients.
The fact that patients appear to have an inherent thrombotic potential after CEA has a number of implications. This potential is conserved with time, and therefore a patient who exhibits significant emboli after a first CEA is likely to be at risk after a second CEA. If no TCD monitoring is available for this patient, then the use of prophylactic, low dose (20ml/hour) 10% Dextran-40 should be considered, if no contraindications exist (eg severe cardiac failure). Alternatively, the procedure could be postponed until a time at which TCD monitoring is available. The use of additional anti-platelet or anti-thrombotic agents may be indicated in those patients who appear to exhibit an increased thrombotic risk at a first procedure.

More significantly, many patients who undergo CEA have other areas of their vascular tree that require surgical or radiological intervention. Are those patients who exhibit high levels of emboli after a CEA at risk of significant thromboembolic complications during further vascular procedures? Do high levels of emboli after CEA indicate a general predisposition to cardiovascular morbidity and mortality? Should patients with high emboli counts be given increased levels of anti-platelet or anti-coagulant therapy? This is an area of cardiovascular research that may throw open a number of intriguing avenues for investigation.
References


CHAPTER SEVEN

A RANDOMISED TRIAL OF VEIN VERSUS DACRON PATCHING DURING CAROTID ENDARTERECTOMY: INFLUENCE OF PATCH TYPE ON POST-OPERATIVE EMBOLISATION
Abstract

Objectives: A recent overview has indicated that while routine patching is safer than routine primary closure following carotid endarterectomy (CEA), there is no systematic evidence that patch type influences outcome. However, most surgeons still perceive that prosthetic patches are probably more thrombogenic than vein. This study tested the hypothesis that there was no difference between different patch types with regard to thrombogenicity.

Materials and Methods: 274 patients undergoing 276 CEAs were randomised to either Dacron patch closure (n=137) or vein patch closure (n=139). All patients with an accessible cranial window were monitored for three hours post-operatively using transcranial Doppler (TCD). The number and rate of embolisation was quantified together with the requirement for selective Dextran therapy to control high rates of post-operative embolisation. All patients were assessed post-operatively and again at 30 days by a neurologist and all underwent Duplex assessment at 30 days.

Results: The 30 day death/any stroke rate was 2.2% for Dacron patched patients and 3.6% for vein patched patients (p=0.72). Dacron patched patients had a higher incidence of post-operative emboli (median 5, interquartile range: 0 - 10.5) as compared with a median of 3 (interquartile range: 1 - 17) for vein (p=0.028). However, the incidence of detecting >50 emboli was virtually identical and patch type had no effect on the incidence of high rate, sustained embolisation requiring Dextran therapy (5.3% for Dacron, 3.7% for vein). No patient had a carotid thrombosis at 30 days.
Conclusions: Sustained, high rate embolisation, previously shown to be highly predictive of progression onto carotid thrombosis, appears to be patient dependent rather than related to patch type.
Introduction

A recent overview of the randomised trials of closure during carotid endarterectomy (CEA) suggested that a policy of routine patching was safer than a policy of routine primary closure in terms of a threefold reduction in peri-operative thrombosis, early and late stroke and late restenosis (1). There is, however, no consensus on the optimal material for patching. The principal advantages of vein include no cost, use of autogenous tissue, resistance to infection and ease of handling. Disadvantages include harvest site complications, vein patch rupture (which is reduced by harvesting the vein from the groin) but which then means the full length of vein is unavailable for future use. The advantages of prosthetic patches include immediate availability, no harvest site complications, the avoidance of rupture and preservation of the saphenous vein. Its principal disadvantage is infection. Although the meta-analysis suggested that there was no significant difference in outcome relative to patch type, there remains a perception amongst surgeons that prosthetic patches are probably more thrombogenic than vein.

Recent work from this unit has shown that up to 70% of patients will have one or more emboli detected in the first three post-operative hours after CEA (2). However, only about 5% will develop sustained, high rate embolisation which has now been shown to be highly predictive of progression onto carotid thrombosis and stroke (3-7). When re-explored, these patients have friable platelet thrombus adherent to the endarterectomy zone, usually with no evidence of any underlying technical error (8). The current study was therefore specifically designed to quantify rates of post-operative embolisation as an indicator of prothrombotic potential in a prospective, randomised trial of vein versus Dacron patches in patients undergoing CEA.
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Materials and methods

The trial was approved by the Leicestershire Area Ethics Committee and patients entered by informed consent. 294 patients undergoing CEA between 1.12.97 and 31.12.99 were considered for inclusion; 20 were subsequently excluded because of refusal to give informed consent (n=10), bilateral varicose vein surgery (n=6), osteomyelitis (n=1), severe claudication (n=1), redo CEA (n=1) and combined common carotid angioplasty plus CEA (n=1). 274 patients were therefore randomised and underwent 276 CEAs. This paper details the results concerning outcome, rate of embolisation and Duplex findings at 30 days.

Randomisation method

Three hundred computer-generated, random treatment methods (vein or Dacron) were consecutively numbered and sealed in opaque envelopes and allocated on a consecutive basis (immediately following induction of anaesthesia) starting with envelope number 1.

Carotid endarterectomy

Antiplatelet therapy was not stopped prior to surgery and the patient’s usual medication administered on the morning of surgery. Carotid endarterectomy was performed by a consultant vascular surgeon (n=154) or supervised trainee (n=122) with normotensive, normocarbic general anaesthesia using loupe magnification, systemic heparinisation (5000u), routine shunting (Pruitt-Inahara, Ideas for Medicine, North Clearwater, Fla) and routine tacking sutures to the distal intimal step to prevent distal intimal dissection. Patients randomised to vein had a segment of long saphenous vein harvested from the groin and anastomosed with 6:0 prolene
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suture (Ethicon Ltd, Somerville, New Jersey, USA). Patients randomised to Dacron had a Hemashield Finesse patch (Boston Scientific Ltd, St. Albans, England.) inserted using 6:0 Prolene (Ethicon Ltd). The vein patch was simply used at the width which it was when harvested (ie the circumference of the vein), it was not trimmed to size. The Dacron patch was a standard 7mm in width.

Blood flow velocity in the middle cerebral artery (MCA) was monitored throughout the procedure using 2MHz pulsed wave TCD ultrasound, a SciMed PC2-64B, with a fixed head probe system (Scimed UK Ltd, Bristol UK) protected by a semi-circular metal headguard with recording onto digital audiotape (DAT) for off-line analysis of emboli (9,10). A 5mm segment of the anastomosis (adjacent to the orifice of the external carotid artery) was left open. The shunt was removed and the lumen vented and irrigated with heparinised saline. Prior to final closure and restoration of flow, the lumen was inspected with a flexible hysteroscope (Olympus 1070-48, Hamburg, Germany) to exclude residual luminal thrombus and intimal flaps (10).

After the procedure, the patient was transferred through to the high dependency unit for further TCD monitoring. All emboli detected in the first three post-operative hours were recorded onto DAT tape and quantified off-line. Previous studies from this unit have shown that post-operative emboli are exclusively particulate (9) and that 3 hours of monitoring is just as effective as 6 hours (11,12). Studies have shown that sustained post-operative embolisation is highly predictive of progression onto thrombosis and stroke (3-7) and that this phase can be abolished by an incremental infusion of Dextran (11,12). Accordingly, any patient who had ≥25 emboli in any 10 minute period or whose emboli distorted the MCA waveform (suggesting they were large) were commenced on an incremental infusion of Dextran 40. An initial bolus of
20ml 10% Dextran-40 was administered, followed by an infusion at a rate commencing at 20ml/hr. If the embolisation rate failed to settle, then the infusion rate was increased by 10ml/hr until embolisation was controlled. The number and rate of embolisation were quantified for every patient, as was the requirement for Dextran therapy.

**Post-operative assessment**

All patients were assessed post-operatively by the same neurologist (HA), who documented any new neurological deficit or cranial nerve injury. Any new neurological deficit lasting >24 hours was classified as a stroke and these patients were investigated by CT scan/autopsy, Duplex assessment of the internal carotid artery (ICA) and TCD assessment of the intracranial vessels. The neurologist reassessed all trial patients at 30 days where those suffering a peri-operative stroke underwent an assessment of stroke severity using the Oxfordshire Handicap Stroke Score (OHS) (13). The advantage of the OHS over the Rankin Score is that it takes into account the disability associated with dysphasia. A minor (non-disabling) stroke scored 0 – 2 on the OHS scale whilst a disabling score scored 3 – 6. All patients underwent a colour Duplex assessment of the operated artery at 30 days by the same vascular technologist (SS) using the ATL Ultramark (Bothel, Wash). Attention was directed towards measuring the maximum diameter of the carotid bulb, excluding carotid occlusion and diagnosing a stenosis ≥70%. At the time of discharge, each patient was asked to complete a visual analogue score (range 1-5) for pain in the groin and neck wounds.
Statistical analysis

Analysis was based on 'intention to treat'. Any patient randomised to vein who then underwent Dacron patching because the vein was inadequate was analysed on the basis that he/she was originally randomised to vein. Similarly, any patient who had already been randomised in the trial and who subsequently required a contralateral CEA received the same type of patch as allocated by the first randomisation. The data in this study were not normally distributed, therefore non-parametric tests were adopted. Continuous variables were analysed using Mann-Whitney U test. Discrete variables were analysed using chi-squared or Fisher’s exact test as appropriate. A “p” value of <0.05 was assumed to represent statistical significance.

Results

Patient demographics

Three patients underwent bilateral CEAs within the trial and each received the same patch allocation as randomised during the first CEA. Table 1 summarises the changes to treatment following randomisation. Five patients (1.8%) underwent reversed saphenous vein bypasses because the ICA was either too thin following endarterectomy (n=2) or had pronounced distal coiling (n=3) thereby precluding safe patch closure. One patient (0.4%) underwent carotid ligation because the distal ICA lumen was narrow (2-3mm) and had adherent mural (but not occluding) thrombus extending up to the skull base on exploration. TCD indicated that MCA flow was adequate to tolerate carotid ligation. Finally, three patients (1.1%) randomised to vein received a Dacron patch because no suitable vein was found following groin exploration. Table 2 summarises the pre-operative patient demographics for the two
groups, which were well matched for all parameters. In particular, there was no significant difference relative to presentation, concurrent medical disease, the severity of the carotid disease, time since the last cerebral event, antiplatelet therapy and dose of aspirin.

Table 3 details the 30 day peri-operative data relative to patch allocation. There was no significant difference regarding length of operation, shunt and clamp times, patch length, angioscopy findings and neck drain losses. Because patients randomised to Dacron did not have a groin wound, the overall drain losses were higher for vein patched patients and there was, inevitably, a higher incidence of groin wound infections (4.3%) and seromas/haematomas (3.6%) in vein patched patients. There were no patch infections or ruptures in any patient in this study.

*Post-operative outcome*

Patch type did not influence the requirement for treating early post-operative hypertension, transfusion requirements, early post-operative re-exploration for neck haematoma and time to hospital discharge (table 3). The median analogue score for neck wound pain was 2 (95% CI 2.4-2.7) for Dacron and 2 (95% CI 2.3-2.5) for vein (p=0.15). The median analogue score for groin wound pain was 1 (95% CI 1.0-1.1) for Dacron and 2 (95% CI 2.1-2.4) for vein (p<0.0001).
Table 4 summarises the 30 day morbidity and mortality data. The incidence of persisting cranial nerve injury at 30 days (excluding the mandibular branch of the facial nerve) was unrelated to patch type (3.6% for Dacron) and 2.9% for vein (p=0.78). No patient in either group recovered from anaesthesia with a new neurological deficit. The 30 day death/disabling stroke rate was 1.5% for Dacron patched patients and 2.9% for patients receiving a vein patch. The death and/or any stroke rate was 2.2% for Dacron and 3.6% for vein patched patients. If the data are re-analysed according to which patch type the patients actually received in the trial (i.e. excluding vein bypasses and correcting for crossovers), the death/any stroke rate was 2.2% in the 133 dacron patched patients and 3.7% for the 134 vein patched patients (p=0.72).

All survivors underwent a colour Duplex scan of the operated ICA at 30 days and no patient in either group was found to have developed a carotid occlusion or stenosis >70%. Four patients randomised to dacron (2.9%) had a 30-69% stenosis detected at 30 days as compared with nine (6.5%) vein patched patients (p=0.26). The median carotid bulb diameter at 30 days was 10.8mm (95% CI 10.3-10.8) for Dacron patched patients and was 12.0mm (95% CI 11.9-12.6) for vein patched patients (p=0.001).

Post-operative embolisation

Seven Dacron patients (5.1%) and 12 patients randomised to vein (8.6%) had no accessible cranial window for TCD. The remaining patients underwent 3 hours of post-operative TCD monitoring. Although the actual difference was small, patients randomised to Dacron had a significantly higher number of emboli detected within the first three post-operative hours (median 5, interquartile range 1-17) as compared to vein patched patients (median 3, interquartile range 0-10.5) (p=0.028). Figure 1 presents the frequency distribution of emboli.
detected in the two groups of patients. Dacron patched patients had a tendency towards having more emboli detected, but above the threshold of 50 emboli, the two curves virtually overlapped. More important, the presence of high rate, sustained embolisation and the requirement for Dextran therapy was unrelated to patch type. Nine dacron patched patients (6.6%) required Dextran therapy as compared with 7 (5.0%) vein patch patients (p=0.62).

If the data are re-analysed relative to the 133 patients who actually received a dacron patch in the trial as compared to the 134 who underwent vein patching (i.e. excluding bypasses and cross-overs) the basic data remains unchanged. The median embolus count for dacron patched patients was 5 (interquartile range 1-17) as compared with 3 (interquartile range 0-10.5) for vein patched patients (p=0.027). Seven patients (5.3%) receiving a dacron patch had sustained embolisation requiring Dextran therapy as compared with 6 vein patched patients (4.4%, p=0.78).

The causes of operation related strokes/deaths are summarised in table 4. Five of the eight deaths/strokes (63%) followed cardiac events or intracranial haemorrhage. The incidence of ipsilateral embolic stroke was (0.7%) for the whole series. No stroke in this trial followed post-operative carotid thrombosis and no patient randomised to vein suffered an ipsilateral stroke secondary to MCA embolism. However, two patients randomised to Dacron suffered focal embolic strokes (sustained embolisation on TCD, focal carotid territory infarct on CT scan). The first developed severe cardiac failure in the immediate post-operative period and required re-intubation and inotropic support. TCD demonstrated high rates of embolisation but adequate doses of Dextran could not be administered because of his severe cardiac failure. He had a disabling hemiparesis following extubation (OHS 3 at 30 days). The second patient had high
rates of embolisation in the immediate post-operative period which were abolished using
Dextran (zero emboli detected at 24 hours). However, he developed an upper limb monoparesis
on day 5. TCD again revealed profuse MCA embolisation which was rapidly controlled by
Dextran. His OHS score at 30 days was 1. When rescanned at 30 days, neither of these patients
had Duplex evidence of occlusion or a stenosis >70%.

Discussion

Meaningful interpretation of this paper is dependent upon the following assumptions; (i) that
the ‘high intensity transients’ detected by TCD following CEA represent particulate emboli
rather than gaseous elements or artefacts, (ii) that sustained embolisation is indicative of an
increased risk of thrombus formation within the endarterectomy zone and (iii) that TCD
diagnosed emboli are clinically relevant.

TCD detected ‘high intensity transients’ were first reported during CEA in 1986 (14) but there
was considerable scepticism as to their nature and relevance. Many clinicians assumed that they
simply represented artefacts. Subsequent work has now clearly shown that TCD can detect very
small embolic fragments and that it is possible to differentiate emboli from artefacts using
consensus criteria (15) or dual gated ultrasound (16,17). Similarly, methods capable of
differentiating gaseous from solid elements have demonstrated that emboli detected in the post-
operative period are exclusively particulate (9).
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A number of studies have now reported that TCD detected emboli are associated with an increased risk of adverse outcome. Embolisation in patients with an asymptomatic carotid stenosis is associated with an increased risk of TIA or stroke (18), while the risk of stroke following acute myocardial infarction is significantly higher in patients with TCD evidence of cerebral embolisation (19). Patients with >10 emboli detected during the dissection phase of CEA have a higher incidence of post-operative cognitive impairment (20) and MRI changes indicative of ischaemia (21), while excess intra-operative embolisation has been associated with an increased risk of operative stroke (22). However, the most knowledge is currently derived from studies on embolisation in the early post-operative period following CEA. Evidence suggests that while a policy of quality control assessment and intra-operative monitoring can virtually abolish the incidence of intra-operative stroke (apparent upon recovery from anaesthesia), the incidence of stroke due to post-operative thrombosis is unaffected and complicates about 3% of CEAs (10). At re-exploration, these patients usually have friable platelet thrombus adherent to the endarterectomy zone (rather than to the patch) with no evidence of underlying technical error (8).

There is now evidence from three continents to show that patients destined to suffer a post-operative stroke due to carotid thrombosis have a preceding 1-2 hour phase of increasing embolisation (3-7). Despite the high flow rates within the carotid artery, platelets start to adhere to the endarterectomised surface within minutes of flow restoration (23) and serve as a source of ongoing embolisation and thrombus accumulation. In a recent study of 500 patients undergoing CEA, up to 70% of patients had one or more emboli detected in the early post-operative period but only 5% developed very high rates of sustained embolisation (2). Levi et al demonstrated that 60% of patients with high rates of post-operative embolisation will suffer a
stroke (5). This is in accordance with our own experience (8) and consistent with published data on the risk of post-operative carotid thrombosis. Embolisation in this high-risk subgroup can be abolished using incremental doses of intravenous Dextran 40 and so prevent progression onto carotid thrombosis (11,12). Thus the available evidence suggests that sustained post-operative embolisation is a high risk predictor for progressing on to carotid thrombosis after CEA and that the rate and magnitude of embolisation could be used as an indirect marker of a prothrombotic tendency.

The randomised trials to date have shown no systematic evidence that vein patches are safer than prosthetic (1) and the findings of this latest study corroborates this. This trial has shown no evidence that the 30 day operative risk is related to patch type. Similarly, patch type had no influence on operation time, drain losses, the need for re-exploration, neck wound pain, cranial nerve injury and time of hospital discharge. Vein patched patients inevitably had a higher incidence of groin wound complications. No patient in this series suffered a vein patch rupture or prosthetic patch infection. A meta-analysis of a mix of non-randomised and randomised trials of vein patching versus synthetic patching concluded that vein was superior with respect to recurrent stenosis and possibly carotid thrombosis (24). This has not been corroborated by the Cochrane overview of randomised trials (1). These issues will be addressed in the long term follow up of these patients.
Despite the findings of the overview of randomised trials, there remains a perception amongst surgeons that prosthetic patches are probably more thrombogenic than vein. In the past, attempts to evaluate the thrombotic nature of the carotid patch using isotope labelled platelets have been fraught with difficulty. This study is the first to have used the rate/magnitude of embolisation as an indirect marker of increased thrombotic potential relative to patch type in a randomised trial. In preparation for this study, we observed three factors that led us to hypothesise that it may, paradoxically, be the patient who is ‘prothrombotic’ rather than the patch. Firstly, patients undergoing re-exploration for post-operative stroke tend to have thrombus accumulating on the endarterectomy zone rather than on the patch, usually in the absence of any underlying technical error (8). Second, it was observed that patients undergoing staged, bilateral CEAs had similar rates of embolisation following each procedure (25). Thirdly, we observed that while aspirin therapy had no influence on the rate of post-operative embolisation, the platelets of patients destined to suffer high rates of post-operative embolisation had an increased sensitivity to adenosine diphosphate (ADP) stimulation (26).

The results of this randomised trial have shown that although Dacron patches were associated with a small but significantly higher rate of embolisation (median 5 versus 3), the difference was primarily evident in patients with the lowest rates of post-operative embolisation. In clinical practice, such low rates of embolisation have never been associated with an increased risk of progressing on to thrombotic stroke. The incidence of detecting more than 50 emboli was almost identical for each patch type. More importantly, the incidence of sustained high rate embolisation requiring Dextran therapy was unaffected by patch type (6.6% for dacron, 5% for vein).
However, two Dacron patched patients suffered ipsilateral embolic strokes despite adjuvant Dextran therapy. In one, adequate doses of Dextran could not be administered because of severe cardiac failure. In the second, Dextran abolished the initial phase of embolisation only for it to recur on day 5. The latter is the only example of delayed re-embolisation to be encountered in over 650 patients. Neither patient occluded nor developed a severe stenosis at 30 days suggesting that the embolic phase was transient. These two patients suggest that Dextran may not be the perfect therapeutic agent for controlling embolisation in all high risk patients since there are limitations about the patients in whom it can be used and also its need to be given intravenously. Alternatives to Dextran, include Rheopro or S-nitrosoglutathione (27), but neither have been evaluated in large clinical series. However, to put things in perspective, our policy of selective dextran therapy has been associated with a 0.7% incidence of embolic stroke for the study group as a whole which is significantly better than our experience prior to implementing the protocol (2). Moreover, it has undoubtedly contributed towards a 0% incidence of early post-operative occlusion in 650 patients.

In summary, the findings of this study support the hypothesis that prosthetic patches appear to be as safe as vein with regard to their thrombogenicity. Future research must be directed at identifying why a small subgroup of CEA patients appears to be at such high risk of developing high rate embolisation thereby enabling other therapeutic strategies (e.g. the pre-operative administration of ADP antagonists) to be considered. This would not only make the operation even safer, but would clearly remove the need for any post-operative TCD monitoring.
Comparison of Dacron and vein patches in terms of actual numbers of emboli

![Comparison of Dacron and vein patches in terms of actual numbers of emboli](image)

Comparison of Dacron and vein patches in terms of number of emboli - best fit curve

![Comparison of Dacron and vein patches in terms of number of emboli - best fit curve](image)

Figure 1
Numbers of emboli for vein and Dacron patches
<table>
<thead>
<tr>
<th></th>
<th>Dacron</th>
<th>Vein</th>
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<tbody>
<tr>
<td>Number of patients randomised</td>
<td>136</td>
<td>137</td>
</tr>
<tr>
<td>Bilateral patients receiving same patch</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total number of procedures</td>
<td>137</td>
<td>139</td>
</tr>
<tr>
<td>Carotid bypass performed</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>ICA ligation</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Unsuitable vein (Dacron patch inserted)</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 7.1:**

**Treatment changes following randomisation**
<table>
<thead>
<tr>
<th></th>
<th>DACRON</th>
<th>VEIN</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=137)</td>
<td>(n=139)</td>
<td></td>
</tr>
<tr>
<td>Male:female</td>
<td>94:43</td>
<td>89:50</td>
<td>0.48</td>
</tr>
<tr>
<td>Median age</td>
<td>71 (95%CI 67.3-70.2)</td>
<td>70 (95%CI 68.9-71.5)</td>
<td>0.30</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>90 (66%)</td>
<td>91 (65%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Previous MI</td>
<td>26 (19%)</td>
<td>33 (24%)</td>
<td>0.45</td>
</tr>
<tr>
<td>Treated angina</td>
<td>39 (28%)</td>
<td>42 (30%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>22 (16%)</td>
<td>21 (15%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Current smoker</td>
<td>37 (27%)</td>
<td>31 (22%)</td>
<td>0.40</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>15 (11%)</td>
<td>16 (12%)</td>
<td>0.9</td>
</tr>
<tr>
<td>TIA/amaurosis</td>
<td>82 (60%)</td>
<td>70 (50%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Stroke</td>
<td>40 (29%)</td>
<td>53 (38%)</td>
<td>0.13</td>
</tr>
<tr>
<td>Unilateral &gt;70% stenosis</td>
<td>85 (62%)</td>
<td>91 (65%)</td>
<td>0.53</td>
</tr>
<tr>
<td>Bilateral &gt;70% stenosis</td>
<td>31 (23%)</td>
<td>24 (17%)</td>
<td>0.37</td>
</tr>
<tr>
<td>Stenosis + contralat occln</td>
<td>21 (15%)</td>
<td>24 (17%)</td>
<td>0.75</td>
</tr>
<tr>
<td>Aspirin therapy alone</td>
<td>108 (79%)</td>
<td>101 (73%)</td>
<td>0.27</td>
</tr>
<tr>
<td>Median aspirin dose</td>
<td>75 (95% CI 90-113)</td>
<td>75 (95% CI 90-110)</td>
<td>0.91</td>
</tr>
<tr>
<td>Aspirin + dipyridamole</td>
<td>16 (12%)</td>
<td>25 (18%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Dipyridamole therapy alone</td>
<td>4 (3%)</td>
<td>5 (4%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Warfarin therapy alone</td>
<td>8 (6%)</td>
<td>8 (6%)</td>
<td>1.0</td>
</tr>
<tr>
<td>No antiplatelet/anticoagulant</td>
<td>1 (0.7%)</td>
<td>0 (0%)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 7.2
Pre-operative patient demographics
<table>
<thead>
<tr>
<th></th>
<th>DACRON</th>
<th>VEIN</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median operation time (mins)</td>
<td>103 (95%CI 102-114)</td>
<td>105 (95%CI 102-115)</td>
<td>0.71</td>
</tr>
<tr>
<td>Median shunt time (mins)</td>
<td>44 (95%CI 43-51)</td>
<td>43 (95%CI 42-48)</td>
<td>0.60</td>
</tr>
<tr>
<td>Median total clamp time (mins)</td>
<td>7 (95%CI 6.2-9.8)</td>
<td>7 (95%CI 7.0-9.9)</td>
<td>0.38</td>
</tr>
<tr>
<td>Median patch length (cm)</td>
<td>6 (95%CI 6.0-6.7)</td>
<td>5.8 (95%CI 5.8-6.3)</td>
<td>0.10</td>
</tr>
<tr>
<td>Normal angioscopy</td>
<td>124 (91%)</td>
<td>126 (91%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Intimal flap repaired</td>
<td>3 (2%)</td>
<td>2 (1%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Luminal thrombus removed</td>
<td>6 (4%)</td>
<td>8 (6%)</td>
<td>0.78</td>
</tr>
<tr>
<td>Median neck drain loss (mls)</td>
<td>40 (95%CI 66-112)</td>
<td>40 (95%CI 51-91)</td>
<td>0.038</td>
</tr>
<tr>
<td>Median groin drain loss (mls)</td>
<td>0 (95%CI 0-1.4)</td>
<td>20 (95%CI 22-42)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Median total drain loss (mls)</td>
<td>40 (95%CI 64-109)</td>
<td>60 (95%CI 77-102)</td>
<td>0.058</td>
</tr>
<tr>
<td>Drainage of neck haematoma</td>
<td>10 (7%)</td>
<td>7 (5%)</td>
<td>0.43</td>
</tr>
<tr>
<td>Patients requiring transfusion</td>
<td>10 (7%)</td>
<td>9 (7%)</td>
<td>0.78</td>
</tr>
<tr>
<td>Post-op hypertension</td>
<td>59 (43%)</td>
<td>53 (38%)</td>
<td>0.39</td>
</tr>
<tr>
<td>Median 3hr embolus count</td>
<td>5 (IQR 1-17)</td>
<td>3 (IQR 0-10.5)</td>
<td>0.028</td>
</tr>
<tr>
<td>Dextran administered</td>
<td>9 (6.6%)</td>
<td>7 (5%)</td>
<td>0.62</td>
</tr>
<tr>
<td>Median day of discharge</td>
<td>3 (95%CI 2.7-3.3)</td>
<td>3 (95%CI 2.7-3.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>Superficial neck infection</td>
<td>2 (1.5%)</td>
<td>1 (0.7%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Groin wound infection</td>
<td>0 (0%)</td>
<td>6 (4%)</td>
<td>0.041</td>
</tr>
<tr>
<td>Groin seroma/haematoma</td>
<td>0 (0%)</td>
<td>5 (4%)</td>
<td>0.074</td>
</tr>
<tr>
<td>Median carotid diameter (mm)</td>
<td>10.8 (95%CI 10.3-10.8)</td>
<td>12.0 (95%CI 11.9-12.6)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 7.3

Peri-operative patient demographics
CHAPTER SEVEN

DACRON VEIN

(n=137) (n=139)

**cranial nerve injury**

- mandibular branch of facial nerve: 1 (DACRON), 2 (VEIN)
- hypoglossal nerve: 3 (DACRON), 2 (VEIN)
- recurrent laryngeal nerve: 2 (DACRON), 2 (VEIN)

**operative deaths**

- acute cardiac failure: 1 (DACRON), 1 (VEIN)
- brainstem stroke: 0 (DACRON), 1 (VEIN)
- intracranial haemorrhage: 0 (DACRON), 1 (VEIN)

**disabling stroke**

- MCA embolism: 1 (DACRON), 0 (VEIN)
- cardio-embolic *: 0 (DACRON), 1 (VEIN)

**non-disabling stroke**

- intracranial haemorrhage: 0 (DACRON), 1 (VEIN)
- MCA embolism: 1 (DACRON), 0 (VEIN)

* post-MI, normal Duplex & TCD

Table 7.4

30-day morbidity and mortality
References


25. Hayes P.D., Patel F., Bell P.R.F., Naylor A.R. Thromboembolism after carotid endarterectomy may have a constitutive component. *Eur J Vasc Endovasc Surg* (IN PRESS)
26. Hayes P.D., Box H., Tull S., Gaunt M.E., Bell P.R.F., Goodall A.H., Naylor A.R.,
Thromboembolic events after CEA are not prevented by aspirin but are due to the platelet

27. Molloy J., Martin J.F., Baskerville P.A., Fraser S.C.A., Markus H.S. S-
nitrosoglutathione reduces the rate of embolisation in humans. *Circulation* 1998;
98:1372-1375.
CHAPTER EIGHT

PATIENTS' THROMBOEMBOLIC POTENTIAL FOLLOWING ENDOTHELIAL DISRUPTION IS RELATED TO THE PLATELETS' SENSITIVITY TO ADP.
Abstract

Background and Purpose: Post-operative microemboli in patients undergoing carotid endarterectomy are a significant risk factor for stroke. These emboli can be detected by TCD monitoring. They are not linked to technical error and are variable between patients. As it is known that platelets play a key role in arterial thrombosis, it was hypothesised that a patient’s risk of post-operative carotid thrombosis was linked to the individual’s platelet response to physiological agonists.

Methods: Samples from 120 patients undergoing CEA were analysed prior to surgery. Platelet aggregation was measured in response to ADP (0.5 - 4 μmol/l), collagen (10 - 50 mg/ml) and arachidonic acid (3 or 6 μmol/l), and fibrinogen binding to GPIIb-IIIa, was measured by whole blood flow cytometry in response to ADP (0.1 - 10 μmol/l) and thrombin (0.02 - 0.16 u/ml). Patients underwent TCD monitoring for 3 hours after surgery and platelet functional data was compared in those who had >25 emboli in this period (n=22) with that in those with <25 emboli (n=88).

Results: The platelet response to ADP was significantly higher in the patients with >25 emboli higher as measured both by aggregometry (p=0.0012) and by flow cytometry (p<0.0001). Platelet aggregation with collagen was also significantly higher in this group (p=0.0018) but the response to thrombin was not statistically different in the two groups. In addition there was no difference in the response to arachidonic acid between the groups.

Conclusion: the platelet response to ADP may be linked to clinical outcome, and thus specific ADP receptor inhibitors may be appropriate for this group of patients.
Introduction

Two international, randomised trials have established level I evidence for the role of carotid endarterectomy (CEA) in the management of selected patients with symptomatic carotid artery disease. However, despite its proven benefit, CEA carried a 5-7% risk of peri-operative stroke (1,2). This includes the true intra-operative stroke (apparent upon recovery from anaesthesia) and the post-operative stroke (occurring sometime afterwards). We have previously shown that intra-operative stroke can be virtually abolished by a policy of intra-operative quality control assessment and monitoring, whilst having no effect on the incidence of post-operative thrombotic stroke (3,4).

The commonest single cause of post-operative stroke is thrombosis / embolism from the endarterectomy zone (5). Platelets play a central role in intravascular thromboembolism, and a number of observations led us to question whether some patients had a greater predisposition to platelet deposition within the endarterectomy zone than others. It had previously been assumed that post-operative carotid thrombosis followed inadvertent technical error, but intra-operative transcranial Doppler (TCD) and completion angioscopy prior to restoration of flow had excluded technical error as the cause (3). Secondly, patients destined to thrombose their operated carotid artery have a 1-2 hour period of increasing cerebral embolisation which precedes onset of symptoms (3,6-12). Approximately 50% of patients with sustained high rate embolisation will progress towards thrombotic stroke (3,9). Thirdly, patients undergoing staged, bilateral CEAs have similar rates of embolisation after each procedure; i.e. those patients that embolised after the first operation also developed emboli following the second procedure, and the converse was also true (13). Finally, in a prospective randomised trial of vein versus prosthetic patch closure,
the incidence of sustained, high rate post-operative embolisation was unrelated to patch type (14).

Given the central role of platelets in intravascular thrombosis, the hypothesis underlying the current study was that increased rates of post-operative embolisation following CEA reflected increased platelet reactivity. We therefore studied pre-operative platelet responsiveness to incremental doses of physiological agonists and correlated the results with the number of emboli detected in the post-operative period using TCD.

Materials and Methods

Patients and blood sampling

The study was approved by the Leicestershire Ethics Committee and all patients gave written informed consent. One hundred and twenty patients were enrolled into the trial. All patients were seen in the single visit out-patient clinic, where they were assessed clinically and by carotid Duplex scanning. Only 5% of the patients required arteriograms. All patients were standardised to 75mg of aspirin for the four weeks prior to carotid endarterectomy (CEA). Any patient taking dipyridamole, thienopyridines or warfarin were excluded. All clinical data was collected prospectively.
Pre-operative blood samples were collected on the morning prior to surgery, between 07.30 and 08.30 to minimise the effect on diurnal variation. Patients had been resting supine for at least 20 minutes and sample collection was performed using a standardised phlebotomy technique designed to minimise platelet activation. Blood was taken without the use of a tourniquet, by clean venepuncture, from an antecubital vein via a 21-gauge butterfly needle, into vacutainers (Becton Dickinson). The first 4.5ml of blood were discarded and subsequent aliquots of 4.5ml were collected into 0.5ml of 3.8% tri-sodium citrate.

Reagents:
ADP, arachidonic acid (sodium salt), human α-thrombin and GPRP peptide were from Sigma (Poole, Dorset, UK). Bovine collagen (Horm, Germany) was from Nycomed. GPIbα and GPIIib/IIIa were identified with FITC-conjugated RFGP37 and RFGP56 Mabs respectively and platelet bound fibrinogen was detected with FITC-conjugated rabbit ant-human fibrinogen (Dako Ltd., High Wycombe, UK) as described previously (15). All antibodies were used at their optimal concentration, determined by titration.

Aggregometry
Platelet aggregation was measured in platelet rich plasma by Born-type aggregometry as previously described (16). Aggregation was measured in response to final concentrations of ADP (0.5, 1, 2 and 4 μmol/l), collagen (10, 20 and 50 mg/ml) and arachidonic acid (3 or 6 μmol/l).
Flow cytometric analysis:

Blood samples were prepared for flow cytometric analysis within 10 minutes of collection, using a whole blood method described previously (15,16). Fibrinogen binding was measured in unstimulated blood samples and in samples stimulated with ADP (0.1, 1, 10 μmol/l) or thrombin (0.02, 0.04, 0.08, 0.16 u/ml), the latter in the presence of GPRP peptide to prevent clot formation. All samples were prepared in duplicate and analysed within 2 hours of collection in a Coulter EPICS Profile II flow cytometer (Coulter Electronics Ltd., Luton, UK). The platelet population was identified from its light scatter characteristics and identity confirmed using the anti-GPIbα Mab, RFGP37-FITC. Five thousand platelets were analysed and the values were recorded as the percentage of cells positive for fluorescent antibody binding. Expression of GPIbα and GPIIb/IIIa were measured as the mean fluorescence (MFI).

Operative procedure

Carotid endarterectomy was performed in a standardised manner throughout the study period. The operation was undertaken with the patient under normocarbic, normotensive general anaesthesia using systemic heparinisation (5000 units unfractionated heparin). A Pruitt-Inahara shunt was used in all cases. The proximal and distal intimal steps were tacked down using 7-0 prolene and all arteriotomies closed with a patch angioplasty. Blood flow velocity in the middle cerebral artery was monitored continuously through the operation with ipsilateral transcranial Doppler ultrasound (TCD) using a 2 MHz fixed, head probe. The lumen was inspected with a flexible hysteroscope (Olympus) prior to flow restoration, to exclude luminal
thrombus or large intimal flaps. All technical errors were corrected prior to restoration of flow. Following recovery from anaesthesia, the patient was transferred to the recovery area of theatre or the high dependency unit for a 3-hour period of TCD monitoring. All TCD data were recorded on to digital audio tape for offline analysis of emboli counts by a technician blinded to the pre-operative blood test results. Dextran-40 (Pharmacia Ltd, Milton Keynes, England) was administered to any patient who had (i) \( > 25 \) emboli in any 10 minute period or (ii) emboli that distorted the MCA waveform which suggested that they were large, according to our previously described protocol (17).

Any new neurological deficit persisting for more than 24 hours within the first 30 days after surgery was classified as a stroke and the severity scored at 30 days by a neurologist using the Oxfordshire Handicap Scale (OHS). A stroke score of 0-2 was classified as non-disabling whilst a score of 3-5 was deemed disabling. Any patient suffering a peri-operative stroke was investigated by CT head scan, Duplex ultrasound plus TCD examination of the extra- and intra-cranial circulation. Patients suffering a fatal stroke underwent autopsy. All patients undergoing CEA in this series were evaluated in the early post-operative period by a neurologist. All data relating to clinical outcome was collected prospectively through the study.
Statistical analysis

Discrete data was analysed using contingency tables (Chi-squared or Fisher’s exact test as appropriate) and continuous data using two-way ANOVA. A “p” value less than 0.5 was taken as significant.

Results

Clinical

One hundred and twenty patients had pre-operative blood samples collected. Of these, 110 (92%) had a cranial window suitable for TCD to determine the magnitude of post-operative embolisation. The 30-day death and any stroke rate was 2.5% (2 deaths and 1 disabling stroke). Five patients (4.5%) developed rates of embolisation in excess of 25 emboli in a 10-minute period and were initiated on an infusion of Dextran-40 according to the unit protocol (14).

Platelet function and post-operative emboli counts.

The results from the pre-operative aggregometry and flow cytometry studies were correlated with the number of post-operative emboli detected in the first three hours after flow restoration across the subendothelium of the endarterectomised vessel wall. The patient population who were suitable for TCD monitoring were split into two groups – a group at increased risk of a thrombotic stroke with >25 postoperative emboli (n=22; 20%) and a low risk group <25 post-operative emboli (n=88; 80%) –
see Figure 1 for the range of emboli counts. The patient risk factors for the low and high risk groups are shown in Table 1.

**Aggregometry**

Platelet aggregation in response to arachidonic acid was used as a marker of the effect of aspirin's inhibition of the cyclo-oxygenase pathway in platelets. Aggregation in response to 3µmol/L arachidonate was blocked by aspirin in all but eight of the patients (6 low risk and 2 high risk), but the majority of patients showed some response to 6µmol/L arachidonate, despite the use of aspirin. There was no difference between the low and high risk groups in terms of their mean aggregation in response to 3 µmol (13.3% [7.5 – 19.1] vs 17.9% [0.6 – 35.1], p=0.583) or 6 µmol (28.6% [23.4 – 33.9] vs 43.2% [26.0 – 61.5], p=0.128) arachidonic acid. Similarly, no difference was noted in the rate of aggregation between the two groups in response to arachidonic acid. Alternate analysis of this data provides a similar result. If the patients are split into quartiles with regard to the percentage aggregation in response to arachidonic acid, the most responsive group do not have significantly more emboli than the group with the least response to arachidonic acid (median number of emboli for each quartile: 3, 3.5, 5 and 3).
In contrast, when ADP was used as the stimulus for platelet aggregation, significant differences were found between the two groups of patients (Figure 2a). In the high-risk group of patients with large numbers of post-operative emboli, the platelets were significantly more sensitive to the effects of ADP. The magnitude of aggregation in the high-risk group was increased significantly (p=0.0012; two-way ANOVA) by 25%, 21%, 14% and 11%, relative to the patients with low thrombotic risk for the four concentrations of ADP (0.5, 1, 2 and 4 μmol/l respectively). The magnitude of aggregation in response to collagen demonstrated a similar pattern to the ADP stimulation (Figure 2b). Relative to the low risk group, the higher rate embolisers had an enhanced aggregation in response to stimulation with the three doses of collagen, of 31%, 19.5% and 10.5% (p=0.0018; two-way ANOVA)

The rates of aggregation with ADP and collagen were also greater in the patients with most emboli (data not shown).

Flow cytometry

The data for the flow cytometry was analysed in a similar manner to the aggregometry data. There was no difference between the two groups with regard to the level of expression (mean fluorescent index) of the platelet surface receptors GPIb (19.2±6.5 vs 19.5±6.9, p=0.87) or GPIIb/IIIa (24.0±7.3 vs 21.7±6.1, p=0.17). The percentage of unstimulated platelets with bound fibrinogen on their surface was not significantly different between the high and low risk groups (2.7±4.2% vs 2.0±3.5%, p=0.417).

Stimulation of the platelets with three physiological concentrations of ADP prior to incubation with the fibrinogen demonstrated that the patients who had most post-
operative emboli bound significantly increased amounts of fibrinogen after ADP incubation (Figure 3), in line with the aggregometry data. For the three concentrations of ADP, the relative increase in the percentage of cells binding fibrinogen in the high-risk group, compared to the low risk group was 36%, 15.5% and 10% for 0.1, 1 and 10 μmol/l respectively (p<0.0001; two-way ANOVA).

In contrast, fibrinogen binding (percentage of cells positive) was not significantly different at any of the four concentrations of thrombin used to stimulate platelets, when comparing the high and low risk groups. The mean percentage differences between the groups were −2%, +22%, +15% and −2% with 0.2, 0.4, 0.8 and 0.16 u/ml thrombin respectively (P≥0.18 for all).

Discussion

The hypothesis of this study was that platelets from patients who developed high numbers of post-op emboli were more responsive to stimulation by physiological agonists than those from patients with a low embolic potential. Identification of the specific platelet stimulatory pathway with the strongest association with post-intervention thromboembolic events may allow targeted anti-platelet therapy, enabling a better balance between haemostasis and thrombotic risk to be found.

The appearance of post-operative emboli was used as the clinical end-point in this study as a surrogate maker for risk of post-operative carotid thrombosis (POCT).
Thrombotic events after arterial intervention can cause significant morbidity or mortality, but they occur at a relatively low frequency and it would therefore be impractical to perform multiple complex laboratory investigations on the very large numbers of subjects that would have to be studied if symptomatic arterial thrombosis was the end point. The association between increasing rates of post-operative embolisation and thrombotic stroke has been documented by this unit and others (3, 6-11), and confirmed in a study of 500 patients undergoing CEA in this unit (18). In the latter study 5% of patients developed the pattern of severe sustained embolisation that has been shown to have a 50% risk of progression onto POCT (3,10).

The level of post-operative embolisation varies considerably amongst CEA patients (17). The hypothesis underlying the current study was that the magnitude of post-operative embolisation reflected an enhanced state of platelet activation and aggregation in susceptible patients. The hypothesis was based on observations that: (i) POCT invariably occurred in the very early post-operative period in the absence of underlying technical error, (ii) POCT appeared to be associated with enhanced platelet deposition and (iii) POCT occurred independently of pre-operative clinical status, concurrent disease or patch type. In contrast, patients undergoing staged, bilateral CEAs had similar rates of embolisation after each procedure suggesting an underlying constitutive aetiology (13).
The hypothesis is, of course, dependent on the fact that these so called ‘TCD high intensity transients’ represent emboli rather than artefact. A number of centres have now established reliable methods for differentiating emboli from artefacts using either dual gated ultrasound (19,20). This unit has also demonstrated that emboli that are observed in the early post-operative period after CEA are exclusively particulate (17). With regard to clinical relevance, the magnitude of particulate embolisation detected by TCD has now been shown to be predictive of the risk of stroke after acute myocardial infarction (21), with cognitive impairment after CEA (8), with ischaemic changes on MRI after CEA (12,22) and with progression onto post-operative carotid thrombosis (3, 6-11).

In previous studies, POCT consistently occurred in patients who were on routine aspirin therapy and in the present study all patients were standardised to low-dose (75mg per day) aspirin for four weeks prior to procedure, and remained on aspirin throughout the peri-operative period. Aspirin has been the mainstay of anti-platelet therapy for two or more decades now, and despite its well proven effectiveness in reducing thromboembolic events aspirin only inhibits one of a number of pathways through which platelets can be stimulated. Thus simply preventing thromboxane A2 release from platelets by inhibiting the cyclo-oxygenase pathway is not adequate to prevent intra-arterial activation and aggregation of platelets (23,24). Our initial hypothesis was that an increased risk of thromboembolisation in a patient could be linked to a reduced sensitivity to aspirin, so-called aspirin resistance. There was, however, no correlation between the magnitude of embolisation and the ability of aspirin to inhibit platelet aggregation induced by arachidonic acid. Thus while aspirin
alone might have prevented some post-operative thrombo-embolic events (ethically we could not withdraw peri-operative aspirin therapy to test this hypothesis), a proportion of CEA patients will continue to have enhanced platelet activation through alternative pathways.

The platelets of patients with >25 emboli in the first three post-operative hours had clear evidence of significantly increased responses to the physiological agonist ADP. In particular, higher rate embolisers had a significant increase in the number of platelets binding fibrinogen (the final common pathway for platelet aggregation) in response to ADP stimulation. The results from the optical aggregometry paralleled those found with the flow cytometry, with the rate and magnitude of platelet aggregation in response to ADP being significantly higher in those with >25 post-operative emboli. This suggests that the platelets of patients with higher rates of post-operative embolisation have an increased sensitivity to ADP. ADP is released from platelet dense granules when platelets are activated and when they adhere to collagen in the damaged vessel wall, or from damaged red cells. The platelet response to collagen is also modulated by ADP (25,26), and therefore the parallel increase in platelet aggregation in response to collagen in the high embolisers may also reflect their sensitivity to collagen. Hence the enhanced platelet responsiveness seen in the patients with the highest levels of thromboembolisation may reflect a specific sensitivity to ADP. This conclusion is supported by the fact that the magnitude of the mean increase in aggregation with ADP was virtually identical to that observed following collagen stimulation. In contrast, there was no statistically significant
association between the magnitude of post-operative embolisation and the platelet response to thrombin.

Platelets have two receptors for ADP; the P2Y_1 receptor that is primarily responsible for platelet shape change, and the P2Y_{12} receptor that amplifies the activation signal through an increase in cytosolic free calcium (25-27). The importance of both receptors has been demonstrated in patients with deficiencies of one or other of these receptors (28), and in studies in knockout mice (29). It is not clear from the present studies whether the increased responsiveness to ADP reflects differences in expression or function of one or both of these receptors, or reflects differences in downstream signalling events within the platelets. It would not appear to be a reflection of the level of expression of the GPIIb-IIIa receptor as this was not different between the high and low embolisation groups. Clearly an understanding of the underlying mechanisms could point to an improved therapeutic strategy in these patients.

The P2Y_{12} ADP receptor antagonist Clopidogrel is proving an effective anti-platelet agent in the context of coronary stenting (30) and unstable coronary syndromes (31,32). In the light of the present findings this agent is currently being tested in a prospective, placebo controlled, randomised trial in our unit. However, a balance must be sought between reducing or abolishing platelet activation against the risks of increased peri-operative bleeding (33). A greater understanding of the factors influencing platelet function in specific patient groups may guide a more directed approach to anti-platelet therapy.
This study provides evidence that individuals' platelet sensitivity to the effect of ADP may influence their risk of thrombosis following vascular intervention. Although this study was performed in patients undergoing open vascular surgery it is likely that the findings are also relevant to other vascular interventions such as angioplasty. The availability of thienopyridine compounds (ticlopidine and clopidogrel) that specifically affect the ADP pathway may provide an avenue for targeted anti-platelet therapy.

Aspirin has been the mainstay of anti-platelet therapy for two or more decades now, and despite its well proven effectiveness in reducing thromboembolic events, many patients suffering an arterial thromboembolic complication are actually on aspirin therapy at the time. Aspirin only inhibits one of a number of pathways through which platelets can be stimulated, and simply preventing thromboxane A2 release from platelets by inhibiting the cyclo-oxygenase pathway is not adequate to prevent intra-arterial activation and aggregation of platelets. Conversely, inhibition of fibrinogen binding to platelets by GPIIb/IIIa receptor agonists would rapidly reduce residual platelet activity to very low levels. This places patients at significant risk of haemorrhagic complications and whilst bleeding following an arterial puncture for an angioplasty can usually be controlled by simple pressure, the results of open surgery in this situation are likely to be very different.
Figure 1: The range of emboli counts following CEA

Range of emboli counts in 110 patients following CEA

Patients ranked in order of emboli numbers

N=88

80% of patients

N=22

20% of patients
### Table 8.1
Risk factors for the high and low risk groups

<table>
<thead>
<tr>
<th></th>
<th>Emboli &gt; 25</th>
<th>Emboli &lt; 25</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=22</td>
<td>N=88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (median yrs)</td>
<td>69</td>
<td>69</td>
<td>0.273</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>15</td>
<td>17</td>
<td>0.447</td>
</tr>
<tr>
<td>Hypertensive (%)</td>
<td>55</td>
<td>61</td>
<td>0.371</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>30</td>
<td>29</td>
<td>0.422</td>
</tr>
<tr>
<td>Previous MI (%)</td>
<td>30</td>
<td>26</td>
<td>0.497</td>
</tr>
<tr>
<td>Angina (%)</td>
<td>35</td>
<td>39</td>
<td>0.391</td>
</tr>
<tr>
<td>Claudication (%)</td>
<td>28</td>
<td>29</td>
<td>0.493</td>
</tr>
<tr>
<td>Aspirin dose (mg)</td>
<td>75</td>
<td>75</td>
<td>0.144</td>
</tr>
<tr>
<td>Carotid stenosis (%)</td>
<td>81</td>
<td>82</td>
<td>0.393</td>
</tr>
</tbody>
</table>

*Presented as:*

|                  |             |             |     |
| Stroke (%)       | 45          | 29          | 0.178 |
| TIA/ Am fugax (%)| 45          | 45          | 0.992 |
| Asymptomatic (%) | 10          | 26          | 0.134 |
Figure 8.1a & 8.1b

The percentage of platelets aggregating in response to the agonists a) ADP (p=0.0012) and b) collagen (p=0.0018).

[Graph showing percentage aggregation vs. [ADP] μmol and [collagen] mg/ml with data points and error bars for <25 emboli and >25 emboli.]
Figure 8.2

Percentage of platelets binding fibrinogen in response to the agonist ADP (p<0.0001).
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Combined therapy with clopidogrel and aspirin significantly increases the bleeding
CHAPTER NINE

DEXTRAN-40 STIMULATES RESTING PLATELETS
AND BINDS MORE AVIDLY TO ACTIVATED
PLATELETS
Introduction

Dextrans are a product of Leuconostoc mesenteroides. These poly-D-glucopyranones are of widely varying molecular weights, but are fractionated to specified molecular weight bands for clinical and scientific use. Dextran was first introduced as a plasma expander in 1944\(^1\). In 1959, it was described as a potential anti-thrombotic agent\(^2\) and in 1961 it was used experimentally to aid in the prevention of post-operative thromboembolism\(^3\). Since then it has been used in a number of clinical and non-clinical settings.

In 1997, the use of Dextran-40 to treat thromboembolic complications after carotid surgery was described by two separate groups\(^4,5\). Carotid endarterectomy (CEA) is performed to reduce the risk of stroke in patients with a stenosis of their carotid artery greater than 70%\(^6\). It involves the opening of the arterial wall and the excision of the intima and some of the medial layer of the wall. This exposes the thrombogenic subendothelium over a length of approximately five to ten centimetres. The defect in the arterial wall is then closed and flow is restored through the lumen of the blood vessel.
Although CEA is performed to prevent strokes, in a number of cases it may actually cause a stroke. In the absence of technical defects, many of these strokes are due to occlusion of the endarterectomised vessel with platelet rich thrombus. Transcranial Doppler ultrasound (TCD) can detect small emboli as they pass through a blood vessel. If it is focused on the middle cerebral artery after CEA, then emboli are detected as they arise from the endarterectomy zone. In patients who are at risk of carotid thrombosis, the numbers of emboli detected increases with time as the thrombus grows in size, until the lumen is completely occluded. If these patients are given low dose (20ml/hour), intravenous 10% Dextran-40 the rate of embolisation falls back towards zero, and the potential carotid thrombosis is prevented (Figure 1).

Using this technique we have now performed over 600 CEAs without a carotid thrombosis. However, it is not clear from the published data how low dose Dextran-40 exerts this dramatic anti-thromboembolic effect. Much of the published literature on the effects of Dextran-40 is from the 1960’s and ’70s and since the publication of many of these studies new techniques for the assessment of platelet function have been developed. We have studied the effects of Dextran-40 on human platelets using flow cytometry and enzyme linked immunosorbent assay, as well as platelet aggregometry, in an attempt to answer a number of questions:

- Does Dextran-40 bind to the platelets’ surface?
- ii) How rapidly does Dextran-40 bind to the platelet surface?
- iii) What effect does Dextran-40 binding exert on the platelet
- iv) Do activated platelets bind more Dextran-40 than resting platelets?
- v) Can Dextran-40 encourage platelet disaggregation?
Methods

Blood collection

Sample collection was performed using a standardised phlebotomy technique to
designed to minimise platelet activation. Two groups were studied, the first were 10
patients due to undergo a carotid endarterectomy and the second were healthy
volunteers from the Departments of Surgery or Pathology at the University of
Leicester. All patients or volunteers had normal platelet counts (>150 x 10^9/ml and
<400 x 10^9/ml). Ethical committee approval was obtained from the local
Leicestershire Health Ethics Committee.

All samples were collected between 08.30 and 09.30 to minimise the effect of diurnal
variation. Blood was taken without the use of a tourniquet, by clean venepuncture
from an antecubtal vein via a 21-gauge needle. The first 4ml of blood were collected
into EDTA and used to determine the full blood count. Subsequent aliquots of 4.5ml
were collected into 0.5ml of 3.8% tri-sodium citrate (Becton-Dickinson, Meylan,
France). Sample processing was commenced within 10 minutes of collection.

Reagents

Antibodies. All antibodies were used at their optimal concentration. Platelets were
identified with RFGP37, a mouse monoclonal antibody (Mab) to Gp Ib (CD42b),
which was conjugated to fluorescein isothiocyanate (FITC) (Cymbus Biotechnology,
Chadlersford, UK), with a molar FITC:protein ratio of 3-4:1. A FITC- conjugated
Mab against Gp IIb/IIIa was also used to confirm the presence of the platelet
population (Cymbus). Platelet bound fibrinogen was detected with a FITC-conjugated polyclonal rabbit antibody to human fibrinogen (Rαfgn-FITC), purchased from Dako Ltd (High Wycombe, UK) \(^2\). Platelet expression of P-selectin (CD 62P) was identified by a R-phycoerythrin (RPE) conjugated Mab (Cymbus Biotechnology, Chandlersford, UK). FITC-conjugated Dextran-40 was obtained from Sigma Chemical Company Ltd (Poole, Dorset, UK).

**Agonists.** Adenosine diphosphate (ADP), arachidonic acid (sodium salt) and human \(\alpha\)-thrombin were purchased from Sigma Ltd (UK). Bovine collagen was obtained from Born (Germany). Samples incubated with thrombin also contained 0.125mmol/l L-glycyl-L-proyl-L-arginyl-L-proline peptide (GPRP) (Sigma), which inhibits fibrin polymerisation and consequent clot formation \(^3\).

**Flow Cytometric Analysis**

Blood samples were prepared for flow cytometric analysis using the whole blood method described by Janes et al \(^2\). Five ul of citrated blood were added to LP3 tubes containing 50\(\mu\)l of Hepes buffered saline (HBS: Na Cl 0.145 mol/l; KCl 5 mol/l; MgSO\(_4\) 1 mmol/l; Hepes 10 mmol/l; pH 7.40) and 5\(\mu\)l of appropriate concentrations of antibodies. In some experiments, 5\(\mu\)l of the following agonists were also included (final concentrations): ADP (10-5 – 10-7M) or thrombin (0.02 – 0.16 units/ml) with GPRP. Dextran-40 was used both in its FITC-conjugated and unconjugated forms (0.05 – 2 mg/ml). After gentle mixing, the samples were incubated for 20 minutes and then diluted with 500\(\mu\)l of 0.2% formyl saline, to inhibit further activation.
Incubations were all carried out at room temperature (22-26°C), and all samples were performed in duplicate.

Samples were analysed, within 2 hours of collection, in a Coulter EPICS Profile II flow cytometer (Coulter Electronics Ltd, Luton, UK). The instrument was aligned daily with “Immunocheck” and “Standard Brite” beads (Coulter Corp., Hialeah, Florida) to calibrate the light scatter and fluorescence parameters respectively. The platelet population was identified from its light scatter characteristics and identity was confirmed using the anti-Gp Ib Mab (RFGP37-FITC). An electronic bitmap was set around the platelet population and adjusted for each sample to ensure that >98% of the particles analysed were positive for Gp Ib. The negative cut-off levels for fluorescence in the unstimulated samples were set at 2%. Five thousand platelets were analysed and the values were expressed as the percentage of cells positive for fluorescent antibody binding, the mean fluorescent intensity (MFI) of each platelet and the binding index (percentage of cells positive for antibody times the MFI).

**Aggregometry**

Samples were analysed in parallel with the flow cytometric samples. Blood was collected as described and then used to perform platelet aggregometry according to the turbidometric method of Born. Three agonists were used, ADP (0.5 - 4 μmol/l), collagen (10 - 50 μg/ml) and ristocetin (0.56 - 1.88 μg/ml).
**Von Willebrand Factor ELISA**

The assay used was a qualitative direct enzyme immunoassay for the detection of vWF activity in citrated human plasma from Shield Diagnostics Ltd (Dundee, UK). This ELISA uses a murine monoclonal IgG antibody which recognises a functional epitope on the vWF antigen\textsuperscript{15}.

**Results**

*Does Dextran-40 bind to the platelets' surface?*

Platelets from 10 healthy volunteers were incubated with five concentrations of FITC-conjugated Dextran-40 for 10 minutes before being processed as described above for flow cytometry. The platelets were then left resting or were stimulated with ADP (1\times10^{-6}M ADP) or thrombin (1.6u/ml). The percentage of platelets binding the Dextran-40 paralleled the rising concentration of the Dextran-40 with which the platelets were incubated. For the resting platelets this increased significantly from 3.7% in the control sample (no dextran) to 12.9% with the lowest dose of Dextran-40 (0.25mg/ml) (p=0.006) and to 63.8% for the highest dose of Dextran-40 (2mg/ml) (p<0.001). Very similar results were seen when the MFI of the platelet bound Dextran-40 were studied. The incubation of the platelets with either of the agonists did not affect the binding of the Dextran-40 to the platelets’ surface. These results are displayed in figure 2.
This experiment was repeated in a further group of 4 healthy volunteers before and then 4 hours after the ingestion of a single dose of 150mg of oral aspirin. The addition of the aspirin did not alter the binding of the Dextran-40 to the platelets’ surface and a similar dose dependent binding pattern was noted as before (data not shown).

How rapidly does Dextran-40 bind to the platelet surface?

A time course study was performed to examine the rate at which Dextran-40 bound to the surface of the platelets, using platelets from 2 healthy volunteers. The platelets, either resting or stimulated (1.6u/ml thrombin), were incubated with Dextran-40 (2mg/ml) for a set period (10,20,30,40,50,60,120,300,600 and 1200 seconds) before the assay was inactivated using 500ul formyl saline. For both the resting and activated platelets the binding of Dextran-40 was maximal (80.8% and 85.4% respectively) at the shortest time point (10s) and did not differ significantly for any of the time points studied.

What effect does Dextran-40 binding exert on the platelet?

Having determined that Dextran-40 binds to the surface of platelets in a dose dependent manner, we further sought to evaluate the effect this binding exerted on platelet activity. Platelets were collected from 10 patients taking 75mg aspirin prior to a carotid endarterectomy using the standardised method described above. The platelet response to Dextran-40 was evaluated using both optical aggregometry and flow
cytometry. For the flow cytometry the platelets were incubated with 4 concentrations of non-FITC conjugated Dextran-40 (0.25, 0.5, 1.0, 2.0mg/ml) or control for 10 minutes. The platelets were then split into 3 aliquots and either left resting or stimulated with ADP (1x10^-6M) or thrombin (0.4u/ml). The ability of the platelets to bind fibrinogen or express p-selectin was then determined using whole blood flow cytometry.

In response to the 4 concentrations of Dextran-40, the percentage of resting platelets binding fibrinogen increased by 266% (p=0.057), 215% (p=0.051), 190% (p=0.066) and 262% (p=0.035). A non-significant increase in fibrinogen binding was seen in platelets stimulated with ADP or thrombin (see Table 1). The percentage of platelets expressing P-selectin on their surface was significantly increased by incubation with Dextran-40, but only when the platelets were activated with ADP (see Table 1). The density (MFI) of fibrinogen binding also significantly increased following incubation with Dextran-40 in the resting platelets (p<0.002 for all) and also for ADP (p= 0.053, 0.023, 0.038 and 0.009), with no change seen in those incubated with thrombin (see Table 2).

For the aggregometry, the platelets (PRP) were incubated with Dextran-40 for 10 minutes before being stimulated with either ADP (4x10^-4M) or collagen (0.2ug/ml). The platelet response was evaluated in terms of the final percentage of platelets aggregating or the mean rate of their response. Incubation with even the lowest dose of
Dextran-40 significantly increased both the total percentage aggregation in response to the agonist ADP (31.3%, p=0.047) as well as the mean rate (43%, p=0.022). A very similar trend was noted for collagen, with all but the lowest dose of Dextran-40 producing significant increases in total aggregation and mean rate of aggregation (see Table 3).

*Do activated platelets bind more Dextran-40 than resting platelets?*

The next stage of the study was to determine whether the platelets’ state of activation at the time it is exposed to the Dextran-40 influences the amount of Dextran-40 that subsequently binds to its surface. Platelets were collected from 9 healthy volunteers and then incubated with 6 different concentrations of FITC-conjugated Dextran-40 (0.0625mg/ml to 2mg/ml) under 3 different conditions. The control group were simply resting platelets, the second group were incubated with one of the concentrations of Dextran-40 for 5 minutes and then stimulated with 1.6u/ml of thrombin, with the final group being stimulated first with the same dose of thrombin and then incubated with the Dextran-40. The platelets were also labelled with PE-conjugated p-selectin to ensure that significant platelet activation had occurred.
The mean activation of the platelets by the 1.6\(\mu\)l/ml thrombin was 93.9\% (range 88.4-95.5\%) for the group incubated with Dextran-40 before activation against 92.9\% (range 90.7-95.3\%) for the group activated prior to incubation with Dextran-40 (\(p=0.50\)). As noted previously, the percentage of platelets binding Dextran-40 increased in a linear manner along with the Dextran-40 concentration (\(R^2=0.88, p<0.001\)).

There was no significant difference between the amount of Dextran-40 bound to the platelets when comparing the resting group and those platelets incubated with Dextran-40 prior to activation, either in terms of percentage of platelets binding Dextran-40 or the MFI (\(p>0.15\) for all). However, when the platelets were stimulated with thrombin prior to the incubation with Dextran-40, the binding of the Dextran-40 increased very significantly (188\%, 174\%, 134\%, 118\%, 90\% and 57\% for the 6 concentrations of Dextran-40, \(p<0.0002\) for all) – see Figures 3a & 3b.

By using 2-colour analysis of the flow cytometer it is possible to identify the proportion of platelets that are both activated (PE positive) and binding Dextran-40 (FITC positive). There is a significant increase in the proportion of platelets positive for both PE and FITC when the Dextran-40 incubation takes place after activation (from 16.7\% to 44.2\%, \(p=0.0001\), for the lowest concentration of Dextran-40 and from 58.9\% to 90.0\%, \(p<0.0001\), for the highest dose of Dextran-40). The other concentrations show similar significant changes (data not shown).
Can Dextran-40 encourage platelet disaggregation?

This section of the study was performed to examine whether Dextran-40 was able to cause the breakdown of formed or forming platelet aggregates. Platelets from healthy volunteers were collected as described. They were then split into 3 aliquots for use in a standard optical aggregometry system. Three agonists were used to stimulate the platelets, ADP (1.5x10-5M), collagen (0.2ug/ml) and ristocetin (2mg/ml). For each of the agonists 4 concentrations of Dextran-40 were added to the platelet rich plasma used for the aggregometry (0,2, 4, 8mg/ml). For each of the agonists the Dextran-40 was added at 2 separate time points, 1 minute after the addition of the agonist and then when 50% of the platelets had aggregated. All of these combinations were tested on an n of 4.

All of the agonists used led to full aggregation of the platelets (>85% in all cases). None of the 3 concentrations of Dextran-40 inhibited aggregation of the stimulated platelets to a greater extent than control (p>0.25 for all) for any of the agonists used. The addition of the Dextran-40 at the start of platelet aggregation or halfway through did not affect the final percentage of aggregation (p>0.18 for all) or the mean rate of aggregation (p>0.22 for all).
Does Dextran-40 block vWF binding?

Plasma samples were collected from 4 healthy volunteers into citrated tubes as described above. Five concentrations of Dextran-40 were added to the plasma samples in the ELISA (0, 0.25, 0.5, 1 and 2mg/ml). All samples were run in duplicate. The mean vWF concentration in the control samples was 1.41 units (SD 0.28) and 1.31 units in the 2mg/ml Dextran-40 samples (p=0.58). No differences were noted for other concentrations of Dextran-40.

Discussion

Dextran-40 is very effective at preventing post-operative thrombotic strokes following carotid endarterectomy. Our unit has now performed over 700 CEAs since we initiated a programme of selectively administering low dose (20ml bolus + 20ml/hour) Dextran-40 to patients deemed to be at high risk of a post-operative thrombotic stroke on the basis of a rising count of micro-emboli on post-operative transcranial Doppler monitoring. Before initiating this policy the unit had a post-operative thrombotic stroke rate of 2.8%. Since using Dextran-40 there has not been a single episode, as opposed to the predicted 19.6 (700 cases x 0.028). At approximately US$8 per litre it is also a very cheap and cost effective therapy.
Our initial hypothesis was that since platelets were one of the central components of a thrombotic process occurring within the arterial circulation, that they would be directly inhibited by Dextran-40. In the clinical setting, the effect of Dextran-40 can be seen within 10-15 minutes of starting the infusion, in terms of the number of microemboli being detected beginning to fall. The time points and incubation times in our study were chosen with this in mind. In addition, the doses used for the in vitro experiments were selected to reflect the normal pharmacological doses used in patients – 20 minutes after initiating clinical therapy the concentration of the Dextran-40 in the circulation is approximately 0.54mg/ml and the range of our in vitro doses was 0.125mg/ml to 2mg/ml.

It is apparent from the data above that Dextran-40 adheres to the platelet surface within 10 seconds of co-incubation. The correlation between platelet bound Dextran-40 and the concentration of Dextran-40 in solution appears to have a strong linear relationship for the doses studied ($R^2=0.88$). Since there is no evidence of saturation occurring, this would suggest that the binding of the Dextran-40 is either non-specific or to a very common platelet surface antigen. The short time period would argue against the release and expression of a receptor from any of the platelet granules. Work from our unit has also shown that Dextran-40 adheres in a similar dose dependent manner to a number of leukocytes, including granulocytes and macrophages (unpublished data) and this supports the hypothesis that the binding is a non-specific phenomenon.
The binding of the Dextran-40 to its surface may stimulate the platelet. There is certainly an increase in the amount of fibrinogen detected binding to the resting platelet following incubation with Dextran-40. This increase does not appear to be related to the dose of Dextran-40 used as it is similar for the highest and lowest doses. This suggests that the binding of Dextran-40 does stimulate the platelet and lead to an increase in true fibrinogen binding sites rather than a non-specific binding of fibrinogen to the surface bound Dextran-40. At the lowest dose of Dextran-40 only 12.9% of platelets are positive for FITC-conjugated Dextran-40, whilst at the highest concentration this increases to 63.8%, and so if the increase seen in fibrinogen binding was non-specific it would be expected to rise in line with the FITC-Dextran-40 expression. These data also indicate that only very low concentrations of Dextran-40 are needed to stimulate the platelet since there is no change in the level of activation seen with increasing doses. A similar non-significant trend is seen in fibrinogen binding following incubation with Dextran-40 and ADP/thrombin. This is likely to simply represent a type II statistical error arising because of the increased variability of results following platelet activation.

P-selectin expression, either percentage of positive cells or MFI, in resting platelets or in those stimulated with thrombin is not affected by incubation with Dextran-40. The percentage of platelets expressing p-selectin increases significantly from control levels when both ADP and Dextran-40 are incubated with the platelets, with the p-selectin levels increasing stepwise with the Dextran-40 concentration (6%, 23%, 23% and 28%).
The process where by Dextran-40 stimulates platelets is unclear at the present. It may represent non-specific binding of Dextran-40 to specific stimulatory platelet receptors. The alternative is that since Dextran-40 is a relatively big molecule, when enough of them bind it is altering the surface charge of the platelet making it more sensitive to activation from other sources.

Platelets that have been stimulated before exposure to Dextran-40 bind significantly more than when they are stimulated after exposure. In fact, stimulating the platelets first with thrombin approximately doubles the percentage of platelets that bind Dextran-40 to their surface for all 6 of the concentrations of Dextran-40 used. This difference is even more marked when the MFI of the FITC-conjugated Dextran-40 is studied, with the highest concentration of Dextran-40 binding almost 7 times more avidly to pre-stimulated platelets relative to those stimulated after exposure to Dextran-40. The explanation for this is likely to be that when the platelets are activated they flip their surface membranes, becoming significantly more negatively charged in the process. This alteration in surface charge then increases the affinity of the platelet for Dextran-40.

This observation taken in isolation could lead to the hypothesis that Dextran-40 may exert its clinical effect by preferentially binding to the negatively charged surface of the activated platelets and then reducing their interaction with other platelets, leukocytes or damaged subendothelium. This would lead the non-activated platelets
relatively unscathed whilst “mopping up” the pro-thrombotic activated ones. However, this does not appear to be the case since aggregation of platelets in the optical aggregometer takes place to a greater extent, and more rapidly, in the presence of Dextran-40. In addition, when Dextran-40 was added to platelets in the midst of aggregating it had no discernible effect on preventing full aggregation occurring, and no significant additional disaggregation occurred when the assay was allowed to continue beyond the time point at which maximal aggregation had occurred.

Dextran-40 remains a clinically effective therapeutic agent. Understanding more about the processes through which it quite clearly influences the thrombotic pathway may allow us to develop more effective novel anti-thrombotic agents which may well have clinical roles outside of carotid endarterectomy.
<table>
<thead>
<tr>
<th>Fibrinogen (Dextran-40 conc)</th>
<th>Mean (%)</th>
<th>P vs control</th>
<th>P-selectin (Dextran-40 conc)</th>
<th>Mean (%)</th>
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**Table 9.1**

The percentage of platelets positive for fibrinogen or p-selectin after incubation with varying concentrations of Dextran-40, +/- agonist.
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<tr>
<th>Fibrinogen (Dextran-40 conc)</th>
<th>Mean (MFI)</th>
<th>P vs control</th>
<th>P-selectin (Dextran-40 conc)</th>
<th>Mean (MFI)</th>
<th>P vs control</th>
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<td>ADP (0.5mg/ml)</td>
<td>4.6</td>
<td>0.660</td>
</tr>
<tr>
<td>ADP (1mg/ml)</td>
<td>28.7</td>
<td>0.023</td>
<td>ADP (1mg/ml)</td>
<td>4.7</td>
<td>0.862</td>
</tr>
<tr>
<td>ADP (2mg/ml)</td>
<td>31.4</td>
<td>0.053</td>
<td>ADP (2mg/ml)</td>
<td>4.6</td>
<td>0.378</td>
</tr>
<tr>
<td>Thrombin (control)</td>
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<td></td>
<td>Thrombin (control)</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>Thrombin (0.25mg/ml)</td>
<td>41.7</td>
<td>0.314</td>
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<td>0.209</td>
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<tr>
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<tr>
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<td>0.137</td>
</tr>
<tr>
<td>Thrombin (2mg/ml)</td>
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<td>0.272</td>
<td>Thrombin (2mg/ml)</td>
<td>5.2</td>
<td>0.156</td>
</tr>
</tbody>
</table>

**Table 9.2**

The MFI of platelets positive for fibrinogen or p-selectin after incubation with varying concentrations of Dextran-40, +/- agonist.
### Table 9.3

The percentage aggregation and mean rates of aggregation for platelets incubated with varying concentrations of Dextran-40 and either ADP or collagen as agonists.

<table>
<thead>
<tr>
<th>ADP (4x10⁻⁴M)</th>
<th>% agg</th>
<th>P vs control</th>
<th>Mean rate</th>
<th>P vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control—no Dextran-40</td>
<td>29.4</td>
<td></td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>0.25mg/ml Dextran-40</td>
<td>38.6</td>
<td>0.047</td>
<td>3.3</td>
<td>0.022</td>
</tr>
<tr>
<td>0.5mg/ml Dextran-40</td>
<td>44.2</td>
<td>0.001</td>
<td>3.4</td>
<td>0.013</td>
</tr>
<tr>
<td>1mg/ml Dextran-40</td>
<td>43.9</td>
<td>0.004</td>
<td>3.1</td>
<td>0.010</td>
</tr>
<tr>
<td>2mg/ml Dextran-40</td>
<td>44.8</td>
<td>0.003</td>
<td>3.0</td>
<td>0.016</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Collagen (0.2ng/ml)</th>
<th>% agg</th>
<th>P vs control</th>
<th>Mean rate</th>
<th>P vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control—no Dextran-40</td>
<td>35.7</td>
<td></td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>0.25mg/ml Dextran-40</td>
<td>45.1</td>
<td>0.053</td>
<td>1.0</td>
<td>0.030</td>
</tr>
<tr>
<td>0.5mg/ml Dextran-40</td>
<td>47.6</td>
<td>0.010</td>
<td>1.1</td>
<td>0.039</td>
</tr>
<tr>
<td>1mg/ml Dextran-40</td>
<td>47.6</td>
<td>0.014</td>
<td>1.1</td>
<td>0.024</td>
</tr>
<tr>
<td>2mg/ml Dextran-40</td>
<td>46.0</td>
<td>0.019</td>
<td>1.1</td>
<td>0.039</td>
</tr>
</tbody>
</table>
Figure 9.1

This demonstrates the change in the number of emboli seen after the initiation of a Dextran-40 infusion in 4 patients who were at high risk of developing a post-operative thrombotic stroke.
Figure 9.2

The percentage of resting or activated platelets binding FITC-conjugated Dextran-40.
Figure 9.3a

This figure demonstrates the significant increase in percentage platelets binding Dextran-40 that occurs if the platelets are incubated with Dextran-40 following activation with thrombin (p<0.0002 for all).
Figure 9.3b

As 9.3a but demonstrating change in MFI for Dextran-40 binding before or after platelet activation (p<0.003 for all).

Density of Dextran-40 binding to platelets

- □ Resting
- □ Dextran-40 before activation
- ■ Dextran-40 after activation

Dextran-40 conc.

0.03mg/ml 0.13mg/ml 0.25mg/ml 0.5mg/ml 1mg/ml 2mg/ml

MFI

16

12

8

4

0
References


CHAPTER TEN

THE PLATELET RECEPTOR HPA-3 (ILE/SER) IS A RISK FACTOR FOR PRE- AND POST-OPERATIVE THROMBOEMBOLIC EVENTS FOLLOWING CEA
Introduction

Platelet receptor polymorphisms exist for both the fibrinogen receptor (GpIb/IIIa) and the collagen receptor (GpIb) \(^1\)\(^2\) – see figure 1. We have studied three of these polymorphisms in order to determine whether they had a clinical effect on patients. We chose to study a cohort of patients undergoing a vascular surgical procedure, carotid endarterectomy (CEA). This particular cohort were chosen as they often have widespread atherosclerotic disease and in the post-operative period it is possible to monitor the artery that has been operated upon allowing us to determine the degree of thrombus formed following arterial injury (unlike coronary angioplasty or coronary artery bypass grafting).

Carotid endarterectomy is performed to reduce patients’ risk of suffering a stroke and involves excising an atheromatous plaque from with the internal carotid artery, the main arterial vessel supplying the brain. Once the plaque has been removed the vessel is repaired and blood flow restored. Unfortunately, this now allows blood to flow over a 5-10cm length of artery that is denuded of endothelium and its surface is now the highly thrombogenic sub-endothelium. Unsurprisingly, platelets begin to cover this section and may begin to form a thrombus. As this thrombus forms, small fragments embolise upstream towards the cerebral circulation. These micro-emboli can be detected as they pass through the middle cerebral artery, a tributary of the internal carotid artery, using trans-cranial Doppler ultrasound (TCD) \(^3\).
As the thrombus increases in size, the number of the micro-emboli detected rises \(^4\). Studies from a number of different centres has shown that the greater the number of emboli detected, the higher is the patient's risk of suffering a post-operative thrombotic stroke secondary to occlusion of the internal carotid artery by thrombus.

Gaunt et al showed that patients with more than 25 emboli in a 10-minute period had a 60% chance of suffering a post-operative stroke, as opposed to the usual risk of 3-5\% \(^5\). Very similar results were produced by Levi et al \(^6\). In a series of 700 CEAs, no patient with low numbers of post-operative emboli developed a thrombotic stroke \(^7\). Spencer et al demonstrated that the commonest post-operative complication in patients who had large numbers of micro-emboli was thrombotic stroke \(^8\). Cantemelo correlated the number of post-operative emboli with ischaemic changes detected post-operatively by cerebral MRI \(^9\).

The TCD can also be used pre-operatively in a similar way, to monitor emboli passing through the middle cerebral artery to the brain. In patients with carotid artery disease, these emboli arise from thrombus overlying unstable or fissured plaques. The number of these emboli detected correlates with a patient's risk of developing a stroke in the pre-operative period.

Therefore, the hypothesis for this study was that if any of the platelet receptor polymorphisms were relatively more pro-thrombotic, that this would result in the pro-thrombotic cohort producing greater numbers of pre or post-operative micro-emboli. To further evaluate platelets' thrombotic tendency in this atherosclerotic cohort of patients, we performed flow cytometry and aggregometry in order to correlate this
with the different platelet receptor polymorphisms. We have also studied the relationship between the polymorphisms and clinical outcomes following CEA.

Methods

Patients and blood sampling

The study was approved by the Leicestershire Ethics Committee and all patients gave written informed consent. All patients were seen in the single visit out-patient clinic, where they were assessed clinically and by carotid Duplex scanning. All patients were standardised to 75mg of aspirin for the four weeks prior to carotid endarterectomy (CEA). Any patient taking dipyridamole, thienopyridines or warfarin were excluded. All clinical data was collected prospectively.

Pre-operative blood samples for platelet function analysis and phenotyping were collected on the morning prior to surgery, between 07.30 and 08.30 to minimise the effect on diurnal variation. Patients had been resting supine for at least 20 minutes and sample collection was performed using a standardised phlebotomy technique designed to minimise platelet activation. Blood was taken without the use of a tourniquet, by clean venepuncture, from an antecubital vein via a 21-gauge butterfly needle, into vacutainers (Becton Dickinson). The first 4.5ml of blood were discarded and subsequent aliquots of 4.5ml were collected into 0.5ml of 3.8% tri-sodium citrate.
**Phenotyping:**

The phenotyping was performed by the Division of Transfusion Medicine, at the University of Cambridge, according to their previously published protocols. The patient phenotype was compared to their laboratory platelet function studies and to their clinical outcomes.

**Reagents:**

ADP, arachidonic acid (sodium salt), human α-thrombin and GPRP peptide were from Sigma (Poole, Dorset, UK). Bovine collagen (Horm, Germany) was from Nycomed. GPIbα and GPIIb/IIIa were identified with FITC-conjugated RFGP37 and RFGP56 Mabs respectively and platelet bound fibrinogen was detected with FITC-conjugated rabbit ant-human fibrinogen (Dako Ltd., High Wycombe, UK) as described previously. All antibodies were used at their optimal concentration, determined by titration.

**Aggregometry:**

Platelet aggregation was measured in platelet rich plasma by Born-type aggregometry as previously described. Aggregation was measured in response to final concentrations of ADP (0.5, 1, 2 and 4 μmol/l), collagen (10, 20 and 50 mg/ml) and arachidonic acid (3 or 6 μmol/l).
Flow cytometric analysis:

Blood samples were prepared for flow cytometric analysis within 10 minutes of collection, using a whole blood method described previously\textsuperscript{10,12}. Fibrinogen binding was measured in unstimulated blood samples and in samples stimulated with ADP (0.1, 1, 10 μmol/l) or thrombin (0.02, 0.04, 0.08, 0.16 u/ml), the latter in the presence of GPRP peptide to prevent clot formation. All samples were prepared in duplicate and analysed within 2 hours of collection in a Coulter EPICS Profile II flow cytometer (Coulter Electronics Ltd., Luton, UK). The platelet population was identified from its light scatter characteristics and identity confirmed using the anti-GPIIb\alpha Mab, RFGP37-FITC. Five thousand platelets were analysed and the values were recorded as the percentage of cells positive for fluorescent antibody binding. Expression of GPIIb\alpha and GPIIb/IIIa were measured as the mean fluorescence (MFI).

Operative procedure:

Carotid endarterectomy was performed in a standardised manner throughout the study period. The operation was undertaken with the patient under normocarbic, normotensive general anaesthesia using systemic heparinisation (5000 units unfractionated heparin). Blood flow velocity in the middle cerebral artery was monitored continuously through the operation with transcranial Doppler ultrasound (TCD) using a 2 MHz fixed, head probe. The lumen was inspected with a flexible hysteroscope (Olympus) prior to flow restoration, to exclude luminal thrombus or large intimal flaps. All technical errors were corrected prior to restoration of flow.
CHAPTER TEN

Following recovery from anaesthesia, the patient was transferred to the recovery area of theatre or the high dependency unit for a 3-hour period of TCD monitoring. All TCD data were recorded on to digital audio tape for off line analysis of emboli counts by a technician blinded to the pre-operative blood test results. Dextran-40 (Pharmacia Ltd, Milton Keynes, England) was administered to any patient who had (i) ≥ 25 emboli in any 10 minute period or (ii) emboli that distorted the MCA waveform which suggested that they were large, according to our previously described protocol.7

Statistical analysis:

Discrete data was analysed using contingency tables (Chi-squared or Fisher’s exact test as appropriate) and continuous data using the Mann-Whitney U test or one-way ANOVA, as appropriate. A “p” value less than 0.05 was taken as significant.

Results

Frequency distribution:

In total 238 patients were studied. The frequency distribution of the three alleles is shown in Table 1. For each polymorphism the most common homozygote (aa) was compared against a combined group of the heterozygotes (ab) plus the less common homozygote (bb). The numbers of emboli (pre-operative and post-operative) in each polymorphism group were compared with respect to the different alleles, as were the tests of platelet function.
Patient risk factors:

The patient risk factors for each allele group are shown in Table 2. The groups were well matched for age and sex. For the HPA-1 polymorphism there were significantly more patients with angina in the more common leu/leu cohort (40.6 vs. 20.7%, p=0.010). There was no excess of previous MI in this group though. The HPA-2 cohorts were not significantly different from one another, although there was a trend to an increase in the numbers of patients with claudication (a symptom of lower limb ischaemia) in the less prevalent thr/met group (21.8 vs. 37%, p=0.094). For the HPA-3 polymorphism there was a significantly greater proportion of smokers (past plus present) in the ser/ser cohort (16.2 vs. 47.6%, p=0.002). There was no difference in the number of current smokers in this group (p=0.218). This was paralleled by a similar increase in the proportion of claudicants in each cohort (16.9 vs. 45.0%, p=0.008). There was no excess of hypertension, angina or MI within the groups though.

Emboli:

The numbers of spontaneous pre-operative micro-emboli detected arising from the carotid artery were monitored for a one-hour period pre-operatively. Of 154 patients who were available for pre-operative TCD monitoring, it was not technically possible to perform this in 14 (no temporal bone window), leaving data for analysis on 140 patients. Overall, 13% of patients had one or more emboli detected pre-operatively. For HPA-2, only 1/27 (3.7%) patients from the thr/met cohort exhibited any spontaneous pre-operative emboli, as opposed to 19/113 (16.8%) patients from the thr/thr cohort (p=0.080). For HPA-3, the mean number of spontaneous pre-operative
emboli increased stepwise from 0.29 (ile/ile) to 0.41 for the heterozygotes and then 2.05 for the ser/ser cohort (p=0.031).

For the post-operative emboli, 203 patients with polymorphism data were available for TCD monitoring, of whom 14 had no temporal window, leaving 189 patients for analysis. The mean number of post-operative emboli per patient is shown in Figure 2. Only the HPA-3 allele significantly increased patients’ risk of developing post-operative embolisation. As with the pre-operative embolisation, the post-operative emboli increased with the changing alleles, 13% (ile/ile), 16% (ile/ser) and 30% with the ser/ser cohort (p=0.040). If the data is analysed in a different manner, splitting the cohorts into those who had significant numbers of emboli (<25 or >25 emboli) in the post-operative period there is still no significant difference between the alleles of the other 2 polymorphisms (HPA-1, p=0.387; HPA-2, p=0.578).

Flow cytometry:

Platelet fibrinogen binding was assessed in 100 patients undergoing CEA who had previously been phenotyped and the relationship between these two functions assessed. For the HPA-1 and HPA-3 cohorts, neither the percentage of cells analysed or the MFI were significantly different with respect to GpIIb/IIIa expression (respectively: 98.6 vs 98.4%, p=0.33 and 98.8 and 99.1%, p=0.49). For the HPA-2 cohort, neither the percentage of cells analysed nor the MFI were significantly different with respect to GpIb expression (98.7 vs 99.1%, p=0.75).

For the resting, unstimulated platelets there was no significant difference in their ability to bind fibrinogen, for any of the alleles within 3 groups of polymorphisms (p>0.15 for all) – see Table 3. After stimulation with ADP none of the different alleles had an increase in the number of platelets positive for fibrinogen or the MFI (p>0.19.
for all) – see Figure 3. Thrombin had no significant effect on the binding of fibrinogen for either HPA-1 or HPA-2 (p>0.31 for all) – see Figure 2. For HPA-3, stimulation with thrombin led to a significant increase in the amount of fibrinogen bound by the serine containing alleles (ser/ser > ser/ile > ile/ile). The ser/ser allele bound on average 32% more fibrinogen than the ser/ile cohort (p=0.044) and 51% more than ile/ile (p=0.024) – see Figure 4.

**Aggregometry:**

Platelet fibrinogen binding was assessed in 94 patients undergoing CEA who had previously been phenotyped and the relationship between these two functions assessed. The platelet response to ADP was not significantly different for any of the alleles with the 3 groups of polymorphisms, in terms of either total aggregation or mean rate of aggregation (p>0.15 for all) – see Table 4. None of the polymorphisms demonstrated any significant difference when stimulated with 3 or 6mmol arachidonic acid (p>0.28 for all).

Neither the alleles of HPA-1 or -2 demonstrated any differences in platelet aggregation in response to collagen. HPA-3 ile/ile aggregated to a significantly greater extent than serine heterozygotes plus homozygotes – see Figure 5. This difference was paralleled by a significant increase in the mean rate of aggregation as well, with ile/ile aggregating 68%, 39% and 24% faster for the 3 concentrations of collagen used (p=0.002, 0.005 and 0.013 respectively). This difference was not maintained when the alleles were further separated into ile/ile vs ile/ser vs ser/ser – see Table 4.
Clinical outcomes:

Data on clinical outcomes was collected prospectively for the phenotyped CEA patients. The outcomes relating to patients' thrombotic/haemorrhagic tendencies and their relationship to the different alleles studied are shown in Table 6. For the pro+ allele of HPA-1, there was a significant increase in the total blood loss in the 24 hours following the operation (81ml vs 129ml, p=0.029). The number of patients requiring a re-operation for post-operative bleeding was also higher for this allele (3.8% vs 13.8%, p=0.020). For the met+ alleles of HPA-2, the use of Dextran-40 to prevent post-operative thrombotic stroke increased from 2.2% (thr/thr) to 7.4% (met+, p=0.160). The proportion of patients requiring a post-operative blood transfusion fell from 10.0% (thr/thr) to 3.7% (met+, p=0.299). For the HPA-3 polymorphism the length of the operation increased stepwise with the changes in alleles (ile/ile 101 mins, ile/ser 105mins and ser/ser 114 mins, p<0.001 with ANOVA). The 24 hour post-operative blood loss also changed significantly for the different alleles (ile/ile 119ml, ile/ser 88ml, ser/ser 64ml, p<0.001 with ANOVA). The proportion of patients requiring re-operation for bleeding complications fell from 9.7% (ile/ile) to 0% (ser/ser).

All the patients were followed up in the out-patient clinic where their operated internal carotid artery was rescanned with duplex ultrasound to look for evidence of restenosis. At 6 weeks, the pro+ HPA-1 allele demonstrated significantly greater evidence of restenosis than leu/leu (6% vs 2%, p=0.049). This was paralleled by a relative increase in the velocity of the blood flow within the vessel (the velocity increases as the degree of stenosis rises), with the velocity of the leu/leu allele being 108cm/s, relative to
160cm/s for the pro+ (p=0.042). At 6 months this difference persisted, with the stenosis in the pro+ cohort being 9%, as opposed to only 4% in the leu/leu group (p=0.038). The velocity differences persisted (p=0.044). For the HPA-2 polymorphism, no difference existed at 6 weeks but by 6 months a similar pattern was seen at clinic follow up. The met+ cohort had a mean of 11% restenosis as opposed to only 3.6% for the thr/thr group (p=0.010). A corresponding pattern was noted with the relative velocities (p=0.045). No differences were seen in either restenosis rates or velocity changes for the HPA-3 cohort at any follow up clinic.

**Discussion**

The allele frequencies are within the range expected for the population. For HPA-1 and HPA-2 there were only small numbers of patients homozygous for the less common allele, 3.4% for pro/pro in HPA-1 and 0% for met/met in HPA-2. Because of this, the data for the most common homozygous allele was compared to that from the heterozygotes combined with the less common homozygous allele (HPA-1 only). This may mean that the influence on platelet function of the less common allele in its homozygous form is underestimated, its effect having been diluted by the more common allele from the heterozygotic phenotype. If the less common allele were to exert any strong influence at a population level it would have to do so through the heterozygote form, the influence of which has been evaluated by this study.
The polymorphism groups were well matched for age and sex. This would seem to indicate that none of the 3 polymorphisms studied, can alone influence patients' longevity. There was an excess of patients with angina in the leu/leu HPA-1 cohort, but this did not manifest itself in an excess of previous MIs. There was an excess of smokers in the HPA-3 ser/ser group, but this included past and current smokers and when analysed alone there was no excess of current smokers in any of the allele groups (p>0.218 for all). The excess of past smokers may well account for the rise in symptoms of claudication in this same cohort, although there was no excess of angina, hypertension or MI.

Spontaneous pre-operative embolisation was significantly associated with the presence of the HPA-3 serine allele. The effect of a heterogeneous serine allele doubled the pre-operative embolisation rate, whilst the ser/ser cohort had a 10-fold increase. There was a weaker, non-significant, association between pre-operative emboli and the HPA-2 threonine allele (p=0.080). This may represent a type II statistical error. The number of post-operative emboli was not influenced by either of the HPA-1 or HPA-2 polymorphisms. The presence of the serine allele of HPA-3 was again associated with significant increases in embolisation, this time post-operative. The heterozygotes suffering 19% more emboli than the ile/ile allele, and the ser/ser cohort 122% more than the ile/ile homozygotes. The fact that a very similar pattern was noted for the increases in pre- and post-embolisation for the HPA-3 polymorphism seems to increase the likelihood that this is a real effect.
The flow cytometry data follows a similar pattern to the emboli data, with HPA-1 & 2 having no effect on fibrinogen binding and the ser/ser HPA-3 allele leading to a significant increase in the ability of the platelet to bind fibrinogen in response to thrombin. For the aggregometry, there were no differences in the platelet response to ADP, but contrary to other findings from the study, the response to collagen was significantly increased in the ile/ile HPA-3 cohort relative a group containing the serine homozygotes and heterozygotes. The reasons for this are unclear but in evolutionary terms it maybe that serine alleles decreased response to collagen offsets its exaggerated pro-thrombotic response to thrombin. If the presence of the HPA-3 serine allele simply led to an increased platelet thrombotic response to all agonists it would be likely to produce a phenotype that suffered from a strong negative selection pressure.

Both HPA-1 and HPA-3 had a significant effect on the blood loss collected into the wound drains in the first 24 hours after the operation. The leu/leu HPA-1 cohort bled significantly less than their pro+ counterparts and this was associated with a marked reduction in the number of patients requiring an urgent re-operation to deal with post-operative bleeding complications from 13.8% (pro+) to 3.8% (leu/leu). A similar pattern was seen with the ser/ser HPA-3 cohort, their post-operative blood loss being half of that for the ile/ile group. None of the ser/ser cohort required a re-operation, whilst 9.7% of the ile/ile patients were returned to theatre. The ability of a given phenotype to reduce haemorrhagic blood loss following a traumatic injury has an obvious positive selection pressure in evolutionary terms.
HPA-1 & 2 appear to have a significant effect on arterial restenosis following arterial injury. For both of the less common alleles there was approximately a three-fold increase in restenosis on follow up ultrasound scanning at 6 months. At the present time only short term data is available and whether these will translate into clinically significant effects in the long term remains to be seen.

Summary

HPA-1 & 2 appear to have a negligible influence on platelet function in patients undergoing carotid artery surgery, and do not seem to be associated with the risk of developing a post-operative thrombotic event. They may play a role in restenosis following arterial injury.

Patients who are homozygous for the serine HPA-3 allele have an increased number of pre- and post-operative micro-emboli detected, and an exaggerated fibrinogen binding response to thrombin. These negative attributes are offset by decreased post-operative blood loss and a reduction in the number of post-operative haemorrhagic complications seen.
Figure 1: The binding sites of the platelet receptor polymorphisms

Sites of platelet receptor polymorphisms

Platelet adhesion

Subendothelium

Thrombin

Epinephrine

ADR

TXA2

Fibrinogen

Collagen

VWF

collagen
Figure 1: Mean number of post-operative emboli for each polymorphism (n=189)
Figure 2: Fibrinogen binding for the 3 polymorphisms in response to ADP and thrombin – results in 100 patients.
Figure 3: Fibrinogen binding for HPA-3 in response to 4 different concentrations of thrombin. The “p” values relate to the difference between the homozygous ile/ile and ser/ser cohorts (n=47[ile/ile], n=43[ile/ser] and n=10[ser/ser]).
Figure 4: The total percentage platelet aggregation following stimulation with 3 doses of collagen for the HPA-3 polymorphism (n=47[ile/ile] and n=53[ser+]).
<table>
<thead>
<tr>
<th></th>
<th>HPA - 1 (a=leu, b=pro)</th>
<th>HPA - 2 (a=thr, b=met)</th>
<th>HPA - 3 (a=ile, b=ser)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aa</td>
<td>157</td>
<td>197</td>
<td>105</td>
</tr>
<tr>
<td>Ab</td>
<td>73</td>
<td>41</td>
<td>102</td>
</tr>
<tr>
<td>Bb</td>
<td>8</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>a:b</td>
<td>0.81 : 0.19</td>
<td>0.91 : 0.09</td>
<td>0.65 : 0.35</td>
</tr>
</tbody>
</table>

Table 1: The frequency distribution of the three polymorphisms studied
Table 2: risk factors (%) for the presence of vascular disease for each of the polymorphism cohorts. The “smoker” group included past and current smokers, when analysed alone there was no excess of current smokers in any of the allele groups.
<table>
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<tr>
<th></th>
<th>Resting SD</th>
<th>ADP 10-7M SD</th>
<th>ADP 10-6M SD</th>
<th>ADP 10-5M SD</th>
</tr>
</thead>
<tbody>
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<td>9.2</td>
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</tr>
<tr>
<td></td>
<td>7.2</td>
<td>5.0</td>
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<td>3.6</td>
</tr>
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<td>0.271</td>
<td>0.235</td>
<td>0.192</td>
</tr>
<tr>
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<td>2.8</td>
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<td></td>
<td>4.5</td>
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<td>1.6</td>
</tr>
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<td>0.426</td>
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<td>HPA-3 ile/ile</td>
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<td>8.1</td>
<td>2.8</td>
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<tr>
<td>p (ile/ile vs</td>
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<td>0.472</td>
<td>0.636</td>
<td>0.855</td>
</tr>
<tr>
<td></td>
<td>ser/ser)</td>
<td></td>
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<tr>
<td>Thrb 0.2u SD</td>
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<td></td>
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<td>p</td>
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<td>0.457</td>
<td>0.451</td>
<td>0.316</td>
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<tr>
<td>Thrb 0.4u SD</td>
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<td>3.6</td>
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<tr>
<td>p</td>
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<td>Thrb 0.8u SD</td>
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<td>12.1</td>
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<tr>
<td>p (ile/ile vs</td>
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<td>0.045</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>ser/ser)</td>
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Table 3: Density of fibrinogen binding on the platelet surface in either resting platelets, or after stimulation with ADP or thrombin – results from 100 patients.
<table>
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<tr>
<th></th>
<th>ADP 5x10^{-7}M</th>
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<th>ADP 2x10^{-6}M</th>
<th>ADP 4x10^{-6}M</th>
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<tr>
<td>HPA-1 leu/leu</td>
<td>14.7 [8.8]</td>
<td>37.6 [14.0]</td>
<td>58.0 [15.4]</td>
<td>73.6 [13.5]</td>
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<tr>
<td>HPA-1 pro+</td>
<td>16.8 [10.7]</td>
<td>37.8 [15.2]</td>
<td>57.3 [20.0]</td>
<td>72.7 [18.1]</td>
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<tr>
<td>p=</td>
<td>0.304</td>
<td>0.938</td>
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<tr>
<td>HPA-2 thr/thr</td>
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<td>38.0 [15.1]</td>
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<td>73.3 [15.6]</td>
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<td>HPA-3 ile/ser</td>
<td>14.7 [9.0]</td>
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<td>58.9 [15.7]</td>
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<tr>
<td>HPA-3 ser/ser</td>
<td>14.9 [8.0]</td>
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<td>72.2 [15.0]</td>
</tr>
<tr>
<td>p=</td>
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<td>0.370</td>
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<table>
<thead>
<tr>
<th></th>
<th>Coll 1ug/ml</th>
<th>Coll 2ug/ml</th>
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<tr>
<td>HPA-1 leu/leu</td>
<td>33.5 [22.7]</td>
<td>54.3 [21.9]</td>
<td>76.5 [17.4]</td>
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<td>HPA-1 pro+</td>
<td>28.8 [22.5]</td>
<td>48.4 [24.9]</td>
<td>73.2 [20.3]</td>
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<tr>
<td>p=</td>
<td>0.343</td>
<td>0.232</td>
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<tr>
<td>HPA-2 thr/thr</td>
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<td>52.2 [22.8]</td>
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<td>HPA-2 met+</td>
<td>30.3 [21.9]</td>
<td>52.7 [21.7]</td>
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<td>p=</td>
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<table>
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<tr>
<th></th>
<th>AA 3mmol</th>
<th>AA 6mmol</th>
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<tr>
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<td>31.8 [26.2]</td>
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<td>HPA-1 pro+</td>
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<td>HPA-2 met+</td>
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<tr>
<td>HPA-3 ile/ser</td>
<td>5.6 [7.1]</td>
<td>28.3 [5.6]</td>
</tr>
<tr>
<td>HPA-3 ser/ser</td>
<td>9.1 [4.7]</td>
<td>41.3 [30.6]</td>
</tr>
<tr>
<td>p=</td>
<td>0.535</td>
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Table 4: Mean percentage platelet aggregation [st dev] in response to ADP, collagen or arachidonic acid (AA) – results from 100 patients.
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<th>HPA-1</th>
<th></th>
<th>HPA-2</th>
<th></th>
<th>HPA-3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>leu/leu</td>
<td>pro+</td>
<td>thr/thr</td>
<td>met+</td>
<td>ile/ile</td>
<td>ile/ser</td>
</tr>
<tr>
<td>Op time – mins</td>
<td>105</td>
<td>103</td>
<td>106</td>
<td>95</td>
<td>101</td>
<td>105</td>
</tr>
<tr>
<td>Dextran used -%</td>
<td>2.8</td>
<td>3.5</td>
<td>2.2</td>
<td>7.4</td>
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<td>1.4</td>
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<tr>
<td>Wound blood loss - ml</td>
<td>81</td>
<td>129</td>
<td>106</td>
<td>59</td>
<td>119</td>
<td>88</td>
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<td>Patient transfused</td>
<td>9.9</td>
<td>7.1</td>
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<td>4.4</td>
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<tr>
<td>Re-operation for bleeding</td>
<td>3.8</td>
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<td>8.9</td>
<td>0</td>
<td>9.7</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Table 5: Clinical outcomes relating to haemostasis for 170 of the phenotyped patients
References


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CHAPTER ELEVEN

FACTORS INFLUENCING PATIENTS' THROMBOTIC POTENTIAL AFTER CEA
Introduction

Major trials have reported stroke rates of around 2-6% following carotid endarterectomy\textsuperscript{1,2}, although the risk may be higher than this in non-specialist centres not involved in the major stroke prevention trials\textsuperscript{3,4}. Those arising in the intra-operative period are usually due to technical error such as failing to maintain adequate cerebral perfusion during the endarterectomy or failure to ensure that the endarterectomy zone was clear of debris or thrombus prior to the restoration of flow\textsuperscript{5}. These patients awake from their CEA with a neurological deficit. Patients who are neurologically intact in recovery and then develop neurological signs have usually suffered either a stroke from sustained embolisation of micro-emboli from the endarterectomy zone or a thrombotic occlusion of the ICA\textsuperscript{6}. These events occur in the first 2-3 hours post-operatively\textsuperscript{7}. Less common causes of post-operative neurological deficits include intra-cranial haemorrhage, hyperperfusion syndrome, emboli from the left atrium and rarely, dissection of the ICA under the distal intimal step. The prevention of technical error by the use of intra-operative quality control measures (intra-operative TCD monitoring and completion angioscopy) can significantly reduce the rate of intra-operative strokes but has no impact on the rate of post-operative strokes\textsuperscript{8}.

Unlike intra-operative stroke, little is known about those factors that place 2-3% of patients at risk of a post-operative thrombotic or embolic stroke. In the hour or so before patients develop a post-operative neurological deficit increasing numbers of micro-emboli can be detected passing through the middle cerebral artery to the brain\textsuperscript{6}. 

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These emboli arise from a forming thrombus adherent to the endarterectomy zone. Patients do not develop a thrombotic stroke without first producing a significant number of emboli\textsuperscript{8}. The prevention of the rise in the number of emboli by the use of an infusion of Dextran-40 reduced the post-operative thrombotic stroke rate to zero in a recent series of 500 consecutive CEAs\textsuperscript{8}. Evidence now exists from 3 continents that the detection of significant rates of embolisation in the postoperative period precedes thrombotic stroke or the appearance of abnormalities on post-operative MRI\textsuperscript{8-11}.

In an attempt to define those factors placing patients at risk of post-operative thrombosis we have compared the number of post-operative emboli detected for each patient with clinical variables that may be associated with post-operative thrombotic stroke.

Methods

The trial was approved by the Leicestershire Area Ethics Committee and patients entered by informed consent. In all, 294 patients undergoing CEA between 1.12.97 and 31.12.99 were considered for inclusion. The following patients were excluded: refusal to give informed consent (n=10), no adequate temporal bone window to allow the recording of post-operative emboli rates (n=23), failure or absence of TCD equipment (n=6), redo CEA (n=3) and combined common carotid angioplasty plus CEA (n=1). This left data on 251 patients available for analysis. This paper details the
results concerning clinical factors and rate of embolisation for the first 24 hour post-operative period.

Carotid endarterectomy

Anti-platelet therapy was not stopped prior to surgery and the patient's usual medication administered on the morning of surgery. Carotid endarterectomy was performed by a consultant vascular surgeon or supervised trainee with normotensive, normocarbic general anaesthesia using loupe magnification, systemic heparinisation (5000u), routine shunting (Pruitt-Inahara, Ideas for Medicine, North Clearwater, Fla), tacking sutures to the distal intimal step to prevent distal intimal dissection and routine patch angioplasty.

Blood flow velocity in the middle cerebral artery (MCA) was monitored throughout the procedure using 2MHz pulsed wave TCD ultrasound, a SciMed PC2-64B, with a fixed head probe system (Scimed UK Ltd, Bristol UK) protected by a semi-circular metal head guard with recording onto digital audiotape (DAT) for off-line analysis of emboli. A 5mm segment of the anastomosis (adjacent to the orifice of the external carotid artery) was left open. The shunt was removed and the lumen vented and irrigated with heparinised saline. Prior to final closure and restoration of flow, the lumen was inspected with a flexible hysteroscope (Olympus 1070-48, Hamburg, Germany) to exclude residual luminal thrombus and intimal flaps.
After the procedure, the patient was transferred through to the high dependency unit for further TCD monitoring. All emboli detected in the first three post-operative hours were recorded onto DAT tape and quantified off-line. Any patient who had ≥25 emboli in any 10 minute period or whose emboli distorted the MCA waveform (suggesting they were large) were commenced on an incremental infusion of Dextran 40. An initial bolus of 20ml 10% Dextran-40 was administered, followed by an infusion at rate commencing at 20ml/hr. If the embolisation rate failed to settle, then the infusion rate was increased by 10ml/hr until embolisation was controlled. The number and rate of embolisation were quantified for each patient, as was the requirement for Dextran therapy.

*Post-operative assessment*

All patients were assessed post-operatively by the same neurologist (HA) who documented any new neurological deficit or cranial nerve injury. Any new neurological deficit lasting >24 hours was classified as a stroke and these patients investigated by CT scan/autopsy, Duplex assessment of the internal carotid artery (ICA) and TCD assessment of the intracranial vessels. The neurologist reassessed all trial patients at 30 days where those suffering a peri-operative stroke underwent an assessment of the stroke severity using the Oxfordshire Handicap Stroke Score (OHS). A minor (non-disabling stroke) scored 0 – 2 on the OHS scale whilst a disabling stroke scored 3 – 6.
CHAPTER ELEVEN

Statistical analysis

As the data in this study were not normally distributed non-parametric tests were adopted. Continuous variables were analysed using Mann-Whitney U test. Discrete variables were analysed using chi-squared or Fisher's exact test as appropriate. A “p” value of <0.05 was assumed to represent statistical significance.

Results

The results are in two main sections, the first relating to factors inherent to the patient such as age and sex, and the second to operative factors that may influence patients’ risk of thrombotic stroke. Table 1 shows the mean number of emboli (+/- st dev) seen for each of the patient related factors. The most striking feature is that women had over 3 times as many emboli as men (p=0.004). If the data is divided into two groups, one with more than and the other with less than 25 emboli in recovery, then 22/47 patients in the first group are female, compared with only 51/206 in the second group (p=0.0026). This data indicates that women’s risk of significant post-operative embolisation is 2.2 times higher than men.

Further analysis of the data relating to the CEAs for men and women was performed in an attempt to identify other differences between these two groups. There was no difference in the mean number of spontaneous pre-operative emboli seen (0.60 vs 0.58, p=0.966). The mean age of women undergoing CEA was 70.2 vs 69.0 for men (p=0.243). There was no difference in their presenting symptoms (p=0.755). There
were equal proportions of smokers (24.7 vs 24.6%, \( p=0.979 \)), diabetics (29.0 vs 28.4%, \( p=0.945 \)), those with a previous MI (19.3 vs 28.9%, \( p=0.169 \)) and angina (46.2 vs 36.4%, \( p=0.251 \)). The proportion of women who were hypertensive was higher than that of men (90.3 vs 62.8%, \( p=0.008 \)). There were more male claudicants (18.1 vs 30.9%, \( p=0.027 \)). The degree of ICA stenosis in both the ipsilateral and contralateral vessels was similar for women and men (80.3 vs 82.1%, \( p=0.119 \) and 61.0 vs 62.7%, \( p=0.878 \), respectively). Women’s operations were significantly shorter at 101 minutes compared to 113 minutes for men (\( p=0.011 \)). The length of time that a shunt was in place was shorter for women (41.3 vs 48.6 minutes, \( p=0.003 \)). The length of patch angioplasty was also significantly shorter (5.4 vs 6.6cm, \( p<0.0001 \)). Significantly more women were given Dextran-40 to prevent the development of a post-operative thrombotic stroke than men (9.7% vs 2.7%, \( p=0.013 \)). There was no difference in the incidence of hyper- or hypotensive episodes in recovery, or in the number of post-operative cardiac events detected.

The age of the patient at operation did not affect their thrombotic risk. The number of post-operative emboli detected did not alter whether the patient presented with a stroke, TIA or amaurosis fugax or was asymptomatic. Factors reliant on the presence of concomitant arterial disease such as previous MI, angina, claudication or significant contralateral carotid artery stenosis did not alter post-operative emboli numbers. All of the statements in this paragraph hold true whether the data is analysed as mean number of emboli in each group, or if the data is split into groups with high and low emboli numbers and the proportion of risk factors in the high and low groups then compared.
Patients who had emboli detected on their pre-operative TCD monitoring were significantly more likely to suffer high rates of post-operative embolisation (64.3 emboli vs 22.1, p=0.027). There is also a significant correlation seen between the number of emboli detected for a patient in the pre-operative period compared with their post-operative monitoring ($R^2=0.17$, $p=0.016$).

Table 2 shows the relationship between operative factors and post-operative embolisation. The length of the procedure itself did not influence emboli counts. The total period of time for which the CCA and ICA were crossed clamped for during the operation and the length of time the blood was flowing through the Pruitt-Inahara shunt did not influence post-operative emboli counts ($p=0.233$ and $p=0.796$, respectively). Finding a technical defect at angioscopy (5 intimal flaps and 14 patients with luminal thrombus) prior to flow restoration that was subsequently corrected, did not influence post-operative embolisation rates ($p=0.569$). The length of the patch angioplasty used to close the arteriotomy site did not affect emboli counts. Whether the procedure was performed by a supervised trainee or an independent consultant did not influence the outcome.

Although the length of the procedure did not influence the risk of post-operative thrombosis, both the start and finish times of the operation did significantly affect emboli counts. For procedures that had a start before 10.30am, the mean number of post-operative emboli was 48.6, as opposed to only 14.7 for later starts ($p=0.004$). If the procedure finished before midday there was a 3.5 fold increase in the number of post-operative emboli detected (53.2 vs 15.1, $p=0.002$). There was a significant
correlation between start and finish times and the emboli counts ($R^2=0.14$ and $0.16$, $p=0.037$ and $0.014$, respectively). The data for the finishing time is shown in Figure 1.

Of the 12 patients with more than 100 emboli in total, 9 had an operation that began in the morning and 3 had an afternoon start (relative risk 1.6 [95% CI, 1.1-2.2], $p=0.063$). Of these 12 patients, 8 had their operation finish in the morning, as opposed to 4 whose operation finished in the afternoon (relative risk 2.0 [95% CI, 1.3-3.2], $p=0.016$). Looking at the series order for the patients with more than 100 emboli (morning starts in bold) it can be seen that the 6 patients with the highest number of emboli are those that had their operations start in the morning (917, 583, 461, 417, 41, 411, 246, 199, 183, 176, 141, 137). The relative risk of a patient being given Dextran-40 to prevent progression onto a thrombotic stroke was 2.2 for morning finishes relative to the afternoon (95% CI, 1.5-3.3 [$=0.007$]). On the other hand, 56 patients had no post-operative emboli detected, 19 had a morning start as opposed to 37 whose operation started in the afternoon (relative risk 0.63 [95% CI, 0.4-0.9], $p=0.011$). Fifteen of the 56 had a morning finish (relative risk 0.64 [95% CI, 0.4-1.0], $p=0.045$).
Discussion

Traditional cardiovascular risk factors did not appear to influence post-operative embolisation in this study. This would seem to indicate that the presence of atherosclerotic disease per se does not act as a stimulus to the platelet/coagulation system to form thrombus. Patients' presenting symptoms, which have been associated with variations in outcome after CEA, did not affect post-operative thrombotic risk. However, the presence of embolisation detected in the monitoring period before the CEA was started was significantly correlated with the development of post-operative embolisation. This may indicate that a common pathway underlies the tendency to form thrombus in the pre- and post-operative periods. The micro-emboli that are generated have a common link despite arising from differing vessel surface coverings (atheroma or the sub-endothelium), ie despite the stimuli initiating the thrombus formation being different the final outcome is similar. Much of the other data from this study supports this supposition that it is not the type of initial stimulus that determines patients' risk of post-operative thrombotic stroke but rather that it is the patients inherent propensity to generate a thrombus in response to vascular injury.

Variation in each of the following factors could be thought of as providing a pro-thrombotic stimulus of variable strength. The length of the operation did not affect post-operative emboli counts. Patients who had a prolonged period with their blood bypassing the operative site through a plastic shunt, which can activate platelets, did not have an excess of post-operative emboli. A long patch angioplasty was not associated with an adverse outcome. The size of the patch inserted is related to the
area of vessel that is stripped of its anti-thrombotic endothelial layer leaving highly thrombogenic sub-endothelium to stimulate thrombus formation, ie the longer the patch the greater the thrombotic stimulus. In keeping with previous studies no relationship was noted between the detection of technical defects at angioscopy and the presence of post-operative embolisation. Again these findings are in keeping with the hypothesis that variation in the degree of pro-thrombotic stimulus does not correlate with the extent of the final thrombotic outcome. No previous studies have reported on the relationship between risk factors and post-operative embolisation so it is not possible to compare these results against published data.

Women suffered more than a three-fold increase in the number of emboli detected in the post-operative period (p=0.004). There was also a significant increase in the proportion of patients suffering high levels of post-operative emboli (>25) that were female. Other factors relating to the operation and potentially to post-operative thrombotic risk were compared between the female and male groups in an attempt to identify any confounding variables. More women than men were being treated for hypertension, but this did not translate into differences in blood pressure in the immediate post-operative period. All of our patients are monitored with intra-arterial blood pressure monitoring in the recovery period and hypertension (>160/90) is actively treated if it occurs. Equal proportions of women and men (42.6% vs 44.8%, p=0.691) were treated for post-operative hypertension. Women actually had shorter operation times, shunt times and patch closure lengths than men. In keeping with women's increased number of post-operative emboli is the fact that they were 3.6
times more likely to be given Dextran-40 therapy than men to prevent progression onto post-operative carotid thrombosis.

Both ECST and NASCET have reported poorer morbidity and mortality figures following CEA for symptomatic women. A recent analysis of the NASCET data showed that the odds ratio for peri-operative stroke in women was 1.7 relative to men. Similar data has also been reported for asymptomatic women from the ACAS study, where the relative risk of 30-day death or stroke was 3.3. The reason for the excess of strokes in the female cohort in each study has not been adequately explained as yet. The data for these studies does not allow analysis of why these events occurred and whether they were intra- or post-operative events. It is possible that one of the reasons for the excess of female strokes in these studies is an increased rate of post-operative carotid thrombosis.

In terms of outcomes from arterial surgery, it is not just following CEA that women fare less well than men. Women suffer significantly decreased one year primary patency rates (61% vs 84%) following iliac angioplasty and stenting. Women's apparent increased tendency to thrombotic arterial occlusion may also explain why there is an excess female mortality following coronary angioplasty for the treatment of acute myocardial infarction, with a relative risk of 2.6 times that of men. In a further study it has been reported that women are more likely to require emergency coronary artery bypass grafting following coronary angioplasty and that their mortality in this situation is 4.3 times higher than the comparable male cohort. A German study examined the 30-day and one year outcomes following coronary artery stenting and
again there was a higher death rate at 30 days for women (hazard ratio 2.0), but this difference disappeared by one year. It is possible that the apparent increase in women’s pro-thrombotic tendency following CEA in our series may well account for the sex differences noted above, with women clotting off their coronary angioplasty or stent sites, or occluding their new coronary grafts.

There was a marked difference between the number of emboli seen for morning procedures against those performed in the afternoon. The link between time of day and platelet function and/or cardiac events is well established but this is the first time that a link between number of emboli and time of operation has been reported. Procedures performed in the morning were significantly more likely to produce high numbers of post-operative emboli (relative risk 2.0), including the 6 patients who had the highest number of emboli, and significantly less likely to have patients who had no emboli after their CEA (relative risk 0.6). This indicates that those patients undergoing their CEA in the morning are at significantly increased risk of post-operative thrombotic stroke relative to patients having an operation in the afternoon. At the present time it is unclear whether this is a function of increased early morning platelet activity or reduced fibrinolytic activity (decreased tPA function) is at present unclear. Whether this increased thrombotic risk exists in other arterial procedures is unclear, but at the present time it may be sensible to make sure that you leave plenty of time for breakfast before embarking upon your carotid surgery.
## Table 1

The relationship between post-operative emboli numbers and patient risk factors

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Mean no. of emboli</th>
<th>St dev</th>
<th>P=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>53.1</td>
<td>143.9</td>
<td>0.004</td>
</tr>
<tr>
<td>Male</td>
<td>17.5</td>
<td>51.9</td>
<td></td>
</tr>
<tr>
<td>Age 70+</td>
<td>32.3</td>
<td>106.7</td>
<td>0.410</td>
</tr>
<tr>
<td>Age &lt;70</td>
<td>23.1</td>
<td>69.1</td>
<td></td>
</tr>
<tr>
<td>Symptomatic</td>
<td>28.9</td>
<td>94.4</td>
<td>0.613</td>
</tr>
<tr>
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<td>15.1</td>
<td>34.6</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>22.0</td>
<td>39.4</td>
<td>0.658</td>
</tr>
<tr>
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<td>96.7</td>
<td></td>
</tr>
<tr>
<td>Hypertensive</td>
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<td>98.1</td>
<td>0.967</td>
</tr>
<tr>
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<td>27.6</td>
<td>74.2</td>
<td></td>
</tr>
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<td>20.1</td>
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<td>0.422</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>30.6</td>
<td>101.7</td>
<td></td>
</tr>
<tr>
<td>Previous MI</td>
<td>37.4</td>
<td>132.5</td>
<td>0.306</td>
</tr>
<tr>
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<td>73.6</td>
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</tr>
<tr>
<td>Angina</td>
<td>13.8</td>
<td>23.4</td>
<td>0.101</td>
</tr>
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<td>No angina</td>
<td>34.1</td>
<td>106.8</td>
<td></td>
</tr>
<tr>
<td>Claudication</td>
<td>32.7</td>
<td>115.4</td>
<td>0.595</td>
</tr>
<tr>
<td>No claudication</td>
<td>26.0</td>
<td>78.7</td>
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</tr>
<tr>
<td>Ipsilateral stenosis &gt;85%</td>
<td>32.9</td>
<td>116.5</td>
<td>0.404</td>
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<tr>
<td>Ipsilateral stenosis &lt;85%</td>
<td>23.7</td>
<td>60.4</td>
<td></td>
</tr>
<tr>
<td>Contralateral stenosis &gt;70%</td>
<td>21.9</td>
<td>40.8</td>
<td>0.420</td>
</tr>
<tr>
<td>Contralateral stenosis &lt;70%</td>
<td>32.1</td>
<td>109.1</td>
<td></td>
</tr>
<tr>
<td>Emboli on pre-op TCD</td>
<td><strong>64.3</strong></td>
<td>108.0</td>
<td></td>
</tr>
<tr>
<td>No emboli on pre-op TCD</td>
<td><strong>22.1</strong></td>
<td>68.8</td>
<td>0.027</td>
</tr>
</tbody>
</table>
## Table 2

Comparing factors relating to the operation and post-operative embolisation.

Angioscopy +ve means that a technical defect was found at angioscopy that required correction.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Mean no. of emboli</th>
<th>St dev</th>
<th>P=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Op time &gt; 2 hours</td>
<td>31.2</td>
<td>83.5</td>
<td>0.752</td>
</tr>
<tr>
<td>Op time &lt; 2 hours</td>
<td>28.3</td>
<td>97.4</td>
<td></td>
</tr>
<tr>
<td>Shunt time &gt; 45 mins</td>
<td>16.6</td>
<td>39.5</td>
<td>0.233</td>
</tr>
<tr>
<td>Shunt time &lt; 45 mins</td>
<td>28.1</td>
<td>85.1</td>
<td></td>
</tr>
<tr>
<td>Clamp time &gt; 6 mins</td>
<td>21.7</td>
<td>64.4</td>
<td>0.796</td>
</tr>
<tr>
<td>Clamp time &lt; 6 mins</td>
<td>24.3</td>
<td>74.0</td>
<td></td>
</tr>
<tr>
<td>Patch length &gt; 6cm</td>
<td>35.9</td>
<td>100.5</td>
<td>0.089</td>
</tr>
<tr>
<td>Patch length &lt; 6cm</td>
<td>15.9</td>
<td>46.5</td>
<td></td>
</tr>
<tr>
<td>Angioscopy +ve</td>
<td>37.5</td>
<td>91.8</td>
<td>0.569</td>
</tr>
<tr>
<td>Angioscopy -ve</td>
<td>27.0</td>
<td>91.8</td>
<td></td>
</tr>
<tr>
<td>Trainee operating</td>
<td>16.3</td>
<td>47.1</td>
<td>0.078</td>
</tr>
<tr>
<td>Consultant</td>
<td>36.8</td>
<td>111.9</td>
<td></td>
</tr>
<tr>
<td>Op start time &lt; 10.30</td>
<td>48.6</td>
<td>136.7</td>
<td>0.004</td>
</tr>
<tr>
<td>Op start time &gt; 10.30</td>
<td>14.7</td>
<td>34.4</td>
<td></td>
</tr>
<tr>
<td>Op finish time &lt; 12.00</td>
<td>53.2</td>
<td>134.7</td>
<td>0.002</td>
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<tr>
<td>Op finish time &gt; 12.00</td>
<td>15.1</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>
The significant inverse correlation between the time of the end of the CEA and the rate of post-operative embolisation

Figure 1

\( p = 0.014 \)
References


PART THREE

SUMMARY
CHAPTER TWELVE

Summary

Carotid endarterectomy for patients with a significant carotid stenosis reduces their risk of stroke over the subsequent years of follow up. This benefit is ameliorated by post-operative strokes and deaths. The higher the complication rate, the more patients that have to be operated on to prevent one stroke. Even for the relatively low complication rates seen during ECST and NASCET six patients required a CEA to prevent one stroke, and there is evidence that complication rates from CEA are significantly higher in non-trial centres. If the post-operative complication rate can be reduced to a minimum then it is possible that patients with lesser stenoses may also benefit from CEA. This body of work has concentrated on identifying factors related to the development of strokes secondary to carotid thrombosis following CEA.

Carotid thrombosis is the most common cause of post-operative stroke after CEA. Traditionally it has been thought that patients developed arterial thrombosis as a result of an event or action during their operation, and that the surgeon himself was the cause of the thrombosis. Findings from this study challenge that concept and have also examined ways in which the post-operative thrombotic stroke rate maybe reduced.

The first part of this study was to see whether patients' risk of thromboembolic events after their primary CEA was maintained if they underwent a subsequent CEA on a different date. There was a significant correlation between the number of post-operative emboli detected at the first and second procedures. It is unlikely that "errors" occurring at the first operation that lead to large numbers of emboli would then be
repeated many months later leading to similar numbers of emboli at the second operation. It seems much more plausible that each particular patient has a given propensity to arterial thrombosis that is preserved over time. It was unclear at this point whether this was due to a single factor or the presence of multiple, additive risk factors.

In our randomised trial of vein patch angioplasty versus Dacron angioplasty we tested the hypothesis that autologous vein would be less pro-thrombotic than Dacron. The results showed that for very low rates of embolisation there were slightly more patients patched with vein than Dacron. However, when the numbers of patients with high rates of embolisation were evaluated (ie those requiring post-operative Dextran-40 therapy) there was no difference between the two types of patch. Again this would support the hypothesis that it is actors related to the patient that predispose them to post-operative strokes.

The next study that was performed had a dual purpose, firstly to identify whether factors relating to patients' platelets, which have a key role in arterial thrombosis, were associated with high rates of post-operative embolisation. The second role of the study was to determine whether it would be ultimately possible to dispense with the units labour intensive programme of post-operative TCD monitoring and replace this with pre-operative anti-platelet medication that would prevent post-operative embolisation. This study demonstrated that those patients with the highest numbers of post-operative emboli had platelets that were significantly more sensitive to the effects of the physiological agonist, ADP, using two different methodological techniques. This

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study also showed that aspirin alone was not sufficient to prevent post-operative embolisation and that the degree of platelet inhibition induced by aspirin did not correlate with post-operative emboli numbers. This study has identified a potential therapeutic target in that an ADP receptor antagonist such as clopidogrel, may help to prevent post-operative thrombotic stroke. If pre-operative administration of clopidogrel could be shown to significantly reduce emboli numbers below the point where no patient requires post-operative Dextran-40 therapy then it would be possible to dispense with the TCD monitoring programme. Many vascular surgical units do not have the manpower and equipment available to them to perform post-operative TCD monitoring and a single pre-operative dose of an anti-platelet agent may be a more acceptable method of preventing thrombotic stroke after CEA.

The administration of low dose Dextran-40 to patients with high rates of post-operative embolisation has previously been proven to prevent post-CEA thrombotic stroke, but despite its efficacy in this area little is understood of its mode of action. Since we had shown that patients' platelets were related to post-operative embolic events the next logical step was to evaluate the effect of Dextran-40 on patients' platelets in vitro. Rather surprisingly Dextran-40 did not directly inhibit the platelets as expected but actually stimulated them. Further work demonstrated the platelets actually bind Dextran-40 to their surface and that this is enhanced when the platelets are activated prior to exposure to the Dextran-40. We were not able to demonstrate any inhibition of platelet function after incubation with Dextran-40. Our working hypothesis is that by preferentially coating the surface of the activated platelets the
Dextran-40 then reduces their ability to interact with other platelets so reducing the ability of the platelets to form a growing thrombus.

As it appeared that platelets were involved in post-operative thrombosis and that patients' propensity to form thrombi was inherent to each individual patient it was a logical step to study inherited differences in platelets' surface receptors for fibrinogen, von Willebrand factor and collagen. These platelet receptor polymorphisms were correlated with clinical events, patient risk factors, platelet function studies and unsurprisingly, post-operative emboli numbers. Of the three polymorphisms studied, only one (HPA-3) demonstrated any consistent effect. Patients with the ser/ser allele were significantly more likely to suffer pre- and post-operative emboli, had increased platelet responsiveness in the laboratory studies and suffered significantly less haemorrhagic complications in the post-operative period. Patients with this allele appear to have a decreased risk of haemorrhage after trauma but at the expense of an increase in their risk of post-operative thrombosis. Although it is not possible to alter a patients phenotype to reduce their risk of thrombotic events, it is possible to screen them before cardiovascular interventions and identify those at high risk. It should then be possible to tailor the management of these patients differently to those with the low risk phenotype – for example all patients with the HPA-3 ser/ser allele could be given prophylactic Dextran-40 therapy to prevent post-operative carotid thrombosis in units where TCD monitoring is not available.
The final body of work studied the risk factors and operative factors for a large cohort of patients undergoing CEA in an attempt to identify factors other than patients’ platelet function that may be associated with emboli. Only three factors were significantly associated with emboli numbers and each of these were patient related rather than factors related to the operation per se. Those patients who had emboli in their pre-operative monitoring period suffered more emboli after their CEA. Again this would imply a constitutive cause for thrombotic events. It has long been known that women fare less well following arterial surgery and this study may have helped to elucidate one of the factors underlying this phenomenon. Female patients were more than twice as likely to need post-operative Dextran-40 therapy to treat very high rates of post-operative embolisation than men. This significantly increased risk of post-operative thrombosis may well explain why women’s stroke rates are higher following CEA, their poorer outcomes following peripheral and coronary angioplasty and their increased mortality after CABG. The final factor associated with post-operative emboli was the time of the CEA. Procedures either starting and/or finishing in the morning had three times as many emboli associated with them relative to afternoon operations. This finding probably relates to the fact that platelets exhibit a marked diurnal variation, with their function being highest in the early morning. This interesting finding offers a very simple possibility to reduce patients’ risk of post-CEA thrombotic stroke – operate on them only in the afternoon.
The findings of this body of work support the hypothesis that it is the patient who is prothrombotic rather than the procedure per se. It has also identified a number of potential targets and therapeutic strategies that may help to reduce the number of post-operative emboli seen after CEA, and thereby reduce patients' risk of thrombotic stroke. The findings of this study may well have relevance to other areas of cardiovascular intervention.