

Asymptomatic Embolisation and Strategies for Treatment in Carotid
Artery Disease

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I hereby declare and affirm that this thesis is entirely my own work and composition.

Date: 18th February 1999 Signature:

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Abbreviations Used

AF	atrial fibrillation
ANOVA	analysis of variance
β TG	beta thromboglobulin
CAS	carotid artery stenosis
cGMP	cyclic guanosine monophosphate
CT	computed tomography
CW	continuous wave
DAT	digital audio tape
dB	decibel
DC	direct current
ECST	European Carotid Surgery Trial
ELISA	enzyme-linked immunoassay
EME	Eden Medizinische Elektronik GmbH
ES	embolic signal(s)
FFT	fast Fourier transform
FITC	fluoro-isothiocyanate
FSC	forward scatter
GMI	geometric mean intensity
GP	glycoprotein
GSNO	S-nitrosoglutathione
HSM	Dr. H.S.Markus
IgG	immunoglobulin G
ICA	internal carotid artery
JM	Dr. J.Molloy
MAP	mean arterial pressure
MCA	middle cerebral artery
MoAB	monoclonal antibody
MRA	magnetic resonance angiography
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NIDDM	non-insulin-dependent diabetes mellitus
NHS	National Health Service
NO	nitric oxide
NRAF	non-rheumatic atrial fibrillation
PE	phycoerythrin
PRF	pulse repetition frequency
PTA	percutaneous transluminal angioplasty
SD	standard deviation
sP-sel	soluble P-selectin
SSC	side scatter
TCD	transcranial Doppler
TIA	transient ischaemic attack
TXA ₂	thromboxane A ₂
TXB ₂	thromboxane B ₂
UK	United Kingdom

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ABSTRACT OF THESIS

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3.5.13)Name of Candidate MOLLOY, JANE ELIZABETH

Address

Degree M. D. Date 15/7/98Title of Thesis ASYMPTOMATIC EMBOLISATION AND STRATEGIES
FOR TREATMENT IN CAROTID ARTERY DISEASENo. of words in the main text of Thesis 25,000 (approx).**Background & Purpose:**

Stroke is the third leading cause of death in industrialized countries. Incidence in the UK is estimated at 2 per 1000, rising dramatically with age. Ischaemic stroke accounts for the majority of these events, and may arise due to embolism, thrombosis or haemodynamic mechanisms. At present, there is no proven accepted treatment for the acute ictus, and management - both medical and surgical - places the emphasis on secondary prevention.

The presence of carotid artery stenosis is one factor known to be associated with increased stroke risk, whether symptomatic or asymptomatic. This may arise indirectly, due to its association with other vascular risk factors, such as hypertension and diabetes, or directly to cause both embolic and haemodynamic stroke. Two large trials have shown significant reduction in stroke risk following carotid endarterectomy in patients who have had recent symptoms. In patients with asymptomatic carotid artery stenosis, surgery is not routinely recommended, on the basis of risk-benefit analysis.

It is possible to monitor for asymptomatic embolic signal using transcranial Doppler ultrasound (TCD) to record from the intra-cerebral vessels. These have been shown to occur in patients with carotid artery stenosis, and correlate with a number of individual markers of clinical risk. However, several areas in the application of TCD to asymptomatic embolus detection are unclear, and will be addressed as below.

Contents:

i) *Assessment of Multi-Gated Doppler* - use of transcranial Doppler ultrasound is hindered by lack of an automated system for analysis of patient recordings. Issues surrounding this will be discussed, and a new method to aid in differentiation of embolic signals from artifact is assessed both in a model and in patients.

ii) *Clinical Significance and Variability of Embolic Signals* - presence of embolic signals as an independent risk factor for stroke is not proven, and studies have assessed their correlation with individual risk markers in small numbers. In a larger patient group clinical assessment of a number of factors is made in combination with transcranial Doppler studies. In a smaller sub-group, the temporal variability of embolic signals is assessed.

iii) *Reduction of Embolisation Using S-Nitrosoglutathione* - though evidence from validation studies in vitro and in animal models supports the particulate nature of emboli detected by transcranial Doppler, no definite conclusions can be drawn regarding the nature of the embolic material. By studying anti-platelet and anti-thrombotic agents in patients with frequent embolic signals it may be possible to reduce these signals - combining intervention studies with a knowledge of the molecular action of such agents may allow a better understanding of the mode of embolisation in such a setting. A study of the use of an anti-platelet agent, S-Nitrosoglutathione, GSNO, to reduce embolisation rates in the setting of carotid endarterectomy is described.

Chapter 1

Stroke - Introduction

1.1 Stroke - Definitions

Stroke is the third leading cause of death in the Western world. It places a major burden on the resources of the National Health Service (NHS) both in terms of acute hospital admissions and long-term support for those left disabled by the initial ictus. The World Health Organisation (WHO) definition of stroke is ‘rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death with no apparent cause other than of vascular origin’¹. The definition includes subarachnoid haemorrhage but excludes transient ischaemic attacks (TIA), subdural haematoma, and haemorrhage or infarction caused by infection or underlying tumour. Clinically, the distinction is drawn between TIA and stroke by defining TIA as causing loss of neurological function lasting <24 hours.

Strokes may therefore be one of two types - ischaemic (infarction) or haemorrhagic. Ischaemia causes the majority of strokes - approximately 80% - and arises either from temporary or permanent occlusion of the arterial supply to an area of brain. Haemorrhage causes approximately 15% of all strokes (10% primary intracerebral haemorrhage, 5% subarachnoid haemorrhage), with the mechanism of the other 5% of strokes being ‘uncertain’. Stroke related to cerebral haemorrhage will not be considered further in this thesis - the term ‘stroke’ will be taken to mean ‘ischaemic stroke’ from this stage on.

1.2 Stroke - Sub-types and Classification

Ischaemic stroke may be classified according to clinical presentation, mechanism or arterial site.

Clinical categorisation was the method used to subdivide patients in the Oxford Community Stroke Project² - with subdivisions into Total Anterior Circulation Syndrome (TACS), Partial Anterior Circulation Syndrome (PACS), Posterior Circulation Syndromes (POCS) and Lacunar Syndromes (LACS). These syndromes predict volume of cerebral infarction, and therefore correlate with outcome - as such they are clinically useful.

Stroke may alternatively be categorised according to the pathophysiologic *mechanism* - such as used in the Stroke Data Bank study³. Pathophysiological classifications used were: large artery atherosclerosis, lacunar, cardiac embolism, tandem arterial pathology, infarct of unknown origin and other. Classification was largely done on the basis of neuroimaging, and in fact it was not possible to categorise 40% of patients assessed⁴.

The National Institute of Neurological Diseases and Stroke (NINDS) classification subdivides according to a combination of *pathological mechanism*, *clinical category* and *arterial distribution*⁵. Again difficulties may be encountered - similar clinical deficits may result from arterial occlusions at different sites, or conversely arterial occlusion may occur without necessarily causing any symptoms.

NINDS Classification

Mechanism: thrombotic; embolic; haemodynamic

Clinical: atherothrombotic; cardioembolic; lacunar

Arterial site: internal carotid; middle cerebral; anterior cerebral; vertebral; basilar;
posterior cerebral

More recently, the Trial of ORG10172 in Acute Stroke Therapy (TOAST) study categorised stroke as atherothromboembolic, cardioembolic, small vessel thrombotic, other and unknown⁶. Here there was failure to clearly categorise in 15% of patients, and on many occasions the imaging diagnosis was at odds with that obtained clinically.

1.3 Stroke Incidence

Incidence of stroke is 1.5/1000/year, rising with age to 10/1000/year by the age of 75. In addition to the burden of mortality, it also accounts for significant morbidity and disability placing both social and financial burdens on carers and caring services. Stroke currently accounts for approximately 5% of UK NHS resources⁷.

There is some evidence to suggest that stroke mortality may be decreasing over time⁸. Potential reasons for this may include decreased reporting rates (death certification), previous diagnostic inaccuracies (improvement with imaging techniques), changing incidence of the different sub-types and improved survival time - hence decreased mortality may be unrelated to stroke incidence.

Reduction of this burden - both on a personal level and on society - will depend on primary preventive measures, secondary preventive measures, and provision of treatment of the acute event - though presently there is no proven

effective treatment in acute stroke. Strategies for stroke prevention will be discussed further below.

1.4 Stroke - Primary Prevention

This refers to prevention of stroke in individuals with no clinical evidence of the disease. As only approximately 20% of strokes occur with any preceding symptoms, then to produce any reduction in the overall incidence of events, effective primary prevention is needed. Preventive measures can be divided into general risk factor reduction and specific pharmacological and surgical therapies. Surgical management, both as a primary and secondary measure, will be considered under the general heading 'Carotid Artery Stenosis'.

1.4.1 Primary Prevention - Risk Factor Control

Hypertension is the most powerful and treatable risk factor for stroke. Both systolic and diastolic blood pressure are independently related to stroke incidence. Reduction of elevated blood pressure significantly lowers stroke risk. Meta-analyses of blood pressure lowering trials found that an average reduction in diastolic blood pressure of 6mmHg produces a 42% reduction in stroke incidence^{9,10}. Treatment of isolated systolic hypertension in people over the age of 60 years has been shown to reduce stroke incidence by 36%, without excessive side effects such as depression or dementia¹¹.

Cigarette smoking is a further significant risk factor in stroke, increasing the risk by a factor of 1.5 to 2.2¹²⁻¹⁴. Risk of stroke correlates with numbers of cigarettes smoked, and risk decreases promptly with cessation of smoking^{12,13,15}.

In diabetic subjects, good glycaemic control is important in risk reduction¹⁶. The role of hypercholesterolaemia in stroke risk has not been clearly defined - a meta-analysis of lipid-lowering trials found no benefit in terms of stroke risk reduction¹⁷. A more recent study of lipid lowering in coronary artery disease¹⁸ showed a reduction in both fatal and non-fatal strokes in patients taking simvastatin - however this was done on post-hoc analysis rather than used as a primary endpoint. There are other lipid-lowering studies reporting a slowing of the progression of atherosclerosis as assessed ultrasonographically^{19,20}. The shared risk factors for coronary and carotid atherosclerosis suggest that risk factor reduction strategies for both should be similar, though there has been a reluctance to generalise studies in one setting to the other.

Excessive alcohol consumption is associated with increased stroke risk - moderate consumption has been shown to have no effect in some studies or to confer a degree of protection^{21,22}. Those taking alcohol to excess should be advised to cut back their intake. Current recommended limits in the UK are ≤ 14 units per week for females and ≤ 21 units per week for males²³.

1.4.2 Primary Prevention - Pharmacological Measures

i) anti-platelet agents

There have been two clinical trials addressing the role of low-dose aspirin in primary prevention of stroke^{24,25} - neither have found any significant difference in the incidence of stroke between the treatment and control groups. Indeed, the British trial²⁵ found a higher incidence of disabling stroke in those taking aspirin.

ii) anticoagulants

Patients with atrial fibrillation (AF) are known to be at increased risk of stroke. In cases where AF is secondary to rheumatic heart disease, or where the patient is about to undergo DC cardioversion this risk appears to be particularly high. Though there are no controlled trials in those specific areas, conventional treatment is to anticoagulate with warfarin.

Non-rheumatic AF (NRAF) has a prevalence in the community of 0.4%²⁶, rising to 2% in those over the age of 60, and further to 10% in those over 70 years of age²⁷. Both the Framingham and Whitehall studies found patients with NRAF to be at a fivefold risk of stroke compared to matched subjects in sinus rhythm, even after controlling for age, sex and associated hypertension^{28,29}. There have been several studies addressing the use of warfarin in prevention of primary stroke events in NRAF patients^{30,31}, showing overall benefit with warfarin treatment. The results of these trials are summarised in **Table 1.1**.

Study	N	PT/INR	Relative Risk Reduction
BAATAF ³⁰	420	1.2-1.5 (PT)	86%
AFASAK ³²	1007	2.8-4.2 (INR)	64%
SPINAF ³³	571	1.2-1.5 (PTR)	79%
SPAF-I ³⁴	1330	2.0-4.5 (INR)	67%
SPAF-II ³¹	1000	2.0-4.5 (INR)	40%

Table 1.1. Summarising the major trials of warfarin versus aspirin or placebo in patients with NRAF in primary stroke prevention. BAATAF - Boston Area Anticoagulation Trial for Atrial Fibrillation; AFASAK - Copenhagen Atrial Fibrillation, Aspirin, Anticoagulation Trial; SPINAF Stroke Prevention in Non-rheumatic Atrial Fibrillation Trial; SPAF-I - Stroke Prevention in Atrial Fibrillation Study; SPAF-II - Stroke Prevention in Atrial Fibrillation II Study.

NRAF has an attributable risk of stroke of 1.5% for those age 50-59, rising to 23.5% for those aged 80-89 years²⁷. In patients younger than 60 years with 'lone' atrial fibrillation (no other heart disease or risk factors) the risk of stroke is low at 0.5-1.0% per annum. With increasing age and presence of a number of other risk factors (including hypertension, diabetes, previous venous thromboembolism, ischaemic heart disease, cardiac failure, atrial enlargement as demonstrated on echocardiography and evidence of an ischaemic lesion on CT scanning) the stroke risk is much higher and therefore benefit of anticoagulation greater³¹. In patients where warfarin is contraindicated, aspirin is superior to no treatment.

1.5 Stroke - Secondary Prevention

By definition, secondary prevention refers to a population with previous TIA or stroke. The same principles will apply as in primary prevention, but the risk-benefit ratios will alter - this will be discussed under each sub-heading.

1.5.1 Secondary Prevention - Risk Factor Control

As discussed above, only about 20% of strokes are preceded by any symptoms, but in these patients risk of future events is of the order of 12% in the following year, falling to approximately 7% per annum in subsequent years³⁵. A similar approach to risk factor reduction as in primary prevention is therefore required. Exceptions to this include over-aggressive treatment of hypertension in the acute phase following stroke and in patients with severe carotid artery stenosis - in both these settings this may lead to hypoperfusion and worsening cerebral ischaemia.

1.5.2 Secondary Prevention - Pharmacological Measures

i) anti-platelet agents

Following observation of platelet thrombi in the retinal arterioles of patients with amaurosis fugax^{36,37}, aspirin, a known anti-platelet agent, was given to patients with recurrent amaurosis and found to be of benefit^{38,39}. Clinical trials of platelet inhibitors were subsequently launched, and there have since been many randomised controlled trials assessing aspirin in secondary prevention - a meta-analysis of these trials showed a reduction in stroke and other serious vascular events by 23%⁴⁰. Aspirin acts by inhibition of the cyclo-oxygenase pathway for the lifetime of the platelet to inhibit aggregation.

A recent study has shown an additive effect if dipyridamole is prescribed in combination with the aspirin, though the dose of aspirin used in this study was small compared to that given in standard practice⁴¹.

Newer anti-platelet agents are emerging, including ticlopidine, clopidogrel and the anti-integrins, such as abciximab.

Ticlopidine is a thienopyridine derivative, and does not affect the cyclo-oxygenase pathway. It inhibits the platelet aggregation induced by adenosine diphosphate and other agonists by altering the platelet membrane to interfere with the membrane-fibrinogen interaction and blocking the platelet glycoprotein IIb/IIIa receptor⁴². Ticlopidine has been shown in two studies to reduce stroke risk - the Canadian American Ticlopidine Study (CATS)⁴³ compared ticlopidine to placebo with reduction in stroke and stroke death of 33.5%. The Ticlopidine Aspirin Stroke Study (TASS)⁴⁴ compared aspirin and ticlopidine directly with a mean patient follow-up of

5.8 years, finding ticlopidine to be as, if not more, effective than aspirin. However, there is an associated risk of neutropaenia - reported in 2% of patients in the TASS - hence monitoring of the full blood count is required. It is presently available in the UK on a named patient basis only.

A similar preparation to ticlopidine without the concomitant risk of neutropaenia is clopidogrel - a second thienopyridine derivative which inhibits platelet aggregation stimulated by adenosine diphosphate. Its role in secondary prevention has been evaluated against aspirin in a large trial⁴⁵ involving 19185 patients with symptomatic cerebrovascular disease, ischaemic heart disease and peripheral vascular disease. A marginal benefit was shown ($p=0.043$).

The final step in platelet aggregation is binding of fibrinogen or von Willebrand factor with the integrin receptor glycoprotein (GP) IIb/IIIa. It is possible to inhibit platelet aggregation by preventing binding to this receptor, either with a blocking antibody (e.g. abciximab), or by synthetic compounds (e.g. integrilin, eptifibatide). Such agents have been used to inhibit platelet aggregation in normal volunteers⁴⁶ and in the setting of unstable angina⁴⁷, acute myocardial infarction⁴⁸ and following coronary angioplasty⁴⁹. There have been no studies of such agents in cerebrovascular disease in humans, though there has been a recent report of the use of TP-9201, a further GP IIb/IIIa antagonist, in prevention of carotid artery re-thrombosis in an animal model⁵⁰.

1.6 Stroke - Surgical Prevention - Carotid Artery Stenosis

1.6.1 Carotid Artery Stenosis - Prevalence

Atherosclerotic change is common at the bifurcation of the common carotid artery in the neck where it may progress to cause initially asymptomatic stenosis of the origins of the internal and external carotid arteries. Complications of internal carotid artery stenosis (CAS) include embolisation or thrombotic occlusion, which may lead to neurological symptoms such as TIA, stroke or retinal ischaemia. Following such an event the lesion would then be termed symptomatic carotid stenosis. It is possible for either embolism or occlusion to occur in the absence of clinical symptoms or signs. It has been estimated that carotid artery disease may be responsible for 20-30% of all new strokes⁵¹.

Asymptomatic CAS may be detected incidentally during investigations for vascular disease during surgical work-up, e.g. in preparation for coronary artery bypass grafting. It may be found during investigation for cause of a neurological event in another vascular territory, or through inappropriate investigation for the cause of some other non-vascular neurological symptom. Auscultation of the neck during clinical examination may reveal the presence of a carotid bruit, which may arise from turbulent flow in the underlying narrowed artery. Bruits are not a reliable indicator of either presence or severity of CAS - one series of 500 patients with asymptomatic neck bruits found degree of CAS of >75% in only 113 of the subjects - this following exclusion of 40 patients with 'non-arterial' neck murmurs⁵². A second study, performed in the UK, examined a series of 331 patients referred to a cerebrovascular clinic, 110 of whom were noted to have a carotid bruit. Of these, 41(37%) were

found to have moderate to severe (30-99%) stenosis of the underlying carotid artery. Investigation of the remaining 221 patients without a bruit found the same degree of disease in 38(17%)⁵³.

There have been three large population-based studies examining the prevalence of carotid artery stenosis (most cases studied being asymptomatic) - this rises from 0.5% in people in their 50's to approximately 10% in those over the age of 80⁵⁴⁻⁵⁶. Risk factors for development of CAS are shared with those causative of other vascular disease, and include hypertension, cigarette smoking, elevated blood lipids and genetic factors. Management of these risk factors has been discussed in detail above, and applies equally in the setting of both asymptomatic and symptomatic CAS.

Diagnosis of CAS can be made using several imaging modalities - non-invasive techniques include Duplex ultrasonography, spiral CT and magnetic resonance angiography. Intra-arterial angiography remains the 'gold standard' yet invasive technique. As centres validate Duplex and MRA results against angiography, non-invasive techniques may replace intra-arterial angiography in the future.

1.6.2 Carotid Artery Stenosis - Natural History

There have been 5 prospective studies of asymptomatic CAS with sample sizes greater than 100⁵⁷⁻⁶¹. For the greater part, stenoses were left unoperated unless they became symptomatic. The rate of development of symptoms attributable to the CAS reported from all these studies would appear to range from 2-5% per annum. Risk tends to increase with degree of stenosis, appearing greatest with reduction of the luminal diameter by 75-90%⁶². There is a close association between CAS and

coronary artery disease, and risk of death from cardiac events appears greater than from stroke in this group^{60,63}.

Once a stenosis has become symptomatic, then the risk of further ipsilateral symptoms increases greatly - this having been demonstrated in two large trials - the European Carotid Surgery Trial (ECST)⁶⁴ and the North American Symptomatic Carotid Endarterectomy Trial (NASCET)⁶⁵. Both these trials randomised patients with symptomatic CAS between medical treatment and surgical carotid endarterectomy. It is from the medically managed arm of each trial that much information can be learned concerning the natural history of the disease. Immediately following ischaemic stroke or TIA due to CAS, risk of further events is highest during the first few weeks to months. In the ECST, the risk of any stroke within 3 years was 21.9% in those receiving medical therapy alone for those with severe (70-99%) CAS. In the NASCET the risk of development of an ipsilateral stroke within 2 years was 26% for those receiving medical therapy alone. There is further evidence to suggest that the risk of further events may be higher following non-disabling stroke than hemispheric TIA which is in turn of higher risk than ipsilateral amaurosis fugax⁶⁶.

1.6.3 Carotid Artery Stenosis - Therapeutic Implications

A. Primary Prevention

As described above, patients with asymptomatic carotid artery stenosis are at increased risk of stroke at the rate of approximately 2% per annum. There has been a trial of carotid endarterectomy in such patients, the Asymptomatic Carotid Atherosclerosis Study, or ACAS⁵⁷ addressing the question of whether carotid endarterectomy reduces the overall 5-year risk of carotid stroke. For the purpose of

this study, CAS was defined as $\geq 60\%$. They reported a significant, though small, stroke reduction in the operative group - in order to prevent one stroke over a five year period 17 operations would be needed. This benefit would be lost if the surgical complication rate were higher - in this study the risk of surgical or angiographic death rate was only 2.3%. The question of identification of a high risk group for whom to target surgery has become a key issue. It has been suggested that in addition to absolute degree of CAS^{58,62}, male sex and coexisting heart disease may confer additional risk⁵².

The UK based Asymptomatic Carotid Surgery Trial, ACST⁶⁷ continues to randomise patients. It is likely that again risk stratification will be of importance here - potential indicators of risk being evaluated include degree of stenosis, presence of a CT infarct, identification of an echolucent plaque on carotid ultrasound and impaired cerebral haemodynamics estimated by transcranial Doppler ultrasound.

At present, carotid endarterectomy is not recommended routinely for patients with asymptomatic carotid artery stenosis in the UK. In the United States, a recent American Heart Association Statement⁶⁸ recommends surgery as of proven benefit for patients with a surgical risk of $< 3\%$ and life expectancy of at least 5 years.

B. Secondary Prevention

As a result of the ECST and NASCET trials as described above, carotid endarterectomy is of proven benefit against best medical therapy alone in patients with 'severe' (70-99%) symptomatic CAS. The surgical complication rates were 7.5% in ECST and 5.8% in NASCET. For ECST surgery conferred a 3-year risk of perioperative death or ipsilateral stroke of 12.3%, an absolute risk reduction of 9.6+/-

3.3%. For NASCET, the risk of an ipsilateral cerebral infarct within 2 years was reduced from 26% to 9%, an absolute risk reduction of 17+/-3.5%.

For mild (0-29%) stenosis, neither study showed any benefit conferred by surgery. In patients with moderate (30-69%) stenosis, both studies have shown a marginal benefit of surgery versus best medical therapy within certain subgroups, and at present surgery is not recommended routinely for this group^{69,70}.

At present, accepted clinical practice in the treatment of symptomatic CAS is therefore carotid endarterectomy. There has also been interest in the possibility of percutaneous transluminal angioplasty (PTA) for treatment of the carotid lesion. This procedure precludes the need for a general anaesthetic or neck incision with the potential complications of both being avoided, and early studies of the procedure produced encouraging results⁷¹. The risks and benefits of PTA versus endarterectomy have been recently addressed in a randomised clinical trial, as part of the Carotid and Vertebral Artery Transluminal Angioplasty Study, or CAVATAS. Final results have not been published, but preliminary data suggest that at 2 years of follow-up the procedures are equivalent⁷²(CAVATAS Collaborators Meeting, London, U.K., October 1998).

1.7 Carotid Artery Stenosis - Embolisation and Clinical Implications

The mechanism by which carotid artery stenosis causes stroke is by either distal embolisation or thrombotic occlusion. It is now possible to detect embolic material within the cerebral circulation by means of transcranial Doppler ultrasound⁷³. The ability to detect circulating cerebral emboli in patients with carotid artery disease is of potential use in the following situations:

i) identifying individuals at highest risk of stroke. Presuming that asymptomatic embolic activity is an indicator of increased clinical risk, in much the same way as TIA's can indicate increased stroke risk, then treatment can be more efficiently targeted. This would be particularly helpful in aiding risk-benefit decisions in patients with asymptomatic carotid artery stenosis, where surgery is as yet only of proven benefit in centres with negligible complication rates⁵⁷, though studies continue of potential risk stratification markers⁶⁷.

A further group in which management is unclear, and where the results of large studies have not been entirely conclusive, is in patients with moderate carotid stenosis, of the order of 30-69%. Results from the NASCET study for this group have been reported in abstract form⁶⁹, suggesting that the risk-benefit ratio may only be favourable in certain subgroups, though by definition such sub-group analysis produces its own statistical difficulties.

ii) in patients with more than one potential embolic source - for example carotid artery stenosis and atrial fibrillation - transcranial Doppler can be employed to localise the embolic source. This may be done both by use of bilateral middle cerebral artery (MCA) monitoring and by recording both above and below the level of the atherosclerotic lesion simultaneously to allow 'tracking' of the embolic signal⁷⁴.

iii) where embolic signals are frequent, it may be used to monitor efficacy of therapeutic measures. As yet it is not possible to distinguish the nature of the embolic material on Doppler spectral characteristics alone - were this to become possible it would allow treatment to be targeted specifically - anti-platelet agents for platelet emboli, anti-coagulants for thrombus.

iv) monitoring of procedures. Emboli have been shown to be associated with adverse outcome in the setting of cardio-pulmonary bypass, carotid endarterectomy and carotid angioplasty. Monitoring in these situations is of use to develop strategies for avoidance of embolisation and to monitor their effectiveness. Further to this it may allow us to draw conclusions regarding the pathophysiology of embolisation in each situation.

In the chapters that follow, the background and history of transcranial Doppler will be described (**Chapter 2**). I will go on to discuss validation and evaluation of the technique both in the clinical setting and in a flow model (**Chapter 3, Chapter 4**). Further work in the clinical setting assessing the incidence and natural history of embolisation rates in CAS will be presented in **Chapters 5 & 6**. In **Chapter 7** the use of transcranial Doppler in monitoring of interventional procedures will be discussed and results of an interventional study using a new anti-platelet agent will be presented.

All patient studies were approved by the King's Healthcare Ethical Committee, with informed consent obtained from each subject.

Chapter 2

Transcranial Doppler Ultrasound and Embolus Detection - Background and History

2.1 Ultrasound

Sound waves are transmitted in longitudinal fashion by propagation of the vibration of air molecules from the sound source (transmitter) to the ear of the listener (receiver). The rate of vibration is measured in cycles per second (Hertz, Hz) and the pitch or frequency of the sound depends on this rate. At greater than 20kHz, the sound is no longer audible to the human ear and is then termed 'ultrasound'.

Ultrasound waves are transmitted as compression waves within a medium, also known as longitudinal waves. The source has a known frequency (f , cycles per second) and periodicity (T , period of time between similar points on consecutive cycles of the waveform). The relationship between these two factors may be described as:

$$f = 1/T$$

The wave is transmitted by causing similar motion of the particles of the insonated medium with the same frequency as the source. As the wave is propagated through the medium there will be compression of the medium as particles move in the same direction as their neighbours, causing a local increase in pressure. As particles then move back in the opposite direction, there will be a local reduction in pressure. This change in pressure is known as the excess pressure. The displacement, velocity and excess pressure of the particle at any point within the medium in front of the source

will vary over time in a sinusoidal fashion with the same frequency as the source. The maximum value of any of these quantities is known as its amplitude. As the wave moves with fixed velocity but varying amplitude, the wavelength (distance between similar points on consecutive cycles of the waveform) will vary. Wavelength and frequency are related to the speed of sound (c) by:

$$c = \lambda/T$$

hence:

$$= \lambda f$$

The speed of transmission of ultrasound depends on both the mass and spacing of the particles and the strength of the forces of attraction between particles. Speed of transmission will increase as the strength of the attractive forces between particles increases (so c is higher for stiffer materials) and decreases as the mass of the particles increases - i.e. the material is more dense.

This relationship can be illustrated as:

$$c = \sqrt{K/\rho} \quad \text{m/s}$$

where ρ = density (kg/m^3)

and K = elastic modulus (stiffness) which is also equal to stress/strain.

$1/K$ equals κ , or compressibility.

The velocity of sound in various biological media is shown in **Table 2.1** overleaf.

Medium	Velocity (m/s)
Air	330
Water (20°C)	1480
Blood	1570
Fat	1460
Muscle	1580
Bone	3500
Soft tissue (mean)	1540

Table 2.1. Velocity of sound in biological media. (Adapted from Fish, P., Diagnostic Medical Ultrasound, John Wiley & Sons, Chichester, England. 1990.)

The velocity with which sound moves through a medium can be related to the excess pressure using the formula:

$$Z = \text{Excess pressure/Particle velocity}$$

normally calculated by $Z = \rho c = \sqrt{\rho/\kappa}$ rayls or kg/m²/s

where Z is the tissue, or acoustic impedance of the material.

Values of the acoustic impedance for a selection of media are tabulated in **Table 2.2.** (Adapted from Evans, McDicken, Skidmore & Woodcock; Doppler Ultrasound. John Wiley & Sons, Chichester, England 1989.)

Medium	Acoustic impedance (kg/m ² /s x 10 ⁶)
Air	0.0004
Water (20°C)	1.48
Fat	1.38
Blood	1.6

Table 2.2. Acoustic impedance values for a variety of media.

The ultrasound source emits the wave from all points on its surface, therefore producing ultrasound throughout a predictable volume in front of the source. The surface joining all similar points on a similar cycle is known as a wavefront. Direction of travel of the ultrasound beam and the wavefront are perpendicular to one another. If produced by a plane transducer, then those wavefronts close to the transducer will be plane, but as the wave travels the beam begins to diverge. As the distance from the transducer increases, wavefronts become part of a spherical surface.

Ultrasound waves used medically are normally produced by an electromechanical transducer contained within a probe which is placed onto the skin surface - the transducer acting as a transmitter. Electrical energy (Joules) is used to drive the transducer to produce mechanical energy. Absorption of this mechanical energy by the insonated medium can generate heat. The rate of generation is also known as the power of the source, measured in Joules/second, or Watts. Power will vary at different points within the insonated medium depending on the width of the beam produced and any focusing effect. This is known as the intensity of the ultrasound beam - the power passing through unit area, perpendicular to the direction of propagation. It is not uniform across the beam, but tends to peak towards the

centre of the beam and reduce towards its outer aspect. The average intensity can be calculated, and is known as the spatial average.

Ultrasound waves may be one of two types - continuous wave and pulsed wave.

2.1.1 Continuous Wave (CW) Ultrasound

CW ultrasound produces a continuous beam of ultrasound into the tissues. The beam thus transmitted is reflected from any acoustic interface encountered. As everything within the path of the beam is insonated it is impossible to estimate the position of the examined site. The only setting in which CW is used routinely is in evaluation of peripheral vascular disease, as CW Doppler using high frequency probes to insonate superficial vessels of known position. The technology involved is relatively simple and relatively inexpensive.

2.1.2 Pulsed Wave Ultrasound

Medical ultrasound mainly utilises pulses, allowing information to be gathered regarding depth of the insonated area and hence localisation. As applied using Doppler to examine flow in insonated vessels it gives information about location and directionality of flow in addition to waveform information.

The intensity of pulsed ultrasound varies over time as well as space - therefore instead of a spatial average there is a temporal average of ultrasound intensity. With pulsed wave ultrasound, the transducer acts as both transmitter and receiver, emitting pulses of ultrasound at a known frequency. The duration of the ultrasound pressure pulse produced is known as the pulse length. Each emitted pulse contains a certain

energy - energy emitted per second, or average transmitted power is equal to the pulse energy multiplied by the pulse repetition frequency (PRF).

As the ultrasound pulse is transmitted into the tissues, it will be altered by the structures insonated. At boundaries between different tissue types it may be reflected or refracted, it may undergo scattering by small tissue structures and also lose energy by absorption. These factors will be considered in further detail below. The time between transmission of the sound wave and detection of the reflected beam allows an indication of the depth of any interface to be calculated.

2.1.3 Ultrasound Propagation

i. Reflection

Any sound beam incident at an interface will undergo modification - part of the beam will be transmitted through the interface, a proportion will be reflected and some energy will be lost by absorption. If the interface is perpendicular to the direction of propagation of the beam, then the amount of ultrasound reflected depends on the acoustic impedance of the two tissues involved. Where the difference in acoustic impedance is high - for example at soft tissue-air interfaces - then there will be a high degree of reflection. At boundaries between differing types of soft tissue, the amount of reflection will be less. Where the angle of insonation is greater than zero, this will influence both the reflected and transmitted intensities. Where ultrasound is incident on a rough surface, or on small objects, then the beam is scattered rather than reflected. The proportion of the beam scattered or reflected determines the intensity of the received signal.

ii) Refraction

Deviation of the ultrasound beam also occurs at tissue interfaces, but is dependent on the difference of the speed of ultrasound transmission, not acoustic impedance. The sound will bend away from the perpendicular if the speed in the second medium is higher than in the first and vice versa.

iii) Scatter

If instead of a smooth interface larger than the wavelength of the insonating beam, the ultrasound is incident on a rough surface, or on small particles of a size smaller than or comparable with the beam wavelength, then the beam will be scattered in all directions rather than reflected. The amount of power lost increases with increased frequency.

iv) Absorption

Some power will be lost secondary to absorption of energy by the insonated tissue, with conversion to heat. Loss due to absorption increases with frequency.

Combinations of the above factors effect a reduction in ultrasound intensity during its passage through any medium - this is termed attenuation of the beam. Attenuation produced by either scatter or absorption is tissue specific, therefore attenuation due solely to these factors can be quantified for any tissue - the fractional rate of change of intensity with distance that would occur if a non-diverging or converging beam were used in a uniform sample of tissue (i.e. with no boundaries allowing reflection or refraction. This is termed the attenuation coefficient (α), and may be calculated for any tissue by the following equation:

$$\alpha = \frac{\Delta I}{I \Delta x} \text{ m}^{-1}$$

where ΔI is the loss of intensity from incident intensity I during passage through a thin layer of thickness Δx and where $\Delta I/I \ll 1$. The overall attenuation coefficient is the sum of the absorption and scatter coefficients.

As the fractional change in intensity is a ratio, it may be expressed in decibels - total decibel attenuation coefficient is defined as:

$$\mu = 4.3\alpha \quad \text{dB/m}$$

which is roughly proportional to frequency. Hence for the frequency of the transducer used the attenuation may be calculated by:

$$\mu \cong kf$$

where k is in dB/m/MHz - or the value of μ at 1MHz, and f is the frequency in MHz.

Typical attenuation coefficients are shown in **Table 2.3**. (From Evan, McDicken, Skidmore & Woodcock; Doppler Ultrasound. John Wiley & Sons, Chichester, England 1989)

Tissue	Attenuation coefficient at 1MHz dB m ⁻¹
Water	0.002
Blood	0.2
Fat	0.6
Liver	0.7
Brain(Adult)	0.8
Soft tissue(mean)	0.7
Castor oil	1.0
Muscle	1.5
Bone (skull)*	10

Table 2.3. Typical attenuation coefficients.

* *Huetner, 1952*

2.2 The Doppler Principle

Ultrasound as described above can be used to study and scan static structures, or structures where there is little movement to produce anatomical or structural information. Movement of any echo-generating tissues can be displayed as a function of time by means of the M-mode display, as used in echocardiography to measure heart valve dynamics.

Movement of reflectors or scatterers within the insonated area will in addition change the frequency of the received signal - the change from the transmitted frequency being known as the Doppler effect, and the magnitude of the change (Doppler shift) being proportional to the reflector or scatterer velocity. By exploitation of this phenomenon, it is possible to monitor the cyclical variation in blood velocity within arteries, and to interpret disease-induced changes in velocity waveform or the increased range of Doppler shift frequencies resulting from disturbed flow. Combination of real-time scanners with Doppler effect instruments may be used to display both vessel anatomy and velocity waveform simultaneously.

The Doppler effect is the change in the observed frequency of a wave due to motion - whether of the source or the observer. When motion is towards the source then there is a perceived increase in frequency as more wave cycles per second are passed. This increased frequency may be calculated according to the formula:

$$f_r = f_t \frac{c+v}{c}$$

where f_t is the transmitted frequency and v is the velocity of the observer.

Conversely, when motion is away from the sound source, the perceived frequency is lower, as fewer wave cycles per second are passed.

If the observer is at rest and the source moves with velocity v in the direction of wave travel, then the wavelengths are compressed and again the perceived frequency is higher.

The Doppler principle was first formulated in 1842 by the Austrian physicist, Christian Doppler, at the Royal Bohemian Society of Sciences in Prague^{75,76}. It was three years later that the theory was experimentally verified, in fact in an experiment designed to prove the theory wrong. The Dutch physicist, Buys Ballot, used a band of horn players on a railroad flatcar moving at 65km/hour to demonstrate that the shift in tone between approaching and receding was perceived to be two steps on the musical 12-tone scale (approximately +/- 6%)⁷⁷.

2.3 Transcranial Doppler Ultrasound

Application of ultrasound for cardiovascular assessment combines this Doppler principle with the radar principle outlined above. It was first suggested in 1960 by two workers in Osaka, Japan⁷⁸; indeed the second of these workers first suggested the possibility of applying the technique to insonate the intracranial vessels⁷⁹. However, the large attenuation effect on the ultrasound beam by the skull was perceived as problematic, and this preconception delayed the development of Doppler ultrasound for transcranial use. It was not until 1981 that Rune Aaslid in Bern, Switzerland made the first transcranial Doppler (TCD) recordings^{80,81}. To do this he used a low frequency (2MHz) transducer, and utilised the naturally-occurring 'acoustical windows' - the transtemporal window, first described by Aaslid and colleagues in 1982⁸⁰, the transorbital window⁸² and the transforaminal window⁸³. The transtemporal approach can be used to examine the middle cerebral, anterior

communicating, distal internal carotid and posterior communicating arteries. Using the transorbital approach, the orbital and distal internal carotid arteries may be studied. From a submandibular approach, the distal carotid may be studied, and via the foramen magnum it is possible to study the vertebral arteries and their continuation into the basilar artery.

Most studies to date utilise the transtemporal approach, and the studies described here are all made from the middle cerebral artery using this approach. The relative thinness of the temporal bone means lower attenuation of the ultrasound beam, but still the acoustical intensity of the ultrasound conducted into the brain tissue is always less than 35% of the original beam⁸⁴. Modifications to the maximum acoustical intensity of the transducer have improved on the possible performance of the technique; however there remains a sub-population in whom it is not possible to obtain an adequate signal - reported failed insonation rates vary between 10 to 23%^{85,86}.

Since 1982, and the first description of the use of transcranial Doppler, there has been a rapid expansion of the application of the technique. It has been used to examine cerebral haemodynamics including demonstration of collaterals, to demonstrate the anatomy of the circle of Willis and also to demonstrate embolic material within the circulation. As the work here is concerned with detection of embolic material within the middle cerebral artery and its clinical relevance, the other applications will not be considered further in this work.

2.4 Detection of Embolic Material

As described above, the intensity of the returned ultrasound signal provides information regarding the proportion of the transmitted beam which is scattered or reflected, which in turn is dependent on the material through which the beam is directed. The degree of reflection, scatter or transmission is determined by the acoustic impedance of the tissue examined. Where there is an interface between two materials of widely differing acoustic impedance the proportion of the ultrasound energy reflected will be high.

Air has a high acoustic impedance compared with the other media (see **Table 2.2**) - hence the ultrasound reflected at an interface between air and a second medium will be high. The proportion of the beam reflected may be determined by the reflection coefficient, defined by the equation:

$$\text{Reflection coefficient, } k = [Z_2 - Z_1 / Z_2 + Z_1]^2$$

where Z_1 is the acoustic impedance of the first medium and Z_2 is that of the second medium. Multiplying the reflection coefficient by 100 gives the percentage reflection at the interface. As the original equation squares the value obtained, the amount of reflection will be the same at the tissue interface irrespective of the direction of movement of the beam, whether from high to low, or from low to high, acoustic impedance.

Of course, the amount of reflection from the interface does not only depend on the above coefficient, but also the size or area of the interface. Where the interface is greater than the wavelength of the insonating beam, reflection will be determined

entirely as above, but where the interface is approximately equal to or smaller than the wavelength of the beam, Rayleigh scattering increases in importance. Here there is scattering of the beam in all directions, and the signal detected will only be a proportion of that reflected towards the receiver.

It can be seen from the above that any embolic material within the circulating blood may act to reflect a proportion of the ultrasound beam. The amount of signal reflected to the receiver will depend to some extent on the composition of the embolus, its size and the wavelength of the ultrasound used. The difference between scatter and reflectance of the beam by the embolus and that produced by the surrounding blood may allow its detection by Doppler ultrasound. Information regarding the intensity of the reflected signal may allow some inference to be drawn regarding the size and composition of the embolic material⁸⁷.

There have been previous studies describing the use of Doppler ultrasound in detection of emboli within the circulation. Detection of gaseous emboli was described as long ago as 1965⁸⁸. They have been detected in the venous circulation of divers in decompression chambers⁸⁹, and were shown to be associated with 'the bends'⁹⁰.

Such signals have also been described in the setting of cardiopulmonary bypass, again first in humans in 1969⁹¹, both in the extracorporeal circuit and in vessels. TCD has since been used to detect air embolism occurring during the time of aortic cannulation, during initiation of the bypass and intermittently during the duration of the bypass⁹². Observation of such signals, and preliminary studies suggesting that such embolic events correlate with poorer outcome as assessed by

neuropsychological testing, have led to development of strategies to avoid such embolic activity^{93,94}.

Although abnormal signals thought to represent fat emboli in the venous circulation of patients undergoing hip replacement were detected as long ago as 1975⁹⁵, this observation was not taken further. Then in 1990, Spencer and co-workers⁷³ reported signals thought to represent solid emboli in the middle cerebral artery prior to arteriotomy in the setting of carotid endarterectomy. There had been previous reports of detection of embolic signals, particularly with relation to insertion of shunts and release of the cross-clamp, felt to be secondary to air emboli⁹⁶, but ES had not previously been noted prior to opening of the artery. From this first observation, Spencer et al described typical features of an embolic signal from presumed solid material as transient (0.01-0.1 seconds), hyperintense (up to 40 dB greater than background) and with an accompanying audible 'chirp'⁷³. A typical embolic signal is illustrated in **Figure 2.1**.

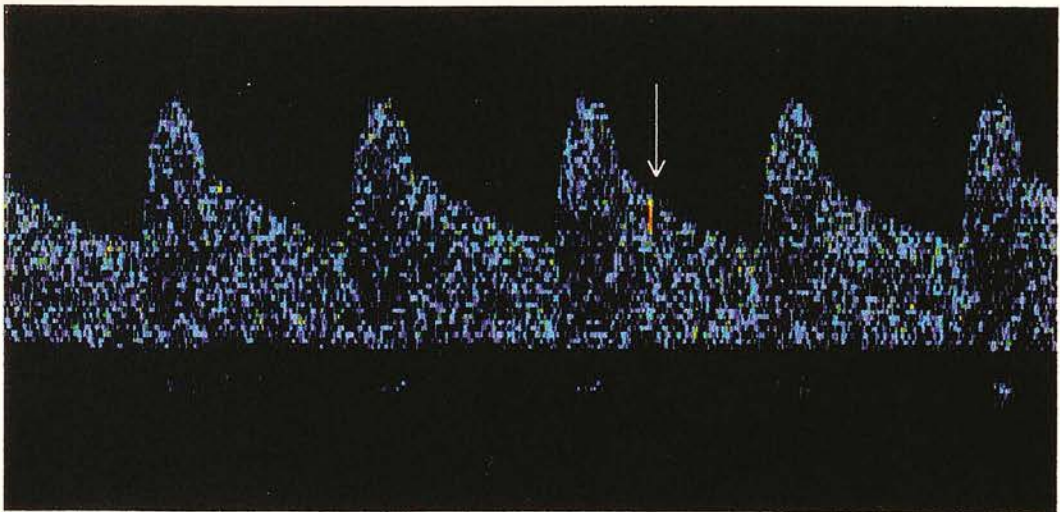


Figure 2.1. Typical embolic signal (arrowed) within the Doppler waveform of a middle cerebral artery. It is of much higher intensity than the surrounding Doppler signal - this may be calculated from an accompanying intensity scale.

Following these observations, reports followed of detection of similar signals in individuals at increased risk of thromboembolic stroke including atrial fibrillation⁹⁷, carotid artery stenosis⁹⁸ and cardiac valve prostheses⁹⁹. However, such initial reports were viewed with caution - artefact such as that produced by probe movement or by movement or speech from the patient also produces transient high intensity signals, and it is important to be certain that these signals do not merely represent artefact or turbulent or abnormal flow patterns.

Validation studies were therefore necessitated, both in vitro and in animal models. Their findings are summarised below.

2.4.1 Embolus Detection - In-Vitro Studies

Markus and Brown⁸⁷ set up a flow model to both validate the technique for embolus detection and to investigate the possibility of discrimination between different embolic materials. Emboli of known size and composition (thrombus, platelet-rich aggregates, atheromatous material and fat) were introduced into the flow circuit and reliably produced transient high-intensity signals as previously described in patients. They found a highly significant correlation between embolus size and maximum amplitude of the reflected signal for all materials used. In addition they suggested that platelet-rich aggregates produce lower intensity ES than seen with atheroma.

A second in vitro study¹⁰⁰ examined reliability of the technique in detection of air emboli and related the intensity of the resulting signals to the volume of air injected. They showed that air volumes of 0.5 μ l were detected 80% of the time,

increasing to 100% for bubbles of $\geq 0.75\mu\text{l}$. Bubble volumes of the order of 30-40 μl resulted in signal saturation.

2.4.2 Embolus Detection - Animal Studies

The first in-vivo validation study was performed by Russell et al in 1991¹⁰¹, in a rabbit model. Embolic material was introduced into the aorta via cannulation of the left renal artery, with insonation of the distal aorta 7cm caudal to the point of cannulation. This was chosen as the rabbit aorta in this portion is of comparable diameter to the human MCA. Though no definitive analysis of the resulting signal intensities was performed in this paper, they reported higher intensity signals resulting from air and fat emboli over emboli composed of clotted whole blood, platelets or atheromatous material.

Further to this work, a second validation study¹⁰² examined embolus detection in a sheep model - allowing insonation of a cerebral vessel with a flow waveform similar to that seen in the human cerebral circulation with preserved diastolic flow. Again they reported a correlation between embolus size and maximum relative intensity of the embolic signal. It was not possible to introduce embolic material of $\leq 400\mu\text{m}$ into the model - hence the lower limit of solid embolus detection could not be defined. Signals from platelet emboli were less intense than atheromatous and thrombus emboli, but only small numbers were studied.

A third study¹⁰⁰ looked further at detection and quantification of air embolisation in the middle cerebral artery of Macaque monkeys, recording via the transtemporal route. As for their in-vitro study described above they found early

saturation of the receiver at the same bubble volumes, with air bubbles reliably producing ES.

In each model introduction of embolic material resulted in production of a short duration high-intensity (high-amplitude) transient signal. In both the rabbit and Macaque models signals were described as having an accompanying characteristic 'chirp'. Combining the findings provides strong evidence that the signals detected in-vivo are consistent with those produced by embolic material in-vitro, and supports the argument that these signals in-vivo do represent embolic activity.

2.5 Factors Influencing Embolus Detection

Preliminary work utilising TCD to record for ES in the setting of carotid artery stenosis produced widely varying results - both regarding percentages of patients found to be ES positive and rates of ES per hour in subjects where ES were detected. In symptomatic CAS the numbers of patients defined as ES positive has ranged from 20-90%¹⁰³⁻¹⁰⁷. The results from these studies are summarised in **Table 2.4** overleaf.

This variability in results can arise from many possible sources - technical factors, patient characteristics, recording protocols used and analysis of any recordings made - whether done manually or by an automated system.

Table 2.4. Summary data from previous studies of ES detection in the setting of symptomatic carotid artery stenosis, highlighting the variability in patient, technical and recording factors - and results.

Authors	Year	Recording Time	Threshold (Decibels)	Sample Volume (mm)	n	ES Positive (%)	Time cut-off (days)	Treatment
Siebler et al ¹⁰⁸	1993	1 hour	9	15	14	100	≤120	H
Grosset et al ¹⁰⁹	1993	30 minutes	-	-	20	100	-	A/W/N
Markus et al ¹¹⁰	1994	20 minutes	4	10	25	24	-	A/A+H/W/N
Siebler et al ¹⁰⁵	1994	1 hour	9	15	33	67	≤121	H/N
Babikian et al ¹⁰⁶	1994	25-30 minutes	9	-	53	28.3	-	H/A/W/T/N
Georgiadis et al ¹⁰³	1994	30 minutes	3	9	50	96	≤2190	A/W/N
Markus et al ¹⁰⁷	1995	20 minutes	4	10	38	21.1	≤182	A/W/N
Siebler et al ¹¹¹	1995	1 hour	9	15	28	89	≤120	-
Valton et al ¹⁰⁴	1995	40 minutes	4	-	20	20	≤81	H/A/T/N/C

'Time cut-off' time in days since last symptomatic from the stenosis used to define whether or not the vessel was 'symptomatic'. H= heparin, W = warfarin, A = aspirin, T = ticlopidine, N = no treatment, C = combination, - = not specified.

2.5.1 Technical Factors Influencing Detection

The most important technical parameters affecting the detectability of ES are: 1) the relationship between the backscattered power from emboli and that from the blood (relative intensity increase); 2) the detection threshold; 3) the size of the sample volume; 4) the fast Fourier transform (FFT) frequency resolution; 5) the FFT temporal resolution; 6) the FFT temporal overlap; 7) the dynamic range of the instrumentation; 8) the transmitted ultrasound frequency; 9) filter settings and 10) recording time¹¹². Both the use of an intensity threshold and recording time will be considered under 'Recording Factors'. In addition, scale, sweep speed and gain settings will influence results.

i) Relative Intensity Increase

This represents the ratio of the acoustic power backscattered from the embolus to that of the surrounding moving blood¹¹³, and is normally measured in decibels (see Chapter 3). The relative intensity increase will be affected to some extent by the transmitted ultrasound frequency and gain settings, and is also dependent on embolus size and composition and the blood volume in the insonated sample.

There are differing methods of determining the intensity increase associated with an ES - different methods being used in different devices. It is also possible to determine the increase manually. For instance, the intensity of the background signal may be expressed as a mean or median value over a certain time period or frequency range, both of which may be variable. Therefore, for any given ES, differing values of intensity increase may be derived, even with all other settings kept constant.

This could potentially have a major influence on ES detection if an intensity threshold is used. This effect is examined in Chapter 3.

ii) Sample Volume

The cross-sectional area of the sample volume at the insonation depth is referred to as the 'beam-width'. For any given probe, the transmitted ultrasound is not uniform, and the beam width will vary with insonation depth - this source of variability is beyond the control of the investigator. It is possible, however, to adjust the axial length of the sample volume. The larger the sample volume, the greater the power output of the transducer. Alterations in axial sample volume will effect a strong influence on measurements of relative intensity increase, and this has been demonstrated in an experimental set-up¹¹⁴. As axial sample volume increases, the number of small ES detected will be reduced - ultrasound reflected from the surrounding blood will be greater and the ratio of signal reflected by the embolus to background signal will be reduced. This ratio may also be described as the embolus: blood ratio, or EBR¹¹³.

iii) Frequency Resolution and Temporal Resolution - Fast Fourier Transformation

TCD systems use a method of analysis known as Fast Fourier Transformation (FFT) to convert the Doppler audio (time domain) signal into the standard time-frequency spectrum display (frequency domain). Intrinsic to FFT processing is that there is an inversely proportional relationship between time and frequency resolution, such that to simultaneously produce high levels of both is impossible, and some compromise has to be made¹¹⁵. As ES are of short duration (often only 10

milliseconds) it would be necessary to produce time resolution of just 1 millisecond to accurately measure their duration. It is not possible to do this, as it would require too great a reduction in frequency resolution - i.e. increasing the time resolution to cope with very short duration ES decreases the frequency resolution such that no useful information on ES velocity can be obtained.

Each FFT is displayed as a column on the frequency display, and execution of each FFT requires a specific amount of time. To obtain a reasonable temporal resolution, the data segment analysed should not exceed 5 to 10 milliseconds¹¹². The greater the number of points used for the FFT, the poorer will be the temporal resolution - an FFT resolution of 64, 128 or 256 points is preferred at present¹¹².

Depending on the speed of the processor, a variable amount of each FFT can be overlapped with the next FFT. If there is no overlap, then it is possible that an ES may be missed by virtue of an embolus passing through the sample volume but arriving between the sampling period of the two time windows and therefore not being displayed on screen. Inadequate FFT overlap may produce variability in the measurement of the intensity increase associated with an ES if it occurs at the temporal edge of the FFT time window¹¹⁶. The FFT settings should be kept constant for all recordings - an overlap of $\geq 50\%$ is essential, as smaller overlaps (e.g. 10%) will risk missing individual ES¹⁰⁷.

iv) Dynamic Range

Large solid emboli or those resulting from gaseous bubbles in the circulation may produce very high-intensity ES with receiver overload and aliasing - precluding assessment of both the intensity increase and ES velocity¹¹⁷. Such signals may appear

similar to those produced by artefact. Determination of the intensity increase may therefore be hindered by a low dynamic range - especially with older machines. To minimise the effect of this restriction in clinical practice, it is best to record at low power and gain settings¹¹⁴.

v) Transmitted Ultrasound Frequency

A 2MHz probe is almost universally applied for TCD recording purposes - use of a different transmitted ultrasound frequency will change both background signal and that reflected from an embolus. Higher frequencies produce lower sensitivity, while reports of use of a lower frequency (1MHz) probe in ES detection in both in-vitro¹¹⁸ and in-vivo¹¹⁹ models have been promising - showing an increased signal:background ratio.

vi) Scale

The scale can be adjusted to alter the velocity range of the Doppler signal. The lowest scale setting will therefore allow the full velocity range of the Doppler signal to be accommodated. The higher the scale setting, the higher the pulse repetition frequency (PRF, number of pulses per second), and therefore higher ultrasound, or power, output. Scale should therefore be kept constant for all recordings. Decreasing the scale may increase the numbers of ES missed as there will be reduced temporal resolution with lower PRF and velocity range. Scale should be kept constant where possible - where the maximal velocity of the Doppler spectrum extends beyond the velocity range it should be possible to alter the position of the zero baseline rather than alter the scale.

vii) Sweep Speed

Adjustments to sweep speed effectively adjust FFT overlap - the faster the sweep speed, the greater the degree of overlap between successive time frames, with effects on ES detection as described above.

viii) Gain

The gain setting may be adjusted to change the amplification of the received Doppler spectrum, such that a clearly defined spectrum is obtained. The intensity scale is colour-coded and adjustable in configurable steps. Recording acquisition should be done at constant low gain settings, such that the Doppler spectrum is just visible - this increases the effective dynamic range over which the intensity increase can be measured¹¹⁴.

2.5.2 Recording Factors Influencing Detection

i) Documentation

The most widely used documentation system presently in use is to record the pre-FFT signal (time domain signal) to digital audio tape¹²⁰. This allows analysis of recordings to be made off-line, blinded to clinical information; also permitting re-examination of regions of interest and exchange of data between centres.

ii) Duration

Studies have shown that both incidence and frequency of ES may vary greatly over time^{107,121,122}. It can be seen in **Table 2.4** that recording times used have varied from 20 minutes to 1 hour. The influence of recording time on ES detection will be considered in greater depth in Chapter 6.

iii) Interpretation of Results

In interpretation of recordings made for ES detection there is great potential for bias and intra- and inter-observer variation. These factors can be minimised by:

- i) analysing all recordings with no knowledge of patient data - i.e. off-line, and
- ii) standardised training of personnel who are to interpret the recordings.

A study in 1996 examined reproducibility between two centres which had different reporting rates of ES positive subjects¹²³. Using a standard tape made of composite recordings from 8 subjects with ipsilateral CAS in whom ES had been detected in a previous study, they assessed both within- and between- centre agreement by examining reporting rates from a total of 4 experienced observers. They reported a high level of agreement, both between all observers (0.90), between three observers in one centre (0.89) and between observers in the two different centres (0.94). Probability of detection was independently related to the relative intensity of the embolic signal ($P < 0.0001$) and also to the position of the ES in the cardiac cycle, ($P = 0.02$) - signals were more reliably detected in systole. The fact that the more intense ES were more likely to be detected led the group to examine the effect of setting a decibel threshold to further define an embolus - though this increased rates of agreement, it had an adverse effect on the sensitivity of the technique.

iv) Use of a Decibel Threshold

The typical characteristics of an ES as first detected have been described previously - with recorded intensity of ES up to 40 decibels. Again, from the table,(2.4) it can be seen that studies have varied in the minimum threshold used to define an ES from 3 to 9 dB. In response to the variable findings among centres, in

1995 the Consensus Committee of the Ninth International Cerebral Haemodynamic Symposium released a statement on the basic identification of Doppler microembolic signals¹²⁴. They recommended that to constitute an ES, the signal should have the following basic features:

1. a Doppler microembolic signal is transient, usually lasting less than 300 milliseconds. Its duration depends on its time taken to pass through the Doppler sample volume.
2. the amplitude of a Doppler microembolic signal is usually at least 3dB higher than that of the background blood flow signal and depends on the characteristics of the individual microembolus.
3. within the appropriate dynamic range of bi-directional Doppler equipment, a signal is unidirectional within the Doppler velocity spectrum.
4. depending on the equipment used and its own velocity, a microembolic signal is accompanied by a 'snap', 'chirp' or 'moan' on the audible output.

The typical appearance of an ES has been illustrated in **Figure 2.1**.

From the technical aspects discussed above, it can be seen that variations in intensity may result from equipment used and recording settings - further differences may be produced by interpretation methods, which will be described below and in Chapter 3. Decibel thresholds need to be determined for each individual centre publishing results.

2.6 Use of Automated Embolus Detection Systems

At present there is no reliable automated ES detection system - all analysis of recordings done off-line is therefore done manually - the recordings may be lengthy and may yield few ES. Some studies have been published having used commercially available automated systems, and the potential problems arising from such systems are considered in further detail in Chapter 4.

Yet further variability may arise due to differences in patient populations and treatment regimes - it can be seen that even within studies there may be great variations in anti-platelet and anti-thrombotic therapy used. This effect can be minimised within study by standardising treatment protocols, but may not always be practical or ethical.

Chapter 3

Methods: Transcranial Doppler Ultrasound

In the previous chapter, technical and recording factors which may produce variability in reported results were discussed, with reference to previous studies in subjects with carotid artery stenosis. In this chapter, general methods used for all TCD recordings reported in this thesis will be described.

3.1 Patient Recordings

All patient recordings were performed using the commercially available TC4040 Pioneer (Nicolet EME Limited, Überlingen, Germany) with software version 2.21, designed for diagnostic cerebral investigation and long-term unilateral or bilateral cerebrovascular monitoring. The 'probe' referred to and used throughout is a 2MHz transducer.

In all cases, unilateral recordings were made from the MCA ipsilateral to the stenosed carotid artery via the transtemporal window. Once a satisfactory MCA signal was obtained the probe was held in place using an external fixation device, and recording commenced for the time selected. For initial studies the fixation device used was a Müller and Möll headset, though an updated headset, (Spencer Technologies, Marc500) was used in studies performed after June 1997.

The MCA was selected as it supplies the majority of the circulation to its ipsilateral hemisphere. It is a direct continuation of the internal carotid artery and is its main branch, coursing in a horizontal plane, laterally and slightly anteriorly. It is the largest branch of the ICA, with a normal mean diameter of 2.5-3.8mm^{125,126}, and

forms an angle of $<20^\circ$ with the insonating probe. Identification of the MCA was made on a number of criteria as described below:

1. ability to insonate the vessel via the transtemporal window.
2. the depth in millimetres from the face of the transducer at which the sample volume is being recorded. This is taken in combination with the ability to trace the vessel along its length.
3. the direction of flow in relation to the transducer - in the case of the MCA, flow will be seen *towards* the probe.
4. the spatial relationship of the insonated vessel to the bifurcation of the internal carotid artery and ability to separately identify the posterior cerebral artery.
5. relative flow velocity of the insonated vessel.

It has been suggested that a sixth criterion should be used - that of the effect of common carotid artery compression manoeuvres on intracerebral flow. We did not apply this to our patient group, as all had significant carotid artery disease, and there are potential hazards associated¹²⁷.

In the case of the recordings made in the dissection phase of carotid endarterectomy, and following carotid PTA, the patient was examined in the supine position. Following carotid endarterectomy, patients were examined while sitting at an angle of 45° . All other recordings were made with the patients sitting upright in a chair.

During the recordings, patients were discouraged from speaking, as this produces artefact which may obscure ES.

Single depth recordings were made onto digital audio tape (DA-R120, TDK Limited, Japan) using a Sony DAT Walkman (Sony Corporation, Japan). Multi-gate recordings were made onto Hi8 videocassette (Maxell, Japan) using a TASCAM DA-88 digital multitrack recorder (TEAC UK Limited, Watford, Herts, UK). All recordings were encoded at the time of their being made, and no patient data were recorded onto the tape packaging. This enabled further coding to be done at a later date and facilitated later off-line analysis to be carried out in a blinded fashion.

3.1.1 Technical Settings

- i) **Sample Volume:** We initially aimed for a sample volume of 4mm, but as this did not provide sufficient power to obtain a satisfactory Doppler spectrum in the majority of patients, this was changed to 5mm at an early stage in the project. In those patients where 5mm proved insufficient, the sample volume was increased step wise to a maximum of 12mm giving a power of 53% (100% power = 675mW/cm²).
- ii) **FFT Resolution:** For all recordings a 128-point FFT was used.
- iii) **FFT Overlap:** An FFT overlap of >50% was used in all recordings.
- iv) **Dynamic Range:** The Pioneer 4040 allows a dynamic range of 64 decibels.
- v) **Filter Setting:** The high-pass filter cut-off used in all recordings was 120 Hz.
- vi) **Scale:** The scale used for all recordings was such that the standard velocity range was 0 to 135 cm/second. Where this was insufficient, the baseline zero was shifted towards the base of the screen to increase the displayed velocity range.
- vii) **Sweep Speed:** This was kept constant at 5.1 seconds for all recordings.
- viii) **Gain:** All recordings were made at low gain settings.

ix) Depth: Depth settings used were between 46 and 56 millimetres in order to achieve the best possible signal. Where two-channel (multi-depth) recordings were made, recordings were standardised with the optimum signal set as the distal channel (proximal MCA) with the reference gate 4 millimetres distally (proximal channel, distal MCA). This protocol was decided on the basis of the multigate Doppler study described in Chapter 4.

3.2 Validating Recording Protocols

3.2.1 Recording Duration The consideration of duration of recording used will be discussed in detail in Chapter 6 - for all studies, recording times chosen will be explained in the relevant chapter.

3.2.2 Recording Interpretation Prior to undertaking any definitive analysis of recordings for the presence of ES, it is first necessary to familiarise oneself with the usual characteristics of ES as recorded onto digital audio tape, and to then validate the analysis technique against a standard recording. Analysis of the standard recording referenced¹²³ in Chapter 2 was therefore undertaken to determine the percentage agreement of my analysis technique against that of an experienced observer.

A total of 1.75 hours of tape were analysed for ES, from a total number of 8 patients. When an ES was detected, the time of onset of this signal was documented, and the Doppler spectrum at that time was saved to the hard drive for later review if necessary. Once analysis was completed the record of all ES detected was compared with that produced by another experienced observer (HSM).

ES were identified on the basis of their characteristic appearance as described in Chapter 2. In total, Observer 1 (JM), observed a total of 89 signals felt to be

embolic in nature. The results for embolus detection were compared with Observer 2 (HSM), who reported 85 embolic signals in all. The results were compared to give a figure for likelihood of agreement between the 2 observers.

Normally comparison between observers is made using Cohen's kappa. This statistic cannot be applied in the situation described here, as analysis of the tape recording relies only on reporting of positive results - there are not any episodes where neither observer records an event (or 'abnormality'). Hence the number of occasions where neither observer records an abnormality is unknown, and is potentially infinite. If we apply the kappa statistic to the proportion of agreement from the results of this tape analysis, we generate **Table 3.1**.

		Observer 1		
Observer 2	+	-		Row Total
+	82	7		89
-	3	0		3
Column Total	85	7		

Table 3.1. Cross-tab table illustrating numbers used to calculate kappa co-efficient for proportion of agreement for the 2 observers as described above. Cohen's kappa from this table is -0.048, indicating extremely poor agreement - it is obvious however that a high degree of agreement had been attained. As there are only 2 observers, there are no 'negative' observations, and this affects the coefficient calculated. This problem is tackled in Bland and Altman, *Statistical Approaches to Medical Measurement*¹²⁸.

Potentially there is an infinite number of 'negative' observations noted by both observers. To look at the effect of adding 'negative' observations to the kappa coefficient we can add a number of theoretical negatives to the tables shown overleaf:

		Observer 1		
Observer 2		+	-	Row Total
+	82	7		89
-	3	18		21
Column Total	85	25		

Table 3.2. Effect of adding a small number of negative observations to the calculations - kappa coefficient here is = 0.726.

The degree of agreement suggested by the kappa statistic will progressively improve as more and more theoretical negative observations are added as illustrated in **Table 3.3** and **Table 3.4**.

		Observer 1		
Observer 2		+	-	Row Total
+	82	7		89
-	3	408		411
Column Total	85	415		

Table 3.3. By increasing the number of negative observations kappa is increased to 0.930.

		Observer 1		
Observer 2		+	-	Row Total
+	82	7		89
-	3	1968		1971
	85	1975		

Table 3.4. Increasing the number of agreed negative observations further, kappa = 0.940 - indicating a high level of agreement.

Instead of using the kappa coefficient, the most meaningful aspect of these observations (of ES) is the probability that if the first observer records an ES then the second observer will concur, because a proportion of agreement regarding negative observations will only be possible if the results are then compared with those of a third observer.

From the original table (**Table 3.1**) the proportion of agreement of Observer 1 (JM) with Observer 2 (HSM) is 0.97 (96.5%); proportion of agreement of Observer 2 with Observer 1 is 0.92 (92.1%). This level of agreement compared favourably with the levels achieved in the paper quoted in Chapter 2. From this it was agreed that tapes from patient recordings could be analysed independently - initially all possible ES identified were saved to the machine hard-drive for review, and after continuing good levels of agreement as to what constituted an ES, analysis was continued independently.

3.2.3 Setting a Decibel Threshold

All studies described in the following chapters also use a decibel threshold, which was set at 7dB or above. The reasons for this are defined below. As mentioned above, one way of improving inter-observer reliability is to implement a decibel threshold to define an ES, though while this improves specificity it does at the same time decrease sensitivity. Use of a decibel threshold was inherent in the early Consensus Statement on embolus detection¹²⁴, where an increase above the background spectrum of 3dB was used. The work that follows focuses on the use of a decibel threshold to aid in increasing the specificity of ES identification - intensity being just one of the criteria used to identify an ES.

Decibel thresholds were used in a number of early studies, varying between 3 to 9 dB^{98,103-111}(Table 2.4). Some of the studies performed were done using intensity thresholds suggested by one investigator on one machine using particular settings - these criteria being then directly applied to either a different machine or to the same machine with different settings, without any re-assessment or validation.

The use of differing decibel thresholds as a possible source of variability in study reports has been discussed (Chapter 2). Arising from concerns over generalisation of thresholds without any within-centre revalidation work, we further examined the effect of use of a decibel threshold and analysis techniques used in assessing intensity of an ES.

The decibel scale is a means of expressing the ratios of intensities or powers - it is a logarithmic scale, therefore compressing the wide range of intensities and powers found in practice into a relatively small range of numbers. It is defined by the following equation¹²⁹:

$$\text{Intensity ratio(dB)} = 10\log_{10}(I/I_0) \text{ dB}$$

Where I is the intensity of the ES and I_0 is the power of the Doppler background spectrum in the absence of any embolic signal - both of these parameters can be measured in a number of ways. If the value of I_0 is a fixed reference level, then intensity or power levels can be quoted in dB. Two of these methods are outlined below, and comparison made of results yielded by the two techniques.



3.2.4 Methods

Method 1

Using ES from the standard tape recordings as used in the interpretation validation, the intensity increase for each ES was calculated from the colour-coded intensity scale on the screen. This can be adjusted so that its intensity can be measured to the nearest decibel¹⁰¹. The gain was reduced until the colour of the adjacent cardiac cycle reached zero, and the intensity of the embolic signal was then determined.

Method 2

Using the same standard recording and the EME Pioneer, the intensity of each ES was calculated with the automatic embolus detection software supplied with the machine. The algorithm determines the power of the ES over the whole spectral line; the background power is also determined over the whole spectral line using a running average of background intensity over the preceding spectral lines. It is not possible to set a threshold using Doppler speckle for the second method, as the software should not detect speckle as opposed to ES.

3.2.5 Results

Comparing embolus intensity as measured by the two methods.

The relationship between ES intensity measured by the two different methods described is demonstrated in **Figure 3.1**. There was a highly significant correlation between measurements made by the different techniques ($r = 0.70$, $P < 0.0001$). However, as seen in the graph (3.1), the absolute values of intensity for the same ES

varied markedly for the different methods, with intensity usually being higher for method 1.

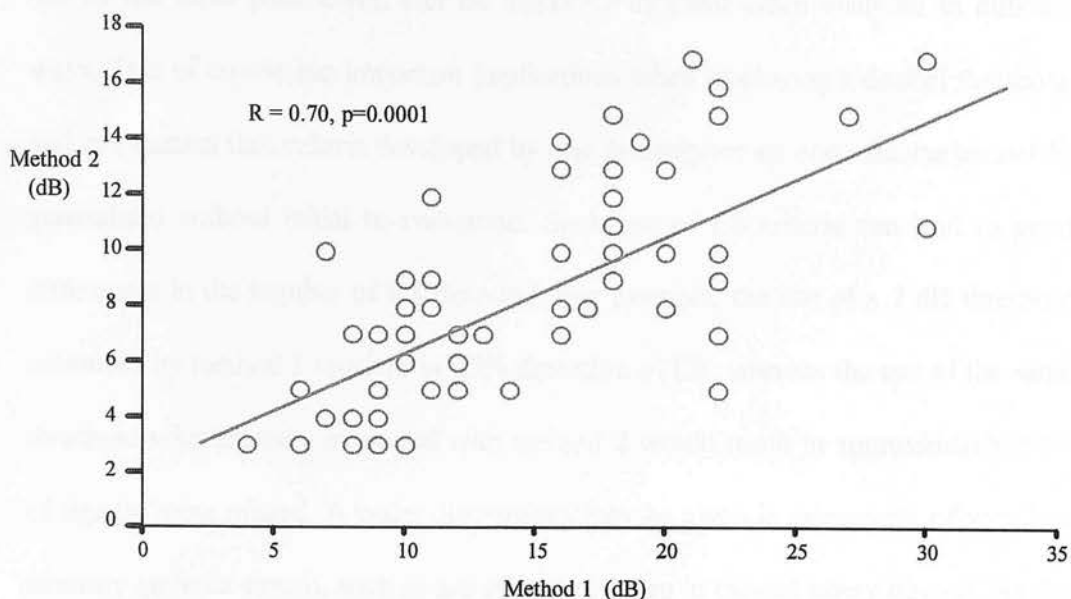


Figure 3.1. Scatterplot illustrating relationship between ES intensity (decibels) as determined by the two reading methods.

We estimated the effect of these differences on the proportion of ES detected using the same decibel threshold but with intensity measured in different ways. This is further illustrated in **Table 3.5**. The percentage of ES detected varied markedly; for example, using a threshold of 7dB would result in only 4.9% of signals being missed by method 1 but 51.4% being missed by method 2.

Analysis Method	<i>Proportion of Embolic Signals Detected Using Each Threshold (%)</i>			
	4dB	5dB	6dB	7dB
1	100	98.8	98.8	95.1
2	91.7	86.1	72.2	48.6

Table 3.5. Demonstrating the effect of measurement of intensity on proportion of embolic signals detected with use of an intensity threshold.

3.2.6 Discussion

The results demonstrate that the intensity of the same ES, recorded with the use of the same parameters, can be markedly different when analysed in different ways. This of course has important implications when employing a decibel threshold, and emphasises that criteria developed by one investigator on one machine cannot be generalised without initial re-evaluation. Such use of ES criteria can lead to great differences in the number of ES detected. For example, the use of a 7 dB threshold measured by method 1 resulted in 95% detection of ES, whereas the use of the same threshold with intensity measured with method 2 would result in approximately 50% of signals being missed. A wider discrepancy may be given in assessment of very low intensity embolic signals, such as are commonly seen in carotid artery disease. As the ES studied were only those previously detected by 4 observers, this in itself may have introduced an intensity threshold.

Using an inappropriately low threshold for the measurement of intensity may lead to inadvertent inclusion of episodes of Doppler speckle as representing ES.

The differences between the results obtained by the two methods may have a number of explanations. The power increase associated with the ES can be measured in a number of ways, including the peak increase at one velocity, the area under the power increase measured across both velocities and across time, or the power increase along the whole spectral line, which will include the power increase of the ES and also of the background Doppler spectrum at other velocities. The background power can also be measured in a number of ways - it can be measured at the same velocity or at all velocities, at the same point of the cardiac cycle or across the whole

cycle, and only within the Doppler spectrum or along the whole spectral line. The background intensity is higher in diastole than in systole, and therefore the position of the ES within the cardiac cycle will alter decibel measurements. If the whole spectral line is used, then for technically poor recordings with artefactual noise outwith the spectrum the background intensity will appear higher, reducing the effective intensity of the ES.

As this study used a standard recording, it is possible to comment on the effect of the reading method on the intensities of the embolic signals recorded. It illustrates that in addition to factors at work in causing variation at the recording stage including sample volume, depth of insonation, patient anatomy and fast Fourier transformation effects, there is also potential for variability in result reporting at the analysis stage.

3.3 Determining a Decibel Threshold for These Studies

It has previously been suggested that an appropriate intensity threshold may be determined by measuring the intensity increase occurring with random episodes of Doppler 'speckle'¹⁰⁷; these variations occur in the normal Doppler spectra and result from a number of factors, including non-uniformity of the ultrasound field and non-uniformity of the red blood cell scatterers. For this reason, the intensity increases associated with 200 episodes of random Doppler speckle using recordings from normal volunteers, made at the same depth, sample volume and gain were calculated. This level of intensity increase above baseline could therefore be expected to be seen at random - i.e. not necessarily in association with presence of an ES.

The histogram demonstrating the intensity distribution of 200 episodes of the saved speckle measured using Method 1 is illustrated in **Figure 3.2**. From this it can

be seen if we apply the recommended decibel threshold of $\geq 3\text{dB}$ to define an ES by this method then many episodes of speckle may be falsely recorded as representing an embolus. Instead the logical conclusion from reading this graph is to use $\geq 7\text{dB}$ as the threshold - thus cutting down significantly on reporting of false positives, though at some expense to technique sensitivity. Accordingly, a decibel threshold of $\geq 7\text{dB}$ is applied in all studies reported in this thesis to define an ES.

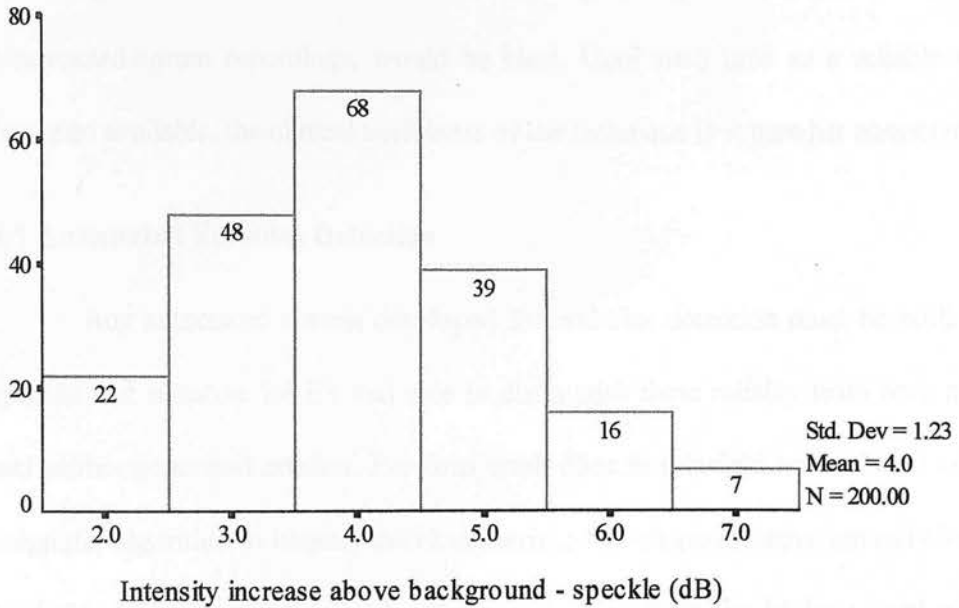


Figure 3.2. Frequency histogram for the 200 episodes of Doppler ‘speckle’, demonstrating increases in intensity above background occurring randomly in normal subjects in the absence of any ES.

Having performed the validation studies above, both in terms of ability to successfully identify ES and the validity of the threshold used, it was possible to go on to perform patient recordings, as described in subsequent chapters.

Chapter 4

Experimental Use of Multi-Gated Doppler

A major hindrance to the wider clinical application of TCD is the lack of an automated embolus detection system - having to review recordings in real time is a lengthy process. A system for embolus detection where segments of interest of the recording are saved for review, as used presently in analysis of 24 hour electrocardiogram recordings, would be ideal. Until such time as a reliable system becomes available, the clinical usefulness of the technique is somewhat restricted.

4.1 Automated Embolus Detection

Any automated system developed for embolus detection must be both highly specific and sensitive for ES and able to distinguish these reliably from both patient- and probe- generated artefact. Previous work done in this field has included use of a computer algorithm to identify the characteristic bell-shaped relative intensity increase occurring with an embolus, and differentiating this from the bi-directional increase seen with an artefact. Using such a system, the investigating group were able to achieve high specificity and sensitivity off-line¹³⁰, but results with on-line systems did not match up to their off-line promise¹³¹. An alternative is to train a neural network, and early work with such a system again showed promise, with a high specificity on-line, but with sensitivity only in the order of 70%¹³².

Unfortunately, artefact, such as may occur secondary to movements of the probe or patient, can also produce embolus-like signals, and therefore ES cannot be

distinguished on the basis of intensity and duration alone. Very high-intensity signals may result in receiver overload and aliasing - i.e. the received signal intensity is beyond the dynamic range or maximum amplitude of the receiver. According to the standard definition of an ES, these cannot be differentiated from artefact¹²⁴.

4.2 Principle of Multi-Gated Doppler

Prior to 1994, multi-gate monitoring of the middle cerebral artery had been described as a method to assess blood velocity profiles and in monitoring autoregulation and reactivity¹³³. The use of a multi-gate device in embolus detection was first proposed by Rune Aaslid at the 8th International Symposium on Cerebral Haemodynamics in Münster, Germany in September 1994. It utilises the 'coincidence method', described by Bothe and Kohlhörster in 1929, that two events triggered by the same stimulus should occur within a certain time window. As emboli pass through the artery being studied, an embolus would be first detected in the deeper (distal) channel and then in the more shallow (proximal) channel after a time delay that should bear a relationship to the distance between the two channels. In contrast, signals of increased intensity resulting from artefact should appear in both channels simultaneously.

Early studies with this technique using a time delay to define an embolus in a flow model gave a sensitivity of 100%. Using the same method in patients gave a sensitivity of 98.1% and specificity of 98.8%¹³⁴. This study used the FFT data, which has a relatively poor temporal resolution, from which to calculate the time delay between two channels. Better results would be expected from using the time domain data which provides much finer temporal resolution. In addition, the in-vitro validation for this study used only air

emboli - there had been no previous validation using solid or formed emboli such as thrombus which result in less intense signals and have been more difficult to detect in previous automated systems. Despite these reservations, the figures quoted were both seen to be an improvement on those produced with previous techniques, and multi-gated Doppler was felt to have some promise for incorporation into an automated ES detection system.

In the following study, a multi-gated Doppler ultrasound system was evaluated in an in-vitro model using both thrombus and air emboli, and then applied to a group of patients with potential embolic sources.

4.3 Evaluation of Multi-Gated Doppler in a Flow Model and in Man

4.3.1 Subjects and Methods

TCD recordings were made using the standard equipment (Chapter 2). The Doppler signal was saved with software that allows the pre-FFT time domain Doppler signal, the post-FFT spectra and the audio signal to be stored on the computer hard disk and replayed (SoundTrak, Nicolet EME, Überlingen, Germany). The initial increase in amplitude at the time of arrival of an embolus or artefact in each of the two channels was measured from the time domain data, allowing a time resolution of 1 millisecond or greater.

4.3.1A In-Vitro Studies

We constructed a flow circuit using polyethylene infusion tubing (4mm internal diameter, Codan Limited), driven by a programmable flow pump (University Developmental Co-operation Flow System) and filled with a proprietary blood analogue - a suspension of nylon filaments in machine oil that had been previously validated as having similar scatter properties to blood (Elf Atochem)¹³⁵. A waveform similar to that

normally obtained from the MCA with relative preservation of diastolic flow was obtained; mean systolic flow velocity was 40 cm/s (peak 95 cm/s, diastolic 30 cm/s). Air was removed from the circuit through an in-built reservoir tank. A length of the tubing was fixed in a water bath and insonated at an incident angle of 35°, such that there was a comparable waveform with equal gain settings at each depth. Channel 1 was set at a depth of 52 mm with the following parameters: sample size 5 mm, power 25% (100% power is equivalent to 675 mW/cm²); and gain, 8. Channel 2 was set at 42 mm depth, at the same settings of sample size, power and gain.

We introduced emboli of 2 types through a transparent side-arm device, allowing visualisation of any accidentally introduced air: 181 air bubbles were introduced using a 1 ml syringe to produce bubbles of maximum volume 0.01 ml; 193 thrombus emboli were prepared from fresh human blood which had been allowed to clot, cut in cuboid shapes with a maximum dimension of 0.5 mm, and then suspended in normal saline. In addition 368 episodes of artefact were produced; 187 of these were produced by tapping the probe and 181 by tapping the tubing to mimic patient artefact.

4.3.1B Patient Studies

The patient studies had 2 aims: (1) to determine in what proportion of patients we could successfully insonate the MCA with satisfactory gate separation, and (2) to determine the sensitivity and specificity of the method in detecting ES and differentiating them from artefact. ES in patients with mechanical prosthetic cardiac valves are more intense than those in patients with carotid stenosis¹⁰⁹ and have been easier to detect by previous automated systems; therefore, we evaluated the method in both patient groups separately. In the carotid group we also included ES recorded during the recovery phase

after carotid endarterectomy and carotid angioplasty; these ES have a similar intensity to those recorded in symptomatic carotid stenosis¹³⁶.

Insonation of 35 MCAs in 24 patients was attempted; (16 men, 8 women; mean [SD, range] age, 66.5 years [10.39, 50-86]). Of these patients, 14 had a known potential source of emboli. Six patients with metallic prosthetic cardiac valves were monitored bilaterally for 20 minutes. Eight patients with symptomatic carotid stenosis (mean stenosis 84.4%, range 60%-95%) were monitored ipsilateral to the stenosis for 1 hour. Four patients later underwent carotid endarterectomy, and 1 patient underwent carotid angioplasty. In these patients more extensive postoperative recordings were made for a total of 300 minutes per patient. All these recordings were made following the procedure, either after catheter removal for percutaneous transluminal angioplasty, or in the recovery room for endarterectomy, and no recordings were made during the procedures when ES could represent air bubbles. In the additional 10 subjects recruited from hospital in-patients, monitoring was continued only for the time it took to obtain a satisfactory signal; this was to determine whether adequate gate separation could be obtained.

Insonation was via the standard approach (Chapter 2). Once the MCA was identified, the depth in the two channels was adjusted, and the axial sample volume reduced while satisfactory visualisation of the Doppler spectra within the two samples was maintained. A standard protocol was used in siting the position of the two sample volumes; we aimed for a sample volume of 5 mm and a distance between the centre points of the sample volumes (i.e. between the two depths) of 10 mm. Episodes of artefact were also recorded for off-line analysis as above. These were produced by requesting that the patient cough, speak or swallow, and by tapping or moving the probe, probe holder, or headgear.

4.3.1C Signal Analysis

For all ES, maximum relative intensity increase was determined according to Method 1 (Chapter 3). The pre-FFT time domain data from the two channels were used to determine whether the intensity increase was present in both channels; if so, the time delay for the onset of the intensity increase in the two channels was measured. The time of 'arrival' of an ES was defined as the point at which the amplitude of the time domain signal was greater than twice background with a concomitant high intensity signal on the colour-coded display. Comparisons between groups were made with the use of Student's *t* test for unpaired data.

4.3.2 Results

4.3.2A In-Vitro Studies

All 374 emboli were detected as high intensity signals. Air emboli resulted in more intense signals than thrombus emboli mean (SD, range) for air emboli 31.5 dB (11.8, 11 to 39) versus 22.5 dB (4.8, 11 to 39) for thrombus emboli, $p < 0.0001$. Forty-three air emboli but no thrombus emboli resulted in receiver overload and a bi-directional signal, as previously reported; these were included in the analysis. All emboli were detected in both channels (**Figure 4.1A, 4.1B**), in all cases first in the more proximal channel, with a mean (SD, range) time delay between the two channels of 17.56 ms (10.31, 3 to 53). The mean (SD, range) delay was 17.32 ms (9.94, 3 to 53) for air emboli and 17.78 ms (10.66, 4 to 50) for thrombus emboli. In contrast artefact appeared simultaneously, or near simultaneously, in the two channels (**Figure 4.1C**) with a mean (SD, range) time delay of -0.01 ms (0.39, -4 to 2); $p < 0.0001$ versus emboli.

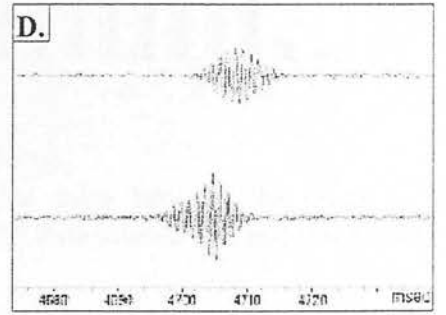
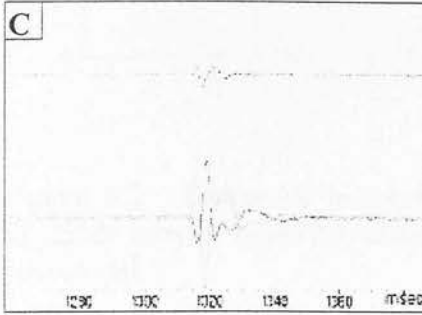
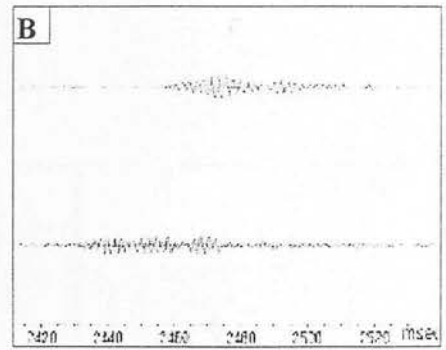
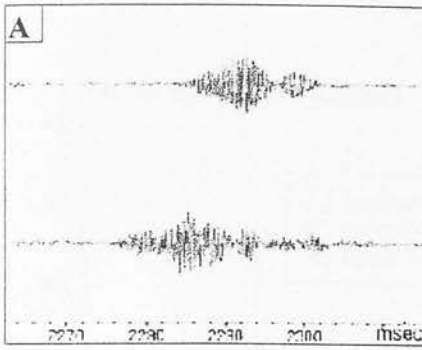
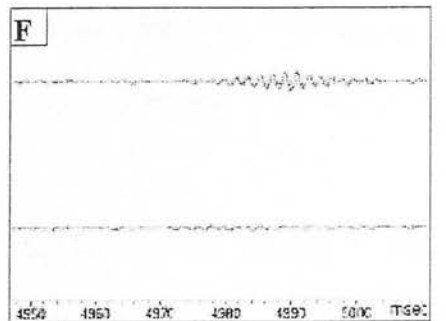
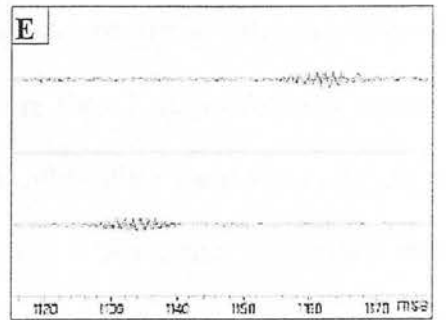


Figure 4.1. Time domain Doppler signals for: **A)** air embolus in flow model; **B)** thrombus in flow model; **C)** artefact caused by probe tapping in the flow model; **D)** embolic signal from patient with prosthetic metallic cardiac valve; **E)** embolic signal from patient with carotid artery stenosis; **F)** lower amplitude signal embolic signal from subject with carotid artery stenosis. In each, the lower of the two traces represents the distal channel (proximal MCA). A time delay is present between the onset of the amplitude increase in the two channels in **A,B,D,E** and **F**. In contrast the artefact in **C** appears simultaneously in the two channels. The amplitude increase in the proximal channel for the carotid embolic signal in (**F**) is of low amplitude compared with the background Doppler signal (see text for further discussion).



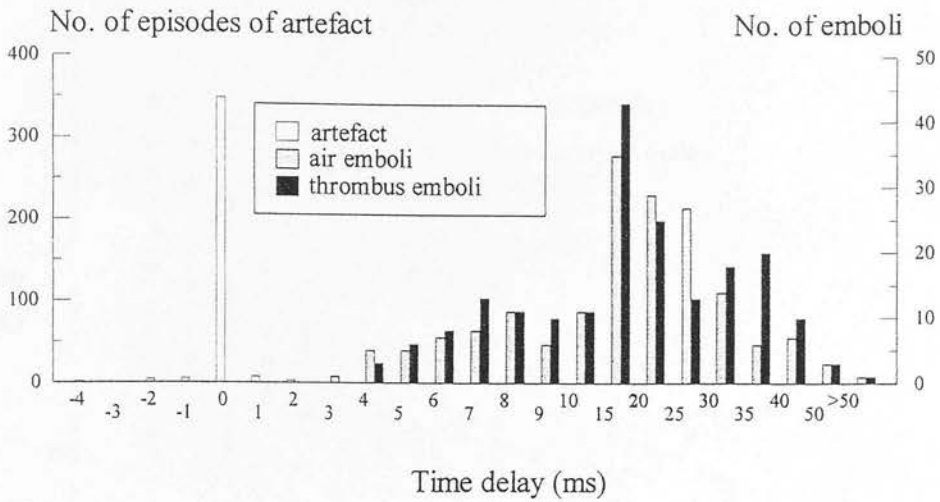


Figure 4.2. Frequency histogram of the time delay between the onset of the amplitude increase in the two channels for air and thrombus emboli and artefact in the flow model.

Time delays for air and thrombus emboli and artefact are shown in **Figure 4.2**. When we specified a cut-off time delay of more than 2 ms to define a signal as an embolus, the method could detect emboli and differentiate them from artefact with a sensitivity of 100% and a specificity of 100%; this was similarly good when either air emboli alone or thrombus emboli alone were considered. These values include the 43 air emboli which resulted in receiver overload and aliasing; for these cases analysed as a separate group, mean (SD, range) time delay between gates was 19.81 ms (9.61, 9 to 53).

There was a highly significant relationship between the velocity at which the embolic signal intensity increase occurred and the time delay between the two channels: air emboli, $r = -0.77$, $p = <0.0001$; thrombus, $r = -0.74$, $p = <0.0001$. (**Figure 4.3a,b**)

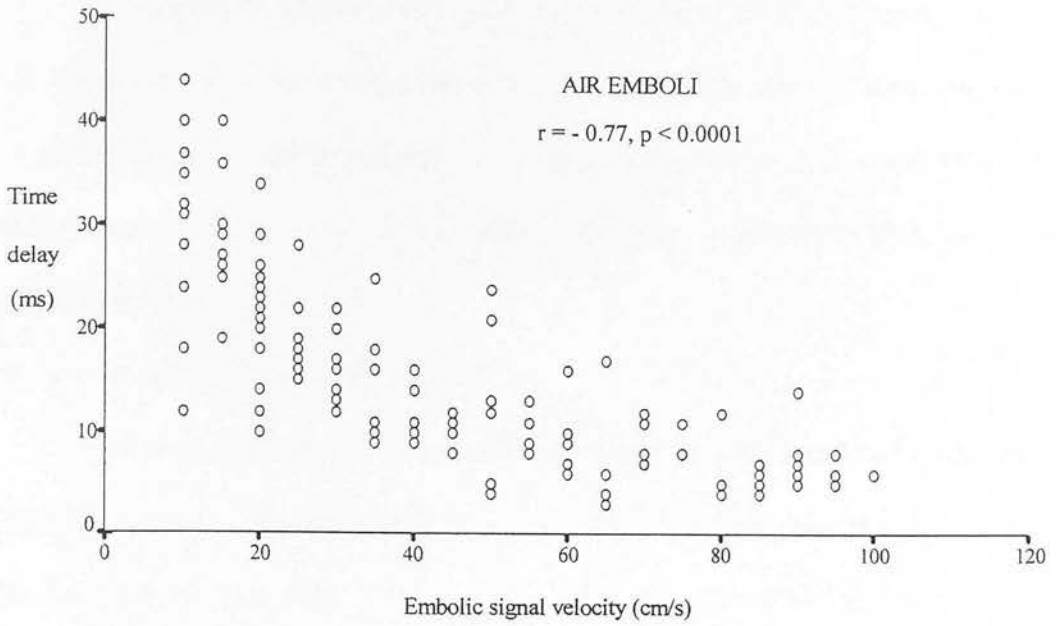


Figure 4.3a.

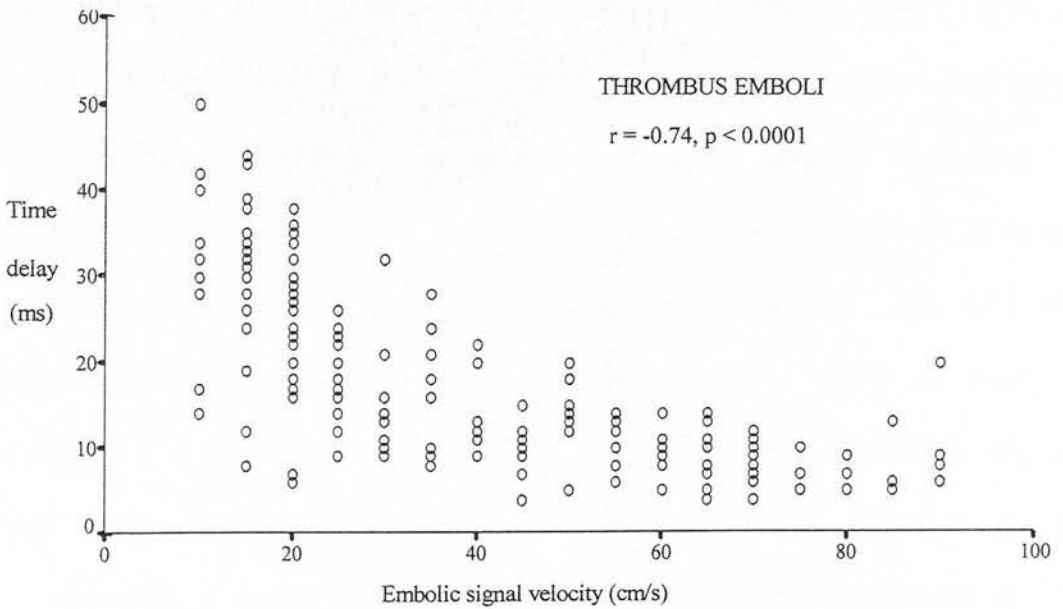


Figure 4.3b.

The two scatter-plots (Figure 4.3a,b) portray the relationship between the time delay seen for each embolus and the velocity of the embolus within the Doppler spectrum.

4.3.2B Patient Studies

Successful insonation of the MCA was possible in 33 of 35 arteries (94.3%). In the two failed cases this was due to unilateral absence of an acoustic temporal window. In all MCAs that could be insonated it was possible to record at 2 depths with a mean (SD, range) sample volume of 4.96 mm (0.20, 4 to 5) and mean (SD, range) gate separation of 4.71 mm (1.85, 1 to 9).

Prosthetic Mechanical Cardiac Valves

We recorded 125 ES, with a mean (SD, range) relative intensity increase of 31.1 dB (7.7, 10 to 55). The mean (SD, range) time delay was 29.6 ms (28.2, 2 to 122). For 30 ES, all in the same patient, the ES were heard and detected in both the time domain data and the post-FFT spectral display in the proximal channel, but were not audible or visible in the distal channel suggesting that some emboli may have passed into a branching artery between the two sample volumes. All other ES were visible and audible in both channels. The mean time delay for ES was significantly longer than that for the 222 episodes of artefact which had a mean (SD, range) time delay of 0.0 ms (0.46, -2 to 3, $p < 0.0001$). Time delays for the ES and artefact are shown in **Figure 4.4**. When only the ES detected in both channels were included, and a threshold of a time delay of 2 milliseconds between the two depths as the defining criterion for an ES were used, the sensitivity was 98.9%, and specificity 99.0%. However, if the ES visible in only one channel were also included the sensitivity fell to 75.2%. On the whole, ES in patients with cardiac valves were clearly visible as a large amplitude increase in the time domain data, in contrast to some of the carotid ES (**Figure 4.1D**)

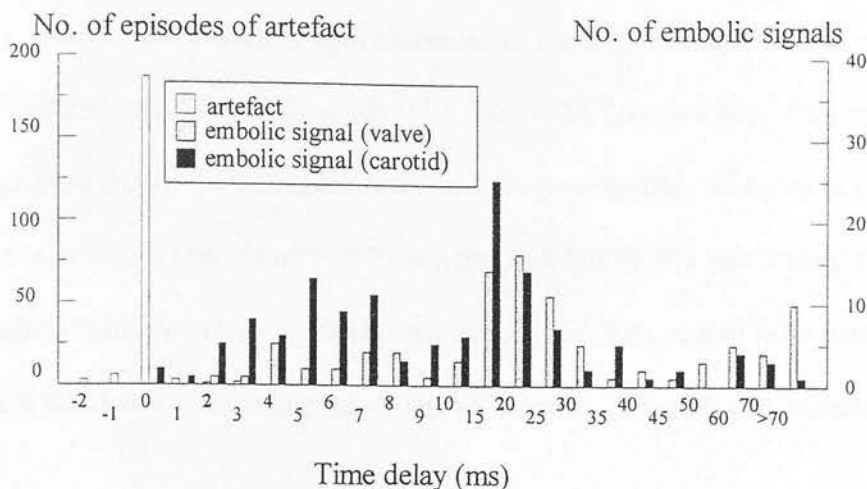


Figure 4.4. Frequency histogram of the time delay between the onset of the amplitude increase in the two channels for embolic signals in patients with carotid artery stenosis and mechanical cardiac valves and artefact in the same patients.

Carotid Artery Stenosis

We recorded 141 ES, with a mean (SD, range) relative intensity increase of 19.3dB (5.9, 5 to 33). No ES were visible and audible in the proximal channel but not in the distal channel, unlike the valve ES. However, two ES audible and visible in the post-FFT spectra in both channels were identifiable in the time domain data of only one channel; one ES was detected only in the proximal but not the distal time domain data, while one ES was clearly audible and visible in the proximal channel post-FFT spectral display but not visible in the time domain data at that depth. This appeared to be a reflection of the fact that the amplitude increase in the time domain data was small for many ES, **Figure 4.1E**, and on occasion difficult to distinguish from the background Doppler signal; a typical example is shown in **Figure 4.1F**. The ratio of the maximum amplitude increase to the background Doppler signal amplitude in the time domain data was small for many carotid ES, and the mean value was significantly smaller than that for valve ES. (5.1[2.5] versus 13.2[5.7]; $p = <0.0001$); individual values are shown overleaf in **Figure 4.5**.

For ES identifiable in both channels, the mean (SD, range) time delay between the two channels was 14.9 milliseconds (15.42, 0 to 90). The time delay was significantly longer than that of the 222 episodes of artefact, $p = <0.0001$. When the same 2-ms cut-off was used, a sensitivity of 94.0 % and specificity of 99.0% was obtained when those signals identifiable in both channels were considered (**Figure 4.4**). The sensitivity fell to 92.6% when all ES, including those only visible in one channel, were considered.

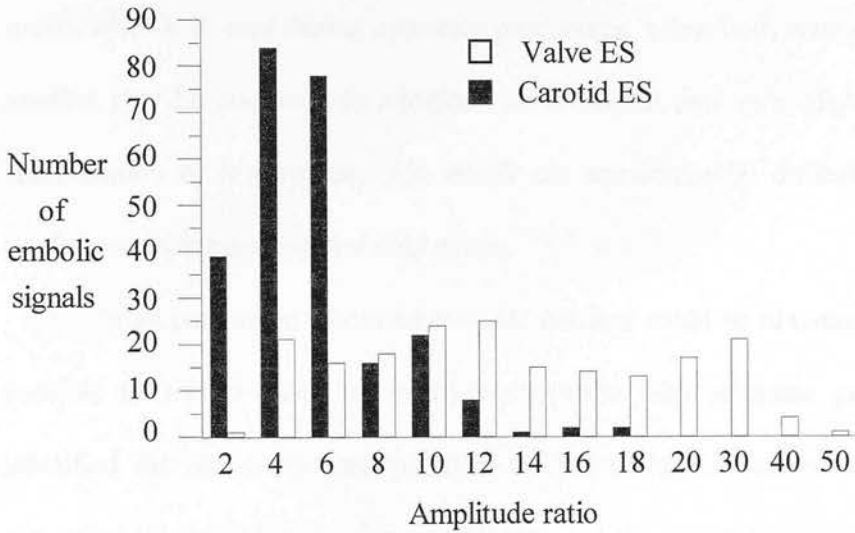


Figure 4.5. The ratio of the amplitude increase in the pre-FFT time domain signal for embolic signals compared with that due to the background Doppler signal in the absence of an embolic signal in both patient groups. Channels at both recording depths have been included.

4.4 Discussion

In this in-vitro model, the use of a multigated technique allowed the identification of ES and their discrimination from artefact with a very high sensitivity and specificity. Of particular note, it allowed detection of ES resulting in receiver overload with a similar high sensitivity and specificity - the first method to be able to identify such ES - which cannot be identified from the frequency spectral data alone either visually or with the use of a neural network¹³² or computer algorithm¹³⁰. This application may be particularly useful when it is used during operative procedures, when both echogenic air emboli and artefact may be common. In addition, the multigate data may allow the unambiguous identification of low-velocity ES, which can sometimes be difficult to separate from artefact on frequency spectral data alone.

In all patients in whom an acoustic window could be obtained, it was technically possible to record using the multi-depth probe with adequate gate separation. We identified the sensitivity and specificity of the multigated system using ES identified according to conventional criteria and compared them with known episodes of artefact. The results in patients were encouraging but not as impressive as the in vitro data for a number of reasons. This was primarily because in some patients the ES did not appear in both channels. In one patient in the valve group, 30 ES (approximately 40% of the signals in this patient) were detected proximally but were not audible or visible distally in either the time domain data or in the post-FFT spectral data. This is likely because between the two sample volumes a proportion of the emboli passed down a branch vessel. Our protocol aimed for a gate separation of 10mm between the centres of each sample volume, and sometimes the distal gate was a fairly shallow depth of 42 mm; the problem

may be reduced with the use of a deeper distal gate, thereby lowering the chance that this gate may be sited beyond the branching of the MCA.

Further studies are required to examine whether reduced gate separation allows such good discrimination between emboli and artefact. On the other hand, gate separation that is too narrow will reduce the time interval between detection of emboli in the proximal and distal gates and will reduce the specificity in differentiating emboli from artefact. Apart from this difficulty in a single patient, the use of a multigated probe in patients with prosthetic cardiac valves allowed detection of emboli and differentiation from artefact with a very high sensitivity, similar to that seen in the in-vitro model. This is a largely a reflection of the higher intensity of the more echogenic mechanical valve ES, as previously reported. This resulted in a large and clear amplitude increase in the time domain signal (**Figure 4.1D**).

In contrast, for the less intense carotid ES, some signals were identifiable audibly and in the post-FFT spectral display but not in the time domain data. This occurred for low-intensity emboli, in which the power or intensity increase was small and lost in the background Doppler signal. It is possible that the total cross-sectional area of the MCA is not fully covered by the sample volume at both depths. This will be less of a problem for more echogenic emboli, which have a larger effective sample volume¹³⁷. Separating the intensity increase at different frequencies by FFT analysis allowed identification of the ES since the intensity increase of an ES is maximal at one frequency. Although only two carotid ES were not detectable in the time domain data, a much larger number resulted in an amplitude increase only slightly greater than that of the background Doppler signal. This is illustrated by the relatively low ratio of the maximum amplitude to background amplitude for many carotid ES in the time domain data (**Figure 4.1E**; **Figure 4.5**). With

the use of off-line analysis in combination with the post-FFT spectral data, it is possible to determine the time delay for most carotid ES. However, it may prove difficult in automated on-line systems based on the time domain data alone to distinguish the small amplitude increase occurring with some carotid ES from amplitude fluctuations in the normal background Doppler signal. Further work is required to improve the sensitivity of the multigated technique for small-amplitude ES.

The predominant factor determining the time taken to travel between the two channels was the velocity at which the ES was maximal, which presumably reflects the speed at which the embolus is travelling. However, in the model that there was a much greater range of time taken between detection in each channel than would be expected by simple mathematical principles. The theoretical distance travelled by an embolus as detected by the probe is 5 mm (i.e. distance between the edges of the axial sample volumes). If we correct for angle of insonation, an embolus travelling at the mean velocity (40 cm/s) would be expected to take 15.3 ms to cross between the two axial sample volumes. Any embolus travelling at the peak velocity for the system (95 cm/s) would be expected to give a time delay of 6.4 ms between the two depths. It is noteworthy that 46 of the emboli we produced traversed the distance in 6 ms or less. This reflects the fact that rather than being cylindrical in shape, with a sharp cut-off of the ultrasound beam at each end of the sample, there is a gradual weakening of the beam at each end. It follows that the effective sample volume will be greater for more echogenic emboli such as air emboli¹³⁷. In patient studies some ES took much longer to travel between the two sample volumes, and this was particularly so for some valve ES. This may be due to their passage being slowed by turbulence, non-laminar flow, and momentary adhesion to the vessel wall.

The results demonstrate that a multigated approach can detect ES and differentiate them from artefact, but, as with other methods, it has some inherent problems which need to be resolved before it is suitable for routine clinical use. The results are similar to those reported previously¹³⁴, but show a lower sensitivity for low amplitude carotid ES. In the previous study carotid ES, a minority of all ES studied, were not separated from those from patients with heart valve replacements or left ventricular assist devices, and no analysis of the intensity of the ES was made. Our results demonstrate that in patients with the more intense mechanical valve ES, high sensitivity and specificity is likely to be obtained; the only major difficulty appeared to be passage of emboli down a branching vessel between the two sample volumes, and this may be improved by reduced gate separation. In patients with carotid ES, the less intense ES may be unclear in one or both channels. For these ES combining the multigated method with a method analysing the post-FFT spectral data may improve sensitivity; use of the frequency spectra may provide greater resolution because the intensity increase associated with an ES is usually centred on a narrow frequency band.

4.5 Further Development

From the above discussion it can be seen that the two situations causing reduced sensitivity of the technique occur: i) in association with a large gate separation distance, and ii) in subjects with low intensity ES, as seen in carotid artery stenosis.

As a direct result from this work, future multi-gate recordings were made as follows: the reference depth was taken as the depth at which optimum spectral recording was possible. The second channel was set 4mm proximal to this (i.e. the reference recording was made from the proximal MCA with the second gate

recording from distal MCA). This was done in an attempt to minimise 'loss' of an embolus via an artery side branch.

The problem with low intensity ES is of a small increase in amplitude in the time domain signal against the background amplitude. A possible method of reducing this effect is to use a lower frequency probe, e.g. 1MHz, which would theoretically improve the background-to-signal ratio¹¹⁹.

Chapter 5

Embollic Signals in Carotid Artery Stenosis (I)

Part I

5.1 Correlation with Markers of Clinical Risk

As discussed, cerebral embolism from extra-cranial internal carotid artery disease is an important mechanism of ischaemic stroke¹³⁸⁻¹⁴⁰, and the increase in stroke risk conferred by presence of a significant internal carotid artery stenosis has been confirmed in two major trials^{64,65}. At present, carotid endarterectomy is not routinely indicated in the management of asymptomatic CAS, on the basis of risk-benefit analysis¹⁴¹. Trials are ongoing to identify indicators of higher risk within this group¹⁴².

For more than 30 years, case reports have existed of direct visualisation of embolic material within the retinal circulation^{143,144}, but it was not until 1990 that it was appreciated that particulate emboli could be detected in the intra-cranial vessels⁷³. Since this time there have been a number of studies monitoring for ES in the setting of CAS, and ES have been shown to correlate with individual markers of stroke risk: they are more common in symptomatic than asymptomatic disease^{105,107}, and are more common soon after the appearance of symptoms^{108,145,146}. ES have also been shown to be more frequent in association with the presence of plaque ulceration as determined both by imaging¹¹¹ and on histological examination¹⁰⁴. There are case reports of ES abating in response to treatment with antiplatelet or anticoagulant therapy^{107,147}, while in a small prospective study in asymptomatic CAS, the presence of two or more ES

of two or more ES per hour was a highly significant independent predictor of stroke risk¹⁴⁸. It may be that the rate of TCD-detected embolism may reflect to some extent the 'disease activity' of the carotid lesion¹⁰⁸.

In this study we determined the incidence and frequency of embolisation in a large series of patients with CAS and correlated the presence of ES with a number of markers of risk in order to further determine the clinical relevance of asymptomatic ES detection.

5.1.1 Methods and Subjects

One hundred and twenty-three (123) patients with >60% CAS as determined on Duplex criteria¹⁴⁹ were recruited to the study. Of these, successful insonation of the ipsilateral MCA was possible in 111 (90.2%). Symptomatic patients (n=69) were defined as having had symptoms (amaurosis fugax, TIA, or stroke) in the territory of the stenosed artery within the previous 12 months. Patients with a possible cardiac source of embolisation (atrial fibrillation, valve disease/prosthesis) were excluded from the study. All patients were maintained on anti-platelet/anti-thrombotic medication as prescribed at their time of entry into the study.

Clinical data, including time lapsed since last symptoms where appropriate, were recorded for all subjects.

In addition, 74 (66.7%) patients underwent carotid angiography for clinical reasons. In 67 patients the resulting films were of sufficient quality as to ascertain presence or absence of plaque ulceration or irregularity. 88 patients (79.3%) had brain imaging by CT scan.

TCD recordings were made from the middle cerebral artery using the standard equipment and protocol (Chapter 2). Target sample volume initially was 4mm - as this

failed in many cases to deliver sufficient power for satisfactory recording this was changed after recruitment of the first 40 patients to 5mm. The axial sample volume was then increased further if necessary to obtain a suitable signal. The sample volume used for each patient was maintained on any subsequent recording.

Analysis of all recordings was performed blind to patient clinical information according to the standard validated methods (Chapter 3).

Each patient underwent recording for 1 hour, and 77 patients (69.4%) attended for a second 1-hour recording on a separate occasion. Mean (SD,range) time between recordings was 17.53 days (20.96, 1-123). Median (range) depth of insonation was 52 mm (46-56) for the first recording made. Median (mean, range) sample volume for all first recordings was 5 mm (5.56, 4-12). Any recording containing 1 or more ES is described as ES positive.

5.1.2 Results

Comparing ES positive to ES negative subjects, there were no significant differences in vascular risk factors between the two groups - see **Table 5.1**.

Factor	ES positive(n=41)	ES negative(n=70)	<i>p</i>
Mean age (SD, range)	66.3 (9.95, 42-81)	67.0 (8.9, 43-82)	0.72
Male sex	30 (73%)	43 (61%)	0.21
Hypertension	29 (71%)	46 (66%)	0.59
Diabetes mellitus	7 (17%)	12 (17%)	0.99
Hyperlipidaemia	18/40 (45%)	38/67 (57%)	0.24
Ischaemic heart disease	15 (37%)	22 (31%)	0.24
Peripheral vascular disease	17 (41%)	23 (33%)	0.36
Current smoker	9 (22%)	22 (31%)	0.28

Table 5.1. Patient characteristics comparing ES positive to ES negative subjects.

5.1.2A ES and Symptom Status

Of the total 111 recordings made, 41(36.9%) were ES positive. For the 69 symptomatic patients, 29 (42.0%) were positive, compared with 12 (28.6%) positive records in 42 asymptomatic subjects, $p=0.15$. ES were more common in recently symptomatic patients: 27 of 57 (47.4%) patients with symptoms in the last 6 months were ES positive ($p= 0.058$ versus asymptomatic). The effect of changing the time cut-off point to define symptom status is demonstrated in **Table 5.2**.

Cut-off Point	n	Percent positive recordings	p (compared with asymptomatic)
1 year	69	42.0	0.15
6 months	57	47.4	0.058
3 months	50	48.0	0.057
1 month	26	53.8	0.037

Table 5.2. To illustrate the influence of selection of the cut-off point in defining symptomatic status in CAS.

In patients in whom ES were detected, there was no difference in the numbers seen between the two groups - median (range) in symptomatic group was 3 (1-45), and in asymptomatic, 4.5 (1-23), $p=0.69$, Mann-Whitney U.

For the 77 patients undergoing two single hour recordings, the two hour period was combined, such that effectively the patients had attended for a 2-hour recording. In this group, there were 47 symptomatic and 30 asymptomatic patients. In the symptomatic group, 28/47 (59.6%) recordings were ES positive, compared with 12/30 (40%) in the asymptomatic group, $p=0.09$. Where ES were detected, median (range) numbers were 2 (1-60) and 1.5 (1-51) respectively, $p=0.76$.

5.1.2B ES and Time Since Symptoms

In symptomatic patients, mean (SD,range) time since last symptom at the time of the first recording, was 79.58 days (85.14, 1-325).

The correlation between ES per hour and time from last symptom was calculated using Spearman's Rho for non-parametric data. This showed a significant negative correlation, with $\rho = -0.2558$, $p = 0.034$ for all first recordings - **Figure 5.1**.

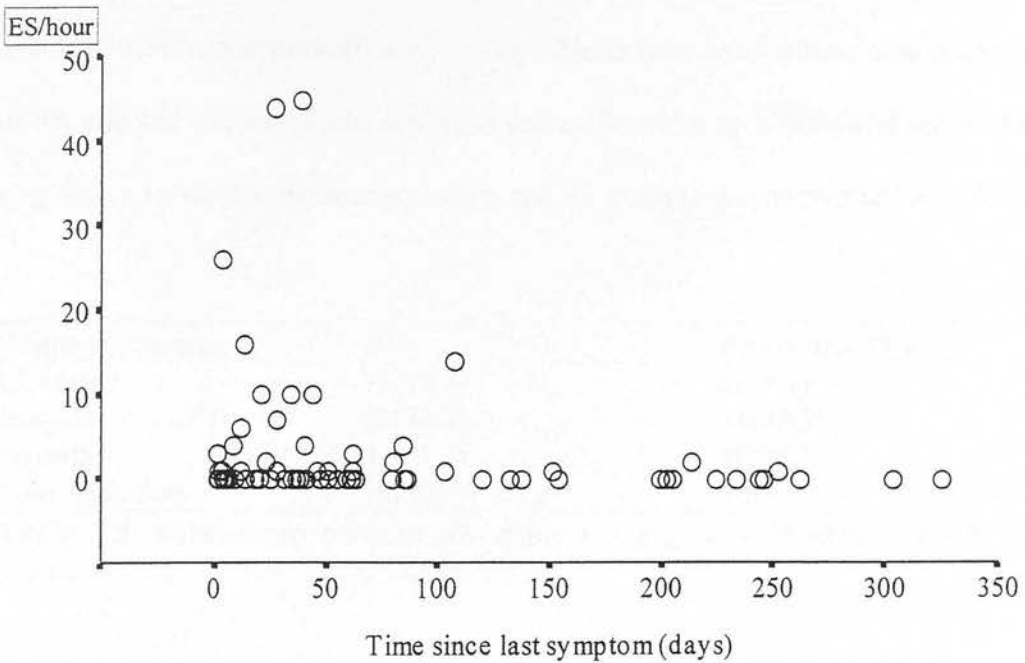


Figure 5.1. Scatterplot illustrating the negative correlation between time since last symptom and ES per hour on first recording (y-axis).

Forty-seven (47) of the symptomatic subjects attended on a second occasion, with documentation of time since last symptom - giving a total of 116 recordings in symptomatic patients. Considering this group, the relationship had increased significance with $\rho = -0.3121$, $p = 0.001$.

5.1.2C ES and Angiographic Appearance

Angiography was performed in 74 patients. Of these films, 67 were of sufficient quality to determine degree of stenosis and plaque characteristics. All films were reviewed by a single observer (HSM), blind to the TCD recording results. Plaques were categorized as ulcerated, irregular or smooth, according to the criteria used in the NASCET trial¹⁵⁰.

Thirteen plaques (19.4%) were classified as ulcerated, 23 (34.3%) as irregular, and 31 (46.3%) as smooth. In addition, 14 subjects were seen to have near occlusion of the affected internal carotid artery, as defined by collapse of the distal vessel. The association between stenosis morphology and ES status is summarized in **Table 5.3**.

Plaque appearance	n(%)	ES positive (%)
Ulcerated	13(19.4)	9(69.2)
Irregular	23(34.3)	10(43.5)
Smooth	31(46.3)	9(29.0)
Near occlusion	14(20.9)	7(50)

Table 5.3. Relationship between ES status and angiographic appearance of the plaque.

Plaque ulceration was associated with ES - $p=0.025$. Presence of ulceration on angiography gave an increased risk of embolisation - Odds Ratio 4.14 (95%CI 1.12-15.26).

5.1.2D ES and Degree of Stenosis

Stenoses were classified on Duplex ultrasound as $\leq 79\%$ ($n=20$) or 80-99% ($n=91$) by standard criteria¹⁴⁹. We found no association between degree of stenosis and embolisation - 6 (30%) and 35 (38.5%) were ES positive respectively - $p=0.48$.

5.1.2E ES and Therapy

Of the total sample, 90 were taking aspirin alone - 44 at 75mg daily, 17 at 150mg daily and 29 at 300mg daily. Two patients were taking no anti-platelet or anti-thrombotic medication. Patients taking any other antiplatelet or antithrombotic medication were excluded from this analysis. There was no correlation between ES and aspirin dose, $p=0.23$.

Aspirin dose(mg)	ES positive (n,%)
75	13(29.5)
150	9(52.9)
300	10(34.5)

Table 5.4. Relationship between aspirin dosage and ES status.

5.1.2F ES and CT Infarct

89 subjects had brain imaging by computed tomography. 38(43%) of these showed a relevant infarct. There was no difference in the numbers ES positive in each group - 42.1% vs. 37.3%, $p=0.64$.

5.1.2G Follow-up and Survival

All study subjects were followed up from the time of the first recording to examine the relationship between presence of ES on MCA monitoring and risk of further events attributable to the studied stenosis. Survival analysis was undertaken, using the Kaplan-Meier log rank test. An event was defined as a focal cerebrovascular event attributable to the stenosis studied. Censor points included carotid endarterectomy, carotid angioplasty, carotid artery occlusion, non-stroke death and asymptomatic at time of last follow-up. Follow-up (days) was for a mean (SD,range) 111.7 (190.13, 1-774).

In the symptomatic group, mean (SD, range) time of follow-up was 22.55 days (20.31, 1-76). Within this group, 9 (13.0%) had recurrent events during the course of follow-up (7 TIA, 2 stroke). Seven (77.7%) of subjects within this group were ES positive at the time of the first recording, $p=0.02$, chi-squared. All had at least one ES positive recording during the total follow-up period.

In the asymptomatic group, mean (SD, range) time of follow-up is 258.1 days (246.9, 2-774). At this time, 32 patients continue to be asymptomatic, with recent follow-up either at Neurology Outpatients ($n=25$), or if not possible then by telephone ($n=7$). In this group, two (5%) have developed symptoms attributable to their stenoses (1 TIA, 1 stroke) - both had ES detected at the time of first examination, $p=0.02$, chi-squared.

The relationship of ES presence to outcome was assessed by Kaplan-Meier log rank test. A highly significant association was found between the two - $p=0.02$ in the symptomatic group and $p=0.07$ for the asymptomatic group (**Figure 5.2A,B**).

Cumulative
Survival

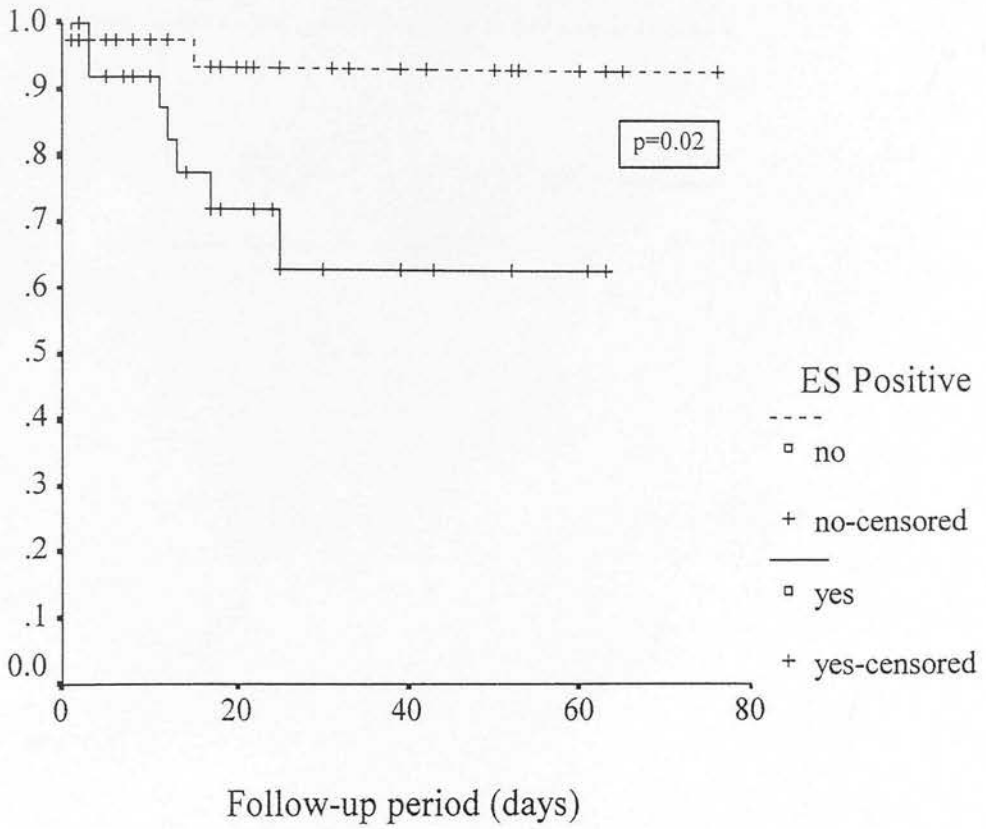


Figure 5.2A.

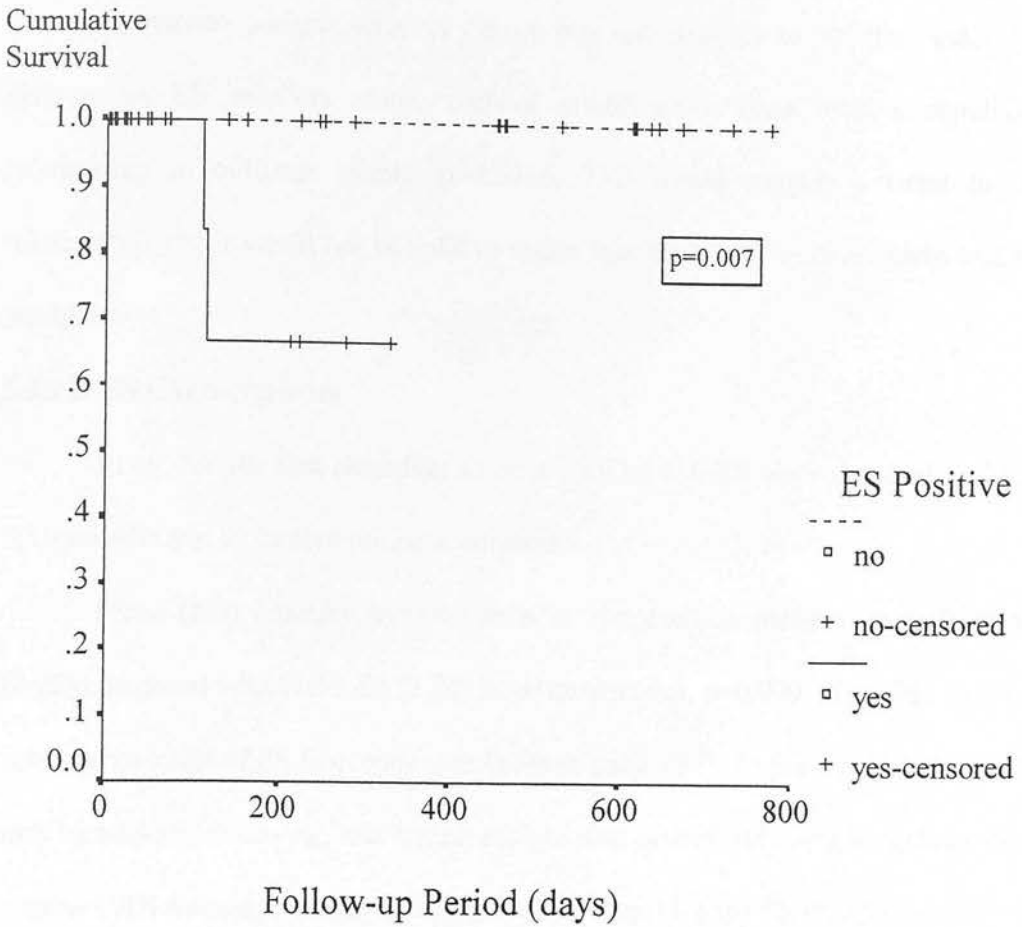


Figure 5.2B.

Figure 5.2. Kaplan-Meier survival plots. **A** - symptomatic subjects. **B** - asymptomatic subjects. For each plot the top (broken) line represents subjects who were ES negative at the time of the first recording. Censored events include carotid endarterectomy, carotid angioplasty, carotid occlusion and non-stroke death. ‘Events’ are all strokes and TIAs occurring in the distribution of the stenosed artery during the follow-up period.

We analysed the data further to examine any possible link between ES ‘load’ and outcome. In studies of ES during and following carotid endarterectomy, ES load has been found to be more important than ES status per se¹⁵¹. To assess numbers of ES as a continuous variable against outcome, a Cox regression model must be fitted, allowing for the possible confounder of symptom status. As there were no events in

the asymptomatic patients with no ES, it was not possible to fit this model. On analysis of ES numbers alone, without stratification, there was a significant relationship to outcome events, $p=0.0006$. This would suggest a trend in the relationship, but it would not be valid to quote this statistic more definitively without stratification.

5.1.2H ES Characteristics

In all, for the first recording alone a total of 316 ES were detected - 223 in symptomatic and 92 in asymptomatic subjects.

Mean (SD) intensity increase seen in symptomatic patients was 12.39 dB (4.25) compared with 11.55 dB (3.54) in asymptomatics, $p=0.099$. However, as there was a large range of ES frequency seen between subjects (1-45 per hour) these results may be subject to skewing, and further analysis was carried out using weighted means - mean (SD) intensity increase in symptomatics was 11.2 dB (2.38), compared with 11.04 dB (2.82) in asymptomatics, $p=0.79$.

Comparison was made between symptom groups: there was no difference in mean intensity comparing patients within the four clinical groups.

Group	n	Weighted Mean (SD) intensity increase (dB)
Asymptomatic	13	10.88(2.76)
Amaurosis fugax	6	11.16(2.34)
TIA	9	10.53(2.77)
Stroke	14	11.66(2.19)

Table 5.5. Relating ES intensity to symptom category.

5.1.3 Conclusion and Discussion

5.1.3A Clinical Factors

The study was undertaken in a large series of patients, evaluating a number of clinical parameters in relation to embolic activity in carotid artery disease, in contrast with earlier studies which have examined individual markers of clinical risk. Small studies have already shown that ES are more common in patients with symptomatic disease and in such subjects are commoner soon after symptoms.

A significant correlation was found between ES per hour and the time lapsed since the patient was last symptomatic, the association increasing in significance when further recordings were added to the analysis.

Surprisingly, we did not find a significant association between symptom status per se and embolisation - an association reported reliably in earlier work. This may be explained in part by the time cut-off we used to define 'symptoms' or absence of symptoms. Previous studies^{104,105,107,108,111} have defined a cut-off point of up to 6 months to determine symptom status, and have found an association between this and embolisation. Indeed by 'shifting' our own cut-off point, first to 6 and then to 3 and 1 month, the results achieve significance, though some of the power of the calculation is lost in this way by reducing numbers in the symptomatic group (as illustrated in **Table 5.2**). The results may have been further influenced by our detection of much higher rates of embolisation in the asymptomatic group than have been reported previously, which was accompanied by a higher level of symptom emergence in the asymptomatic group. This is reflected in the survival data which will be discussed in more detail below.

There was a highly significant association of embolisation with plaque ulceration as demonstrated angiographically. Again this is in keeping with previous studies^{104,111}, and provides further indirect evidence of the importance of local factors at the level of the plaque influencing embolus formation. We found no correlation between embolisation and degree of Duplex-determined ipsilateral stenosis.

Aspirin dose did not appear to significantly influence embolisation - if anything there was a trend towards higher incidence of ES on the higher doses of aspirin. It is possible that subjects at higher risk of recurrence may be maintained on a higher aspirin dose, with asymptomatic subjects more likely to be prescribed a lower dose. In addition, splitting the study group dosewise does give small numbers in each group which will itself influence the results.

There was no association found between presence of a relevant infarct on CT and ES status. However, we did not exclude patients from this study who had had temporally distant symptoms, which may act to confound results. To investigate any relationship fully, a prospective study in the setting of asymptomatic CAS with initial normal CT brain scan would be necessary.

5.1.3B Prospective Data

It has already been discussed that we found much higher rates of embolisation in our asymptomatic patients than have been previously reported by other groups. To examine further the prognostic significance of ES, we looked at the prospective data in more detail, and found that of our series of 111 patients, 11 (9.9%) had recurrent symptoms attributable to their CAS. Both in the symptomatic and the asymptomatic groups, there was a highly significant relationship between detection of ES at the first recording and symptom recurrence.

Taken in combination, the association of known clinical risk factors with the presence of ES, and the demonstration that ES predict future events provide considerable evidence for the clinical importance of ES in patients with CAS. Further reports from NASCET and ECST regarding allocation to endarterectomy of patients with moderate CAS suggest that there may be only certain subgroups who will benefit from surgery^{69,70}. It may be implied from our data that those with ES as recorded using transcranial Doppler may form an important subgroup in which early intervention may be necessary, underlining the importance of incorporation of TCD in prospective studies of asymptomatic CAS. However, the numbers in our prospective study were relatively small and the findings need to be replicated in a larger multicentre study.

Part 2

Embolie Signals in Carotid Artery Stenosis (I)

5.2 Platelet Activation and Carotid Embolisation

5.2.1 Background

Previous clinical and pathologic studies have suggested that increased levels of platelet activation, or more reactive platelets may be found in the setting of acute or transient cerebral ischaemia and in cerebral infarction^{152,153}. Such platelet activation has also been described in the setting of other vascular disorders including unstable angina¹⁵⁴, myocardial infarction¹⁵⁵, cardiopulmonary bypass¹⁵⁶ and following coronary angioplasty¹⁵⁷ and thrombolysis¹⁵⁸. Platelet hyperreactivity has been reported in association with vascular risk factors including hypertension¹⁵⁹, cigarette smoking¹⁶⁰, diabetes mellitus¹⁶¹, and hyperlipoproteinaemia¹⁶². Increased platelet reactivity has also been described in stress¹⁶³ and following strenuous exercise¹⁶⁴.

Having implicated platelets in thrombo-embolism, one may also expect to find a relationship between asymptomatic embolisation and platelet activation. Demonstration of such a relationship would provide further evidence of the clinical significance of asymptomatic ES.

5.2.2 Measuring Platelet Reactivity - Relevance to Cerebrovascular Disease

i. Platelet Aggregation

The first available method for evaluation of platelet reactivity came in the 1960's, with the development of the platelet aggregometer by Professor Born¹⁶⁵, allowing ex-vivo investigation of platelet responses to physiological agonists. Post-

mortem studies have revealed circulating platelet aggregates in intra-myocardial vessels¹⁶⁶ - this observation led to the development of the 'Wu and Hoak' platelet count ratio method to assess the presence of circulating platelet aggregates¹⁶⁷. Platelet hyperreactivity as detected by both these methods has been described in the setting of both acute ischaemic stroke¹⁵³ and transient ischaemic attack^{152,153}.

ii. Platelet Specific Proteins

Platelet activation can also be measured indirectly by assays of platelet specific proteins released on activation. These include beta thromboglobulin (β TG) and platelet factor-4 (PF-4)¹⁶⁸. However measurement of these markers is subject to in-vitro activation of platelets during phlebotomy and plasma processing. More recently it has been possible to measure urinary β TG, which is not subject to such influence, but it has not been assessed on the scale of a large clinical or epidemiological study. Plasma β TG has been found to be elevated in the acute phase of stroke, and independently related to increased mortality¹⁶⁹.

iii. Non-specific Platelet Proteins

Platelet activation is accompanied by thromboxane A₂ synthesis. TXA₂ is released into the circulation and is converted to TXB₂, which is then further broken down into a series of compounds including 2,3-dinor-TXB₂ and 11-dehydro-TXB₂, which undergo urinary excretion. It is possible to measure urinary 2,3-dinor-TXB₂ by gas chromatography-mass spectrometry - useful information regarding in-vivo TXA₂ synthesis by activated platelets. Levels of urinary 2,3-dinor-TXB₂ are increased in cardio-, cerebro- and peripheral vascular disorders^{170,171}. Use of serum TXB₂ itself as a marker of platelet activation has not been examined in detail.

The non-specific platelet alpha-granule protein, soluble (circulating) P-selectin (sP-sel) may also be assayed in plasma using an ELISA. There are some limitations to interpretation of results from such assays, as a proportion of sP-sel is endothelium-derived - therefore elevation may represent either primary platelet activation, activation secondary to tissue damage or endothelial damage per se. It is argued that the failure of sP-sel levels to correlate with von Willebrand factor, an established endothelial cell marker, would imply that sP-sel is platelet derived¹⁷². One advantage of this assay is that it is not subject to large variations by sampling technique¹⁷³.

One recent study in the setting of acute ischaemic stroke, and in patients with recently (<1 week) symptomatic CAS found sP-sel levels to be significantly elevated¹⁷⁴. A second study of sP-selectin levels in patients with symptomatic CAS found no relationship - in contrast to subjects with extensive peripheral vascular disease.¹⁷⁵ This study did not define any cut-off point to define 'symptomatic', and this may provide a basis for explaining the difference in results.

iv. Direct Markers

There are activation-dependent surface membrane changes which occur on platelet activation, such that by assessing surface glycoprotein expression it is possible to distinguish activated from resting platelets. By using monoclonal antibodies (MoAbs) specific for these glycoproteins it is possible to detect activated platelets in vivo by flow cytometry^{176,177}. Changes include down-regulation of the glycoprotein Ib receptor, up-regulation and conformational change of the glycoprotein IIb-IIIa receptor¹⁷⁸, and with release of platelet granule contents the alpha-granule membrane proteins are incorporated into the platelet outer membrane. To study activation

specific proteins, the two most commonly studied types of MoAb are those directed against the conformational change in the GPIIb-IIIa protein and those directed against granule membrane proteins.

The GPIIb-IIIa complex (also known as CD41/61) acts as a receptor for fibrinogen, von Willebrand factor, fibronectin and vitronectin, and is essential for platelet aggregation¹⁷⁹. It is possible to assess the amount of this antigen expressed at the platelet surface using most MoAbs directed against this antigen, or to use an MoAb (PAC1) directed against the fibrinogen binding site exposed by the conformational change in this complex¹⁷⁸. Other GPIIb-IIIa-specific activation-dependent MoAbs are directed against either ligand-induced conformational changes in the complex (ligand-induced binding sites - LIBS)¹⁸⁰ or receptor-induced conformational changes in the bound ligand - fibrinogen, (receptor-induced binding sites - RIBS)¹⁷⁷. As well as GPIIb-IIIa-specific MoAbs, fluorescein-conjugated fibrinogen can also be used in flow-cytometric assays to detect the activated form of the platelet surface GPIIb-IIIa complex¹⁸¹.

More widely studied are MoAbs directed against granule membrane proteins, particularly against P-selectin (also known as CD62P; formerly known as GMP-140, or PADGEM protein). P-selectin is involved in mediation of adhesion of activated platelets to neutrophils and monocytes, and is a component of the α -granule membrane of resting platelets only expressed at the platelet surface after α -granule secretion¹⁸².

Fluorescence-activated flow cytometry is advantageous above the other measures of platelet activation described, in that platelets are studied within their

physiological milieu and the test is specific for and sensitive to presence of activated platelets, able to detect a sub-population of as few as 1% partially activated platelets¹⁸³. The technique of flow cytometry is discussed in further detail in **Appendix 1** (page 156).

v. Other markers

Shortened platelet survival time may be used as a marker of increased platelet reactivity, as assessed by platelet scintigraphy¹⁸⁴ - this technique has been used in acute ischaemic stroke, confirming marked platelet activation in this setting¹⁸⁵. Increases in platelet volume have been reported in acute cerebral ischaemia^{186,187}. Another marker, glyocalicin (the extramembranous portion of GPIb) has also been assayed, and may have some potential use as a marker of platelet damage¹⁸⁸. The largest case series found no association between glyocalicin level and previous TIA¹⁸⁹.

Table 5.6 below provides a summary of platelet activation markers and detection methods (taken from Wu, K.K.. Platelet activation mechanisms and markers in arterial thrombosis. *Journal of Internal Medicine* 1996;239:17-34).

Categories	Methods
<i>Platelet aggregometry</i>	
SPA	Aggregometry
Agonist-induced aggregation	Aggregometry
Shear stress-induced aggregation	Viscometer-flow cytometry
Platelet aggregates	Platelet-count ratio or flow cytometry
<i>Platelet activation products</i>	
Beta-thromboglobulin (plasma or urine)	RIA or EIA
11-dehydro-TXB ₂ (urinary)	RIA
2,3-dinor-TXB ₂ (urinary)	GC-MS
Glycocalicin (plasma)	EIA
<i>Activated platelets</i>	
Activated GPIIb-IIIa	Flow cytometry
P-selectin	Flow cytometry
<i>Platelet turnover</i>	
Platelet size	Electron sizing
Platelet survival	¹¹¹ In or ⁵¹ Cr platelet test

EIA, enzyme immunoassay; GC-MS, gas chromatography-mass spectrometry; GPIIb-IIIa, platelet membrane glycoprotein IIb-IIIa; RIA, radioimmunoassay; SPA, spontaneous platelet aggregation; TXB₂, thromboxane B₂

5.2.3 Platelet Activation and Carotid Artery Stenosis

From the above discussion, it can be seen that systemic platelet activation has been described in the setting of cerebral ischaemia and infarction and in conjunction with a number of vascular risk factors. Anti-platelet agents, such as aspirin, are proven in secondary prevention of stroke. By combining this knowledge it is reasonable to hypothesise that systemic platelet activation may occur in the setting of symptomatic CAS and furthermore that platelet activation may relate to development of asymptomatic ES - whether this occurs locally at the level of the plaque or as a systemic phenomenon.

In a subgroup of CAS patients recruited to the study detailed in Part I of this chapter, we investigated platelet activation using both fluorescence activated flow cytometry and sP-selectin assay (ELISA), comparing a group of symptomatic patients with CAS to a group of population controls. The relationship between platelet activation levels and detection of asymptomatic ES was also investigated.

5.2.4 Methods

5.2.4A. Flow Cytometry

40 subjects were recruited - 26 with CAS (18 symptomatic, 8 asymptomatic) from the above study and 14 normal controls.

After discarding the first 2.5ml of blood, a 1.8ml blood sample was obtained for each subject, uncuffed, from an ante-cubital vein and drawn into a syringe containing 0.2ml of 3.15% trisodium citrate. Samples were processed immediately for flow cytometric analysis as over.

Samples were prepared into 5 separate test-tubes: tube 1 acted as a double negative control; tube 2 was a single-stained (FITC) preparation of MoAb to glycoprotein Ib (CD42b) to allow compensation settings to be made; tube 3 contained a single-stained (PE) preparation of MoAb to glycoprotein IIb/IIIa (CD41) allowing further compensation settings of the PE detector. Tubes 4 and 5 contained double stained preparations of FITC-labelled IgG to CD42b with PE-labelled CD41 and PE-labelled MoAb to P-selectin (CD62P) respectively. All MoAbs were supplied by Serotec Limited, Oxford, UK. After a 5-minute incubation period at room temperature, 50µl of cold HEPES buffered saline was added, and the samples incubated for 20 minutes longer at room temperature. Finally, samples were fixed with 500µl of 0.2% formol saline, and flow cytometric analysis performed within 2 hours of collection.

Sample analysis was performed using a FACScalibur flow cytometer (Becton Dickinson Limited, Oxford, UK) with CellQuest software (Becton Dickinson Ltd.). The cell sorter was calibrated monthly using fluorescent microbead standards (Becton Dickinson Ltd.) using FACSCompv2.0 software (Becton Dickinson Ltd.). The platelet population was analysed at a high flow rate, and identified on the basis of forward- and side- scatter characteristics obtained with gain settings in the logarithmic mode (see **Appendix 1**). As dual-colour analysis was used, a fluorescence threshold was set to analyse only those cells that had bound FITC-labelled anti-CD42b. Because erythrocytes and white blood cells do not bind this platelet-specific antibody they were effectively excluded from the analysis. Platelet-bound PE-labelled anti-CD41 or anti-CD62P was then determined by analysing 10,000 platelets for PE fluorescence.

CD62P (P-selectin) is expressed only at the surface of activated platelets - platelet activity as assessed by degree of CD62P expression can therefore be taken as the absolute percentage of platelets stained with PE at a level of >98% of those platelets incubated with non-specific PE-labelled antibody. CD41 is expressed on all platelets, active or resting, but the degree of CD41 expression increases per platelet on activation. Platelet activation as determined by CD41 expression (MoAb binding) was therefore taken as the geometric mean fluorescence intensity per platelet (in arbitrary fluorescence units), again for those cells having PE fluorescence >98% of those cells incubated with negative control MoAb.

For both studies described below, subjects with CAS underwent a one-hour TCD recording on the day that blood was taken for assessment. Where results are related to presence or absence of ES, this refers to the recording done on the same day.

5.2.4B. ELISA

53 patients with $\geq 70\%$ symptomatic CAS from the main study described in Part I had blood drawn at the time of their TCD recording. Data from these patients were compared with those from 56 population controls, closely matched for age, sex and vascular risk factors.

Blood Sampling

Both patients and controls were asked to donate 35ml of blood for study use. Venesection was from the brachial vein, using a 21-gauge needle, with blood drawn using the Vacutainer system. Blood for this study comprised 4.5ml drawn into 0.5ml of 0.129 mol trisodium citrate. Samples were transferred immediately to a refrigerator

and within 4 hours were spun down for 10 minutes at 4°C at 1500g. Plasma was aspirated and stored at -80°C until analysis. Analysis was then performed in duplicate at the end of the study by a single observer blind to group category.

Laboratory Assays

Soluble P-selectin levels were measured using a quantitative sandwich enzyme-linked immunoassay (R & D Systems, Abingdon, UK) according to the manufacturers instructions. Assays were performed by N. Smith, Department of Medicine, King's College School of Medicine & Dentistry.

Statistics

Percentages of CD62P-positive platelets and geometric mean fluorescence intensity determining CD41 expression were not normally distributed, and hence a log transformation was carried out to produce a normal curve. Comparisons between groups were then performed by One-way ANOVA.

sP-selectin levels as determined on ELISA were found to be normally distributed. Comparison of means therefore uses Student t-test unless otherwise indicated. Correlations are made using Pearson's test. Where multiple comparisons are to be made, one-way ANOVA is used as detailed in the text.

5.2.5 Results

5.2.5A. Flow Cytometry

Subject characteristics are summarised in **Table 5.6**.

Factor	Symptomatic CAS	Asymptomatic CAS	Normal Control	p
n	18	8	14	
Age(mean,SD)	67.17(8.98)	71.62(6.27)	62.71(7.49)	0.05
Male sex	16	5	6	0.02*
Hypertension	10	6	3	0.14
Diabetes	3	1	0	0.29
Ischaemic Heart Disease	5	3	3	0.72
Peripheral Vascular Disease	4	3	0	0.06
Current smoker	5	0	2	0.21

Table 5.6. Clinical data for the three compared groups. There was an excess of males in the symptomatic group.

There was no relationship between gender and platelet activation - for CD41 logGMI, mean (SD) in males was 2.32 (0.14) versus females 2.37 (0.17), $p=0.36$, *t*-test; for CD62P, log percentage positive patients mean (SD) was - 0.22 (0.47) for males versus 0.17 (0.26) in females, $p=0.17$, *t*-test.

Comparison was made of antigen expression in the three groups as measured by labelled antibody expression - the results are illustrated in **Table 5.7** below. Further comparison was made of platelet activation among ES positive and ES negative subjects - again shown in table form, **Table 5.8**.

Antibody Expression	sCAS mean(SD)	aCAS mean(SD)	Controls mean(SD)	p (one-way ANOVA)
CD62P	0.07(0.51)	0.07(0.34)	-0.1(0.36)	0.87
CD41	2.32(0.15)	2.26(0.09)	2.39(0.17)	0.12

Table 5.7. Comparing platelet activation as determined by flow cytometric analysis between the three groups - figures given are in log units.

Antibody Expression	ES +ve mean(SD)	ES-ve mean(SD)	p (t-test)
CD62P	0.11(0.60)	0.04(0.33)	0.69
CD41	2.30(0.16)	2.31(0.13)	0.79

Table 5.8. Comparing platelet activation as determined by flow cytometric analysis between ES positive and ES negative groups - figures given are in log units.

5.2.5B ELISA

The demographic characteristics of the two groups are summarised in

Table 5.9. There were no significant differences between the groups.

	Control Group	Symptomatic CAS	p
n	56	53	
Mean (SD) Age (years)	66.25(7.04)	67.04(8.94)	0.43†
Sex M:F	40:16	36:17	0.69‡
Diabetes*	6(11%)	7(13%)	0.69‡
Hypertension**	32(57%)	35(66%)	0.34‡
Current smoker	14(25%)	12(23%)	0.77‡

Table 5.9. Characteristics of patient and control groups. ** - includes both insulin-dependent and non-insulin dependent diabetes mellitus; † Mann-Whitney U; ‡ chi-squared.

There was no difference in sP-selectin levels between patients and controls: mean (SD, range) sP-selectin level in normal controls was 151.6 ng/ml (69.08, 33.19-298.78), versus 135.71 ng/ml (55.57, 59.26-281.24) in patients, p=0.12.

In 48 patients with symptomatic CAS in whom TCD recordings were performed, ES were detected in 20 (41.7%). There was no difference found between those with a positive versus those with a negative recording - in ES positive patients, mean (SD) sP-selectin 134.64 ng/ml (48.03) versus 140.73 ng/ml (63.25), p=0.72.

The relationship between sP-sel levels found in the CAS group by symptom category - amaurosis fugax, hemispheric transient ischaemic attack and completed stroke was examined. The results are shown in **Table 5.10** - by one-way ANOVA there was no detectable difference according to symptom categorisation, $p=0.68$.

Symptom Category	Mean (SD) sP-selectin (ng/ml)
Amaurosis fugax (n=8)	119.7(40.9)
Hemispheric TIA (n=16)	138.8(65.5)
Completed Stroke (n=29)	138.4(54.1)

Table 5.10. sP-selectin level by symptom category.

For the patient group, time lapsed in days since last symptom was recorded - mean(SD, range) 89.55 (93.95,1-325). There was no correlation between sP-selectin levels and time since last symptom, ($r = -0.002$, $p= 0.99$).

5.2.6 Discussion

Both sets of results - flow cytometry and ELISA - are consistent with a lack of systemic activation in CAS. We did not find any evidence of raised sP-selectin levels in patients with symptomatic CAS. This is supported by one previous study¹⁷⁵, and may be due to atherosclerosis in this group being less widespread, or due to the fact that subjects were well-matched for other vascular risk factors. We found no relation with other markers of clinical risk in CAS including time from symptoms, presence of an infarct on CT scan, symptom category or presence of ES on TCD examination. The range of time since last symptoms in our study however was variable, ranging from 1 to 325

days (mean 89.55, SD 93.95), and this may have had an influence on results. It is unclear whether the main source of sP-selectin in humans is the endothelium, where it is stored in the Weibel-Palade bodies of endothelial cells, or platelets, where it is released from the alpha-granules on platelet activation¹⁹⁰. As sP-selectin levels appear to correlate with extent of atherosclerotic disease, then either source would be a possibility. It has been suggested however that the failure of sP-selectin levels to correlate with von Willebrand factor, an established endothelial cell marker would imply that sP-selectin is mainly platelet-derived¹⁷².

It is likely that local factors at plaque level are of greater importance in embolisation in CAS - it may be that a more representative picture of platelet activation at plaque level could be obtained by sampling from the vessel in the region of the plaque, as has been the case in flow cytometric studies in coronary artery disease reported previously¹⁹¹.

Chapter 6

Embollic Signals in Carotid Artery Stenosis (II)

6.1 Variability

In Chapter 5, the importance of ES detection in CAS with regard to their value as a predictive factor in stroke was demonstrated and their presence correlated with indirect markers of stroke risk. As discussed in Chapter 3, previous studies have reported very different proportions of patients with CAS in whom ES can be detected. One reason for this may be the differing recording times used, which have ranged from 20 minutes to 1 hour - see **Table 2.4**.

There has been little work done previously examining variability of embolic activity, though that which exists suggests that the presence of ES within an individual may be variable^{107,121}. If there are to be any prospective studies of the significance of ES in predicting stroke risk, it is vital that recording protocols be standardised. Any such study would require large numbers of patients as there would be relatively few outcome events - by definition more than one centre would need to be involved, and again the importance of standardisation is great. Furthermore, to determine numbers required to power such a study, more information is needed about the degree of variability expected.

To elucidate further the variable nature of embolisation, we determined the effect of both repeating and extending recording times in patients with symptomatic and asymptomatic carotid stenosis in a subgroup of the patients recruited as described in Chapter 5.

6.1.1 Subjects and Methods:

Twenty symptomatic and twenty asymptomatic patients with >60% CAS as determined by Duplex criteria¹⁴⁹ were recruited and completed the study. Symptomatic patients were defined as having had symptoms (amaurosis fugax, transient ischaemic attack, or stroke) in the territory of the stenosed carotid artery within the last year. Patients with potential cardiac sources of emboli were excluded. Seven were considered but not included for the following reasons: 5 had no acoustic window, 1 underwent carotid endarterectomy before completion of the protocol, and 1 had a major stroke while awaiting surgery.

The demographic characteristics and treatment of the 40 patients are summarized in **Table 6.1**.

<i>Factor</i>	<i>Symptomatic</i>	<i>Asymptomatic</i>
Number	20	20
Mean Age (SD, range)	66.75 (9.73, 42-82)	70.4 (8.41, 43-79)
Male Sex	75%	65%
Hypertension	55%	70%
Diabetes	15%	20%
Hyperlipidaemia	40%	30%
Ischaemic Heart Disease	25%	25%
Peripheral Vascular Disease	20%	40%
Smoker	85%	70%
Positive Family History	60%	60%
Treatment - Aspirin*	95%	95%
Treatment - Warfarin**	-	5%

Table 6.1. Clinical data - patients completing variability study. * for symptomatic group on aspirin, doses are as follows: 75mg,n=9; 150mg,n=4; 300mg,n=6. For the asymptomatic group: 75mg,n=13; 150mg,n=3; 300mg,n=3.

**only one patient was taking warfarin regularly, INR 2.0-3.0, as he had repeated TIA's on aspirin alone.

For 39 of the 40 patients anti-platelet and anti-coagulant medication was left unaltered. The 40th patient suffered a gastrointestinal bleed and aspirin was stopped between the first and second recordings. There was no difference in the degree of carotid stenosis between the two groups of patients: symptomatic, 60%-79%, n=5; symptomatic, 80%-99%, n=15; asymptomatic, 60%-79%, n=3; asymptomatic 80-99%, n=17 ($\chi^2=0.63$, p= 0.43).

TCD recordings were made from the middle cerebral artery according to the standard protocol (Chapter 3). Each patient was present on three occasions for a 1-hour recording, and on one occasion (but not always the first occasion) this was extended to a 2-hour recording. Mean (SD) time between recordings was 11.48 days (10.09) in the symptomatic group and 18.78 days (10.09) in the asymptomatic group. Mean (range) depth of insonation was 52.6 mm (48-56). Sample volume was kept constant for each patient for all three recordings.

All recordings were analysed off-line, blind to clinical information and patient group, and ES identified on spectral and acoustic characteristics (Chapter 3). A second observer (NK, see Acknowledgements) performed some of the analysis in this study - hence interobserver reproducibility studies were performed for the two observers analysing the tapes. This was performed on a 2-hour recording comprising six 20-minute recordings from the ipsilateral middle cerebral artery in six patients with symptomatic carotid stenosis. Observer 1 (JM) detected a total of 89 ES, compared with Observer 2 who detected a total of 95 signals. The probability of agreement¹²⁸ of observer 2 compared with observer 1 was 0.93.

6.1.2 Statistical Analysis

All statistical analyses were performed on a PC with the use of SPSS for Windows and Genstat. The number of ES per tape was recorded, with a positive recording defined as one containing one or more ES. We evaluated the effects of repeating or prolonging recordings in two ways. First, we determined the cumulative yield resulting from extending and repeating the recordings. Second, we determined which recording protocol resulted in the greatest agreement between different recordings. For this analysis we treated the data as if they were two reproducibility studies: one with two consecutive 1-hour recordings (the 2-hour recording) and one with two non-consecutive recordings (two 1-hour recordings repeated on different days). Kappa statistics¹²⁸ were calculated, and their 95% confidence intervals were derived with the use of the standard error of the kappa (κ) statistic provided by SPSS. The agreement was considered excellent for $\kappa > 0.75$, fair for $\kappa = 0.4$ to 0.75 , and poor for $\kappa < 0.4$.

Neither numbers of ES or their intensity were normally distributed, and therefore differences were analysed with the use of non-parametric statistics. To compare the number of ES per hour in the symptomatic and asymptomatic subjects, we attempted to fit a Poisson distribution, but because the variance was much greater than the mean, it was more appropriately fitted by a super Poisson distribution to allow for a dispersion parameter. We therefore applied the Wald test¹⁹² with the addition of a heterogeneity factor into the model to compensate for the variance.

6.1.3 Results

6.1.3A Effect of Repeating and Extending the Recording on the Cumulative Yield

For the purpose of this analysis, the first hour section of the 2-hour recording is treated as an individual 1-hour record. In this way each patient effectively attended on three separate occasions for 1-hour recordings.

In symptomatic subjects, ten (50%) were ES positive at the first examination. After two and three recordings, the cumulative proportion of ES-positive patients increased to 12 (60%) and 15 (75%) respectively. Detailed results for each patient and each recording are shown in **Table 6.2**.

<i>Patient</i>	<i>Recording 1</i>	<i>Recording 2</i>	<i>Recording 3</i>	<i>2 hour recording</i>
1	0	0	0*	0
2	6	13*	2	31
3	0*	0	1	2
4	1*	0	0	3
5	16	4	1*	1
6	4	11*	9	23
7	0*	0	13	0
8	0	0	1*	2
9	0*	1	0	0
10	2	0*	2	1
11	1	0*	0	0
12	0*	1	1	0
13	0*	0	0	0
14	0*	0	0	0
15	0	0	0*	0
16	0	0	0*	0
17	10	0*	0	0
18	2	1*	0	2
19	1	0	0*	0
20	1	0*	0	0

Table 6.2. For all 20 symptomatic patients, showing number of ES detected per recording, where recording 1 is the first and recording 3 the third 1 hour recording made. An asterisk(*) denotes the first hour of the 2 hour recording.

In asymptomatic subjects, four (20%) had a positive first recording. Single repetition of the recording gave a cumulative yield of 5 ES-positive patients (25%). A third recording provided no further increase in yield. As for symptomatic subjects, some subjects who were ES-positive on one recording were ES-negative on the next recording. Again, results per patient are tabulated in detail in **Table 6.3**.

<i>Patient</i>	<i>Recording 1</i>	<i>Recording 2</i>	<i>Recording 3</i>	<i>2 hour recording</i>
1	0*	0	0	0
2	0*	0	0	0
3	0*	0	0	0
4	0	0	0*	0
5	23	2*	7	6
6	1	0*	1	1
7	0	0*	0	1
8	0*	0	0	0
9	14*	1	0	26
10	0*	0	0	0
11	0	0*	0	1
12	0*	0	0	0
13	1*	1	0	1
14	0*	0	0	0
15	0*	0	0	0
16	0	0*	0	0
17	0	0*	0	1
18	0	1	0*	0
19	0*	0	0	0
20	0	0*	0	0

Table 6.3. For all 20 asymptomatic patients, showing number of ES detected per recording, where recording 1 is the first and recording 3 the third 1 hour recording made. An asterisk(*) denotes the first hour of the 2 hour recording.

The effect of extending the recording time (by 15-minute increments up to 2 hours) on the yield of ES-positive patients is shown in **Table 6.4**. In symptomatic patients during the 2-hour recording, 6 patients (30%) were ES-positive by 1 hour; extending the recording for a further hour increased the yield to 8(40%). In asymptomatic patients during the 2 hour recording, 3 patients (15%) were ES-

positive by 1 hour; extending the recording for a further hour increased the yield to 7 (35%). The values for the yield of the first hour of the two hour recording are not necessarily the same as those for the first single-hour recording because the 2-hour recording was not always performed on the first occasion (see “Subjects and Methods”).

<i>Time from start of recording (minutes)</i>	<i>Number of positive recordings (%) Symptomatic</i>	<i>Number of positive recordings (%) Asymptomatic</i>
15	3 (15%)	2 (10%)
30	5 (25%)	3 (15%)
45	5 (25%)	3 (15%)
60	6 (30%)	3 (15%)
75	7 (35%)	4 (20%)
90	7 (35%)	4 (20%)
105	8 (40%)	4 (20%)
120	8 (40%)	7 (35%)

Table 6.4. For both symptomatic and asymptomatic patients showing cumulative percentage positive recordings broken down into 15 minute segments.

6.1.3B Comparison Between the Different Recording Strategies

This analysis was performed by treating the data as a “reproducibility” study. The comparisons between different recording strategies are presented in **Figure 6.1** and assessed by the kappa statistic. The greater the κ value, the greater the agreement between the two recordings, and therefore the less informative the second recording.

There was an excellent level of agreement between 2 consecutive hours of recording in symptomatic subjects ($\kappa=0.78$, $p=0.0003$)(**Figure 6.1A**) while there was no significant agreement between two single hour recordings performed on different days in symptomatic patients ($\kappa=0.22$, $p=0.27$)(**Figure 6.1C**).

In contrast, in asymptomatic patients there was a fair level of agreement between two consecutive 1-hour recordings ($\kappa=0.49$, $p=0.01$)(**Figure 6.1B**) and between two single hour recordings performed on different days ($\kappa=0.48$, $p=0.03$)(**Figure 6.1D**).

Using a similar method of analysis, we looked at levels of agreement between a two-hour recording performed at one visit and a two hour recording composed of two single hour recordings made at two separate visits. This was done to determine which recording strategy produces higher numbers of ES positive patients and therefore which may yield most information. For asymptomatic stenosis there was a fair level of agreement between the two methods of recording ($\kappa=0.53$, $P=0.01$)(**Figure 6.1F**), whereas for symptomatic recording there was poor agreement ($\kappa=0.15$, $p=0.44$)(**Figure 6.1E**).

6.1.3C Comparison Between Symptomatic and Asymptomatic Patients

When we considered all 1-hour recordings, a higher proportion of recordings with ES was found among symptomatic recordings (24/60 versus 10/60; $\chi^2=8.04$, $p=0.005$). However, when we considered only those recordings in which ES were detected, there was no difference in the median (range) of the total number of ES in the two groups: symptomatic, 2 (1-16); asymptomatic, 1 (1-23) $p=0.7$.

We found a significant difference in the intensities of ES found between the two groups - median (mean, range) in symptomatic subjects 12 dB (13.7, 7-30) versus 11 dB (11.7, 7-30), $p=0.01$, Mann-Whitney U. As the number of patients positive for ES was small, with a range from 1-23 per hour, intensity data are subject to skew, and the analysis was repeated using weighted means. Weighted mean data were normally

distributed - comparison was therefore made using *t*-test: for symptomatic subjects mean (SD) 12.26 (3.37) versus 10.84 (2.90) for asymptomatic group, $p=0.253$.

A. Symptomatic Consecutive 1 hour Recordings Kappa = 0.78, 95% CI 0.50-1.06, $p=0.0003$				B. Asymptomatic Consecutive 1 hour Recordings Kappa = 0.49, 95% CI 0.11-0.87, $p=0.01$			
		2nd hour				2nd hour	
		ES +	ES -			ES +	ES -
1st hour	ES +	6	0	1st hour	ES +	3	0
	ES -	2	12		ES -	4	13

C. Symptomatic Inconsecutive 1 hour Recordings Kappa = 0.22, 95% CI 0.17-0.61, $p=0.27$				D. Asymptomatic Inconsecutive 1 hour Recordings Kappa = 0.48, 95% CI -0.02-0.98, $p=0.02$			
		2nd hour				2nd hour	
		ES +	ES -			ES +	ES -
1st hour	ES +	5	6	1st hour	ES +	2	2
	ES -	2	7		ES -	1	15

E. Symptomatic 2 hour Recordings Kappa = 0.15, 95% CI -0.22-0.53, $p=0.44$				F. Asymptomatic 2 hour Recordings Kappa = 0.53, 95% CI 0.14-0.93, $p=0.01$			
		inconsecutive				inconsecutive	
		ES +	ES -			ES +	ES -
consecutive	ES +	6	2	consecutive	ES +	4	3
	ES -	7	5		ES -	1	12

Figure 6.1. Evaluation of the agreement between different periods of recording. For each comparison, the 2x2 tables show whether ES were detected (ES+) or not detected (ES-) during each of the recording periods, which are then compared with the kappa statistic, which is shown with its 95% confidence intervals. Top, agreement between the first hour and second hour of the consecutive 2-hour recording is shown for (A) symptomatic and (B) asymptomatic stenoses. Middle, agreement between two nonconsecutive 1-hour recordings (made at 2 separate visits) is shown for (C) symptomatic and (D) asymptomatic stenoses. Bottom, agreement between the consecutive 2-hour recording and the nonconsecutive 2-hour recording (made up of two single-hour recordings at different visits) is shown (E,F).

6.1.4 Discussion

The study demonstrates that the recording protocol is crucial in determining the proportion of patients in whom ES can be detected. In common with previous pilot studies^{107,121}, our results confirm the variability of asymptomatic embolisation over time. By repeating recordings on three separate occasions, the proportion of ES-positive patients increased from 50% to 75% in symptomatic patients and 20 to 25% in asymptomatic patients.

If ES were detected in symptomatic stenosis patients during a 1-hour period, they were usually detectable in the same subjects during a second consecutive 1-hour recording, as reflected by excellent agreement between the two recordings ($\kappa=0.78$). Therefore, extending the recording time from 1 to 2 hours in symptomatic patients is of limited benefit. In contrast, there was no significant agreement between which patients were positive for ES on 1 hour of recording compared with a non-consecutive second hour separated by a number of days; the mean time between recordings was 11 days in the symptomatic group. These results demonstrate the variability of asymptomatic embolisation in patients with symptomatic stenosis; they also demonstrate that although within a 2-hour period the process may be relatively constant, a few days later embolisation status may well have changed.

In asymptomatic patients there was fair agreement between which patients were positive for ES during 1-hour recordings compared with those positive during a second consecutive hour ($\kappa=0.49$ versus $\kappa=0.78$ for symptomatic stenosis). This was a level of agreement similar to that seen between two non-consecutive hours of recording separated by a few days ($\kappa=0.48$). This reflects in part the lower frequency of patients with asymptomatic stenosis in whom ES can be detected and demonstrates

the usefulness of either increasing the recording time or repeating recordings in patients with asymptomatic stenosis.

We found a significantly higher proportion of recordings with ES in symptomatic than in asymptomatic patients, as reported previously^{105,107}, though at odds with the findings reported in Chapter 5. This may be explained on the basis that this study considered only the first 40 CAS patients recruited for detailed analysis of recording protocols, forming the basis for further recordings made. When we considered only those recordings in which ES were detected, there was no difference in the frequency of ES between asymptomatic and symptomatic patients. Comparing individual emboli, there was a highly significant difference between the intensity increase of ES in the two groups, with those in symptomatic recordings having a higher relative intensity. Comparing mean ES intensity on a patient-by-patient basis, this significance was lost, though there was still an apparent trend which may be explored further with a larger sample size. Although there are many technical difficulties in deriving embolus size from the intensity of the ES as discussed earlier⁸⁷, this difference may be consistent with emboli in symptomatic patients being larger or possibly of more echogenic material.

In conclusion, in asymptomatic stenosis, recording for 1 hour would appear to be the minimum reasonable period, and detection of ES-positive patients will be increased to a similar extent by prolonging recording to 2 hours or repeating the recording. In contrast, in patients with symptomatic disease a single hour of recording at one time is probably sufficient, but repeating the recording on a second occasion will identify additional subjects in whom embolisation is occurring.

Chapter 7

Models of Embolisation

There are a number of mechanisms for thromboembolism in stroke, as was discussed in Chapter 1, and the composition of emboli varies. As aspirin, an anti-platelet agent, is proven in secondary prevention of stroke¹⁹³, it is not unreasonable to assume that many emboli are composed of platelet aggregates, and visualisation of platelet emboli in the retinal circulation supports this¹⁹⁴.

Many strokes and thrombo-embolic events occur despite the use of aspirin, but other agents are now available, including ticlopidine, clopidogrel, anti-glycoprotein IIb/IIIa antagonists and nitric oxide donors. Current methods available to evaluate these agents' efficacy are not ideal - ex-vivo studies such as platelet aggregation provide an indicator of potential effectiveness but may not be totally representative of biological effectiveness in-vivo¹⁹⁵. Currently available animal models¹⁹⁶ are not always representative of the situation in man. Large multi-centre trials using vascular events as primary endpoints are required. However, the number of outcome events in such studies may be small, hence recruitment of large numbers is needed, and may not always give a definitive result⁴⁵.

It can be seen that what is needed is a reliable model in which to evaluate the efficacy of new agents in-vivo in man, using small patient numbers. This would be useful in bridging the gap between laboratory studies and clinical trials. Patients with frequent ES as detected by TCD may provide us with a 'model' of embolisation in which anti-platelet and anti-thrombotic agents may be trialled.

7.1 Potential Models

Testing of a new anti-platelet agent was initially proposed in four distinct situations, all arising in patients with carotid artery stenosis. Each situation and possible modes of embolisation are considered below:

7.1.1 Severe Carotid Artery Stenosis

Intact endothelial cells play many physiological roles¹⁹⁷ including provision of a non-thrombogenic surface and maintenance of vascular tone by release of small molecules such as nitric oxide.

It is widely accepted that atherosclerosis develops as a response to injury. Endothelial cell dysfunction may result from various sources of endothelial injury, including exposure to agents such as oxidated low density lipoproteins(oxLDL), shear stress, hyperhomocysteinaemia, immunologic mechanisms and infection¹⁹⁸. Branch points of the arterial tree are particularly susceptible to this process, such as at the carotid bifurcation. Endothelial cell dysfunction may be manifest as increased trapping of lipoprotein within the artery and the appearance of adhesive glycoproteins on the endothelial cell surface. As this process continues, there is development of the fatty streak, composed of foam cells and accompanying lymphocytes¹⁹⁹⁻²⁰⁴. As the process continues, there is continued cell influx and proliferation leading to more advanced lesions and progressing ultimately to the fibrous plaque. The fibrous plaque increases in size and by projection into the arterial lumen may impede blood flow. They are covered by a dense cap of connective tissue with embedded smooth muscle cells usually overlying a core of lipid and necrotic debris. Endothelial cell loss means that there is decreased production of molecules such as prostaglandin I₂(PGI₂), nitric

oxide (NO) and heparan sulphate. Platelet thrombi may form at sites of endothelial disjunction and activated platelets release mitogenic factors perpetuating the development of the lesion¹⁹⁸.

Platelet activation may take place by exposure to sub-endothelial elements, particularly collagen, and via thrombin generated by interaction between the platelet and the vessel wall²⁰⁵. Platelet aggregate formation may then occur either at sites of endothelial loss - either by exposure to subendothelial elements or thrombin, by loss of the endothelial anti-thrombotic properties or by shear-stress induced aggregation.

With the more advanced fibrous plaque there is more extensive endothelial loss, with the potential for ulceration, intra-plaque haemorrhage and thrombosis. There may be eventual plaque rupture with release of atheromatous debris as described above. Data have shown that most sudden deaths from myocardial infarction are due to ruptures or fissures, particularly at the margins of the fibrous cap, resulting in haemorrhage into the plaque, thrombosis and artery occlusion²⁰⁶. Extrapolation of these data into the carotid artery setting might account for transformation from 'inactive' to 'active' plaque, with development of emboli. This would be supported by the finding that embolisation is seen more frequently in association with high grade¹⁴⁸ and ulcerated¹¹¹ lesions.

7.1.2 Carotid Endarterectomy - Dissection Phase

The 'dissection phase' of carotid endarterectomy includes the time from skin preparation to clamping of the internal carotid artery - during this time the arterial system has not been entered, and therefore any emboli detected can be assumed to be particulate. The first ever description of a particulate embolus being detected by

transcranial Doppler ultrasound was in this setting⁷³. Further work has shown that asymptomatic embolic signals can indeed be detected during the dissection phase of carotid endarterectomy, and these have been shown to correlate with post-operative cerebral focal ischaemia. In one study, >10 ES during the dissection phase were shown to correlate with a significant deterioration in postoperative cognitive function²⁰⁷. In a second study, >10 ES during this phase was shown to correlate with postoperative cerebral complications and with the appearance of new hyperintense lesions on postoperative T2-weighted magnetic resonance imaging²⁰⁸.

Prior to arteriotomy, the situation as regards formation of platelet aggregates with the potential for embolisation is as described above at the surface of the advanced atherosclerotic plaque. In addition, many ES seen during the dissection phase occur in association with handling of the artery²⁰⁹, such as occurs during slinging - these may in part represent emboli produced by dislodgment of plaque rather than platelet aggregates alone, and may be particularly important with friable, ulcerated plaques or those with associated thrombus²⁰⁹.

7.1.3 Carotid Endarterectomy - Post-Operative Phase.

Asymptomatic embolic signals are frequent following carotid endarterectomy^{145,207}, and recent studies demonstrate that a high frequency of embolic signals during the early post-operative phase correlates with early stroke risk^{51,210,211}. One of the earlier studies published also demonstrated a correlation between persistent particulate embolisation and development of neurological deficit²⁰⁷. It is important to note that these studies have all made recordings following skin closure, and hence record beyond the time when air embolisation is a possibility.

Technically successful carotid endarterectomy involves removal of the plaque at the level of the superficial media. Hence post-operatively there is a large area denuded of endothelium - presumably the mechanism of platelet aggregate formation in this situation is via the same pathways as at the intact plaque, but with the previous microscopic areas of endothelial loss magnified to a macroscopic scale.

7.1.4 Carotid Angioplasty

Though carotid endarterectomy is the accepted treatment for symptomatic carotid artery stenosis, there has been a recent trial examining the safety and feasibility of applying percutaneous angioplasty to the carotid and vertebral arteries⁷². Possible advantages of percutaneous transluminal angioplasty (PTA) include a shorter admission time, avoidance of a general anaesthetic and surgical incision and an ability to dilate surgically inaccessible lesions such as stenoses high in the internal carotid⁷¹.

The mechanisms by which PTA increases the size of the luminal diameter have been studied in both animals and cadavers. Balloon-induced barotrauma causes endothelial denudation; cracking, splitting and disruption of the atherosclerotic plaque; dehiscence of the intima and plaque from the underlying media; and stretching and tearing of the media and adventitia with resultant aneurysmal dilatation^{212,213}. Balloon inflation may also be deleterious, causing plaque haemorrhage, extensive dissection (causing luminal compromise), platelet deposition or thrombus formation^{213,214}. The endothelial loss causes increased thrombogenicity, which in animal models has been shown to return to near normal thrombogenicity within 24 hours²¹⁵.

It can be seen that both the therapeutic aims and unwanted effects provide a situation for development of embolisation - of dislodged atherosclerotic material, platelet aggregates and thrombus. In one study of patients treated by PTA with use of a distal occlusive balloon, debris released after deflation of the proximal angioplasty balloon was retrieved, and cholesterol crystals found in over 60% of cases²¹⁶.

One previous study found evidence of embolisation by transcranial Doppler monitoring in 90% of patients immediately after balloon inflation - this figure fell to 80% after femoral catheter removal and 20% 48 hours following the procedure¹³⁶.

There is no published evidence relating asymptomatic ES to clinical outcome in this setting.

Having postulated that much of the embolisation in the above four 'model' situations as detected using TCD arises secondary to platelet adherence and subsequent aggregation, we aimed to influence embolisation rates using a new anti-platelet agent - S-Nitrosoglutathione, GSNO. To prove any effect of this agent, we needed subjects in whom ES could be reliably detected in sufficient numbers to provide the power needed to demonstrate a therapeutic effect.

Before any study could start in looking at modification of the embolisation process, it was first necessary to look at the natural incidence of embolisation in the proposed groups, such that decisions could be made concerning feasibility. As a result, the first of the models described above was rejected as unsuitable, the reasons for this being detailed below.

7.1.1A Severe Carotid Artery Stenosis - Comment

We recruited 114 patients with >60% CAS on Duplex ultrasound, each of them undergoing at least 1 hour of TCD recording from the ipsilateral MCA. This work is described in Chapter 5. In a smaller group of patients (n=40), we also investigated the variability of ES over time and within recordings, as described in Chapter 6.

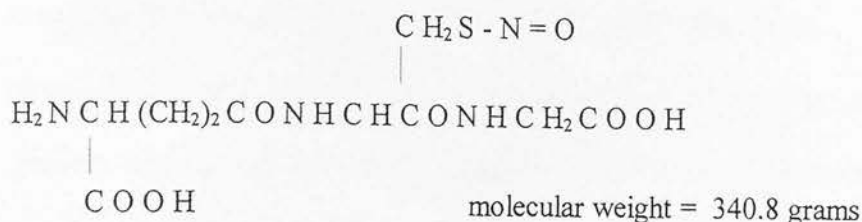
From our studies we concluded that ES in CAS may be infrequent, and are highly variable. Though information about embolisation in these patients may be of interest, and possible application in longitudinal studies of risk, we concluded it would not be feasible to undertake longitudinal or crossover studies of efficacy of therapy in this group in the small numbers permitted by my time-scale.

We therefore went on to study embolisation in the setting of carotid endarterectomy - both dissection phase and post-operatively - and carotid angioplasty (PTA). In these situations the use of GSNO was investigated as a possible means of reducing the incidence of ES, which have been shown to be clinically relevant as above.

7.2 S-Nitrosoglutathione, GSNO

The agent we used - GSNO, S-Nitrosoglutathione - is a possibly targeted nitric oxide (NO) donor with specific anti-platelet action. In addition to its effects on vascular tone, nitric oxide inhibits platelet aggregation by stimulating soluble guanylate cyclase, thereby increasing cyclic guanosine monophosphate²¹⁷ which leads

to reduced platelet adhesion and aggregation²¹⁸. Its molecular structure is shown below:



Organic nitrates, which act through releasing NO, reduce platelet deposition and thrombus formation after angioplasty in pigs, but often at doses causing hypotension^{219,220}. Similarly in man organic nitrates induce hypotension at doses required for an anti-platelet effect²²¹.

S-Nitrosoglutathione (GSNO) is a stable S-nitrosothiol from which NO is released by the action of enzymes associated with platelet membranes²²². In animals and humans GSNO has significant anti-platelet action at doses which cause less haemodynamic effect than conventional NO donors²²³. In a previous study it has been demonstrated that platelet activation occurs following coronary angioplasty and that this activation can be prevented by administration of GSNO¹⁹¹. GSNO has also been shown to inhibit platelet activation in the setting of acute myocardial infarction and unstable angina²²⁴.

7.3 Carotid Endarterectomy Study

7.3.1 Subjects & Methods

The methods described below were used for all patients undergoing carotid endarterectomy, whether recordings were made during the dissection phase, post-operatively or both.

We studied a total of 37 patients undergoing carotid endarterectomy for symptomatic internal carotid artery stenosis of >70% determined angiographically using the ECST method of measurement⁶⁴. Of these, 30 patients had recordings made during the dissection phase, and twenty-four patients had recordings made during the post-operative phase. Demographic characteristics for the two groups are detailed in the Results section. There was an overlap between the two groups, with 17 patients having studies performed during both phases of the surgery.

All patients were pre-treated with aspirin 300mg once daily for at least one week prior to operation and all were given 5000IU sodium heparin intravenously 2 minutes to carotid clamping.

7.3.1A Methods - GSNO

GSNO was supplied in powder form (prepared by Dr. Dave Madge, Wolfson Institute for Biomedical Research, London). Infusions were prepared for each individual patient on the morning of surgery according to the weight of the patient. The amount used was 5.4×10^{-3} g/kg, reconstituted with 100ml of normal saline, prepared under sterile conditions and drawn into 50ml syringes via a 0.2 μ m filter (Gelman Sciences, Michigan, USA). Once prepared, both syringes and the infusion tubing were wrapped in aluminium foil, as exposure to light accelerates spontaneous release of NO, and hence loss of GSNO activity.

For each group, GSNO was administered as an intravenous infusion, commenced at induction of anaesthesia at 2.2nmol/kg/minute, and, if tolerated, increased to 4.4nmol/kg/minute at 10 minutes and continued until the arteriotomy was made. The criterion for terminating the infusion was a drop in mean arterial pressure (MAP) of 10mmHg or greater. If blood pressure returned to the pre-GSNO level a

further test infusion was given and maintained at the lower rate. If once more there was a blood pressure drop of 10mm or greater in MAP, the infusion was stopped permanently. The dose used was chosen from work done showing effective reduction of platelet activation in the setting of acute myocardial infarction and unstable angina using GSNO²²⁴. Using a standardised weight of GSNO for each patient facilitated the use of constant infusion rates of 7.5ml/h rising to 15ml/h if tolerated.

7.3.1B Methods - Dissection Phase

Patients were allocated to treatment with either GSNO (n=15) or no additional treatment (n=15). For the first 17 patients allocation was done on an open basis due to concerns over potential side effects of the GSNO (hypotension, bleeding) - however, side effects seen in this initial phase were minimal, and subsequently patients were randomised between GSNO or normal saline, drawn up and administered under the same protocol as the active treatment, with both the surgeon and anaesthetist blind to the treatment group.

The GSNO infusion was commenced at the time of induction of anaesthesia and stopped at the time of cross-clamping, excepting those patients who also had recordings done for the post-operative phase. In this group 12 received GSNO, which continued as per the protocol below.

In all patients a one hour recording was made in the 24 hours preceding surgery. Further TCD recording was started at the start of the procedure ('knife to skin') and stopped at the time of cross-clamping of the common carotid artery, i.e. before any arteriotomy was made. All data analysis was done by the treatment administrator,(JM), off-line and blind to treatment group, at a later date.

7.3.1C Methods - Post-Operative Phase

Patients were allocated to treatment with either GSNO (n=12) or no additional treatment (n=12). In view of the potential side effects of the GSNO (hypotension, bleeding), and our limited experience of its clinical use, allocation to treatment or control group for the first 17 subjects was done on an open basis as described above.

GSNO was commenced as per the above protocol at the time of induction of anaesthesia and continued until 2 hours from skin closure.

Post-operative TCD recordings were made for 3 hours commencing 30 minutes from skin closure, and for 1 hour at 6 and 24 hours after skin closure.

7.4 Carotid Angioplasty Study

7.4.1 Subjects and Methods

From January 1996 to April 1997, a total of 6 carotid angioplasties were performed at our centre. Of these, 1 patient had no acoustic window, and was unable to be included in TCD studies. For the remaining patients, 4 were allocated to no additional treatment, and 1 received GSNO.

GSNO was prepared as detailed above - commencing at the time of femoral puncture at a rate of 7.5ml/hour, increased at 10 minutes to 15ml/hour to continue to 2 hours from catheter withdrawal.

All patients had TCD recordings made for 1 hour in the 24 hours preceding the procedure from the ipsilateral MCA. On the day of the angioplasty, recordings were made for 3.5 hours commencing at 30 minutes from catheter withdrawal, then for a further hour at 6 and 24 hours from catheter withdrawal.

All TCD recordings were made and analysed according to the methods described in Chapter 3.

For all 3 studies, all patients were taking aspirin 300mg daily for at least one week prior to the procedure. Patients taking any other anti-platelet or anti-thrombotic medication were excluded. In addition, all patients received 5000IU of heparin during the procedure - during endarterectomy 2 minutes prior to the cross-clamp being applied, during angioplasty 2 minutes prior to first balloon inflation. Angioplasty patients were also given 300-600 μ g of atropine immediately prior to the first balloon inflation.

7.5 Statistical Methods

As the numbers in the carotid angioplasty group are so small, results are merely presented in table form, and no statistical analysis is made. For both carotid endarterectomy studies, the numbers and intensity of ES in each group were not normally distributed and therefore comparisons between groups were performed using the Mann-Whitney U test for non-parametric data.

7.6 Results

7.6.1 Results - Dissection Phase

All 30 patients underwent technically successful carotid endarterectomy. Their clinical data are summarised in **Table 7.1**.

Of the 15 patients allocated to receive GSNO, 12(80%) tolerated the full dose with no clinically apparent side effects - in one patient the infusion was continued at half dose, in the other 2 the infusion had to be stopped - all due to hypotension which reversed on stopping the infusion. When applying the same criteria to patients

receiving normal saline prepared by the same method, the infusion had to be stopped in one subject on the advice of the anaesthetist, again secondary to hypotension.

Factor	GSNO group (n=15)	Control group (n=15)	p value (chi-squared unless specified)
Sex M:F	13:2	10:5	0.20
Age (mean,SD) years	65.1(8.5)	62.7(10.6)	0.50 (t-test)
Diabetes	3	0	0.07
Hypertension*	7	9	0.46
Current smoker	3	6	0.23
Percentage stenosis [†] mean(SD)	87.3(9.2)	85.5(10.8)	0.64 (Mann Whitney)
Time since symptoms,days mean(SD)	93.2(103.6)	67.7(80.2)	0.52 (Mann Whitney)
Symptom category Am.fug/TIA/Stroke	2/6/7	4/3/8	0.42
Dissection time, minutes mean(SD)	49.0(15.5)	50.6(17.0)	0.77 (Mann Whitney)
ES per hour, dissection median(range)	3.6(0-28)	0(0-62.3)	0.17 (Mann Whitney)

Table 7.1. Patient characteristics - control and GSNO-treated groups - dissection phase. *Hypertension is defined as systolic pressure of >160mmHg or diastolic >90mmHg or on anti-hypertensive treatment. [†]Defined by ECST criteria. All cases of diabetes were of NIDDM type.

In the control group receiving either no GSNO or normal saline, the median (range) number of ES detected per hour was zero (0-62.26) versus 3.6 (0-28) in the treatment group, $p=0.17$, Mann Whitney U. In the control group, 6 patients were ES positive, compared with 12 in the GSNO group, $p=0.03$. A summary of the results for each patient illustrating dissection time and ES detected per hour for each patient is shown in **Table 7.2**.

Patient number	Group	Dissection time(minutes)	Total ES detected	ES/hour
1	Control	27	0	0
2	Control	75	0	0
3	Control	32	0	0
4	Control	77	8	6.23
5	Control	66	0	0
6	Control	32	0	0
7	Control	40	0	0
8	Control	36	2	3.3
9	Control	41	0	0
10	Control	60	0	0
11	Control	53	55	62.26
12	Control	70	1	0.86
13	Control	43	27	37.67
14	Control	66	0	0
15	Control	41	22	32.2
16	GSNO	60	5	5
17	GSNO	30	14	28
18	GSNO	38	12	18.95
19	GSNO	92	6	3.91
20	GSNO	43	1	1.4
21	GSNO	48	3	3.75
22	GSNO	50	1	1.2
23	GSNO	61	1	0.98
24	GSNO	50	0	0
25	GSNO	53	5	5.66
26	GSNO	48	1	1.25
27	GSNO	30	0	0
28	GSNO	52	0	0
29	GSNO	50	3	3.6
30	GSNO	30	5	10

Table 7.2. For each patient monitored during dissection phase - according to group - showing the length of the dissection period and numbers of ES per hour detected.

From the scatterplot, **Figure 7.1**, it can be seen that one patient in the control group had particularly high numbers of ES, which may effect some skewing of results.

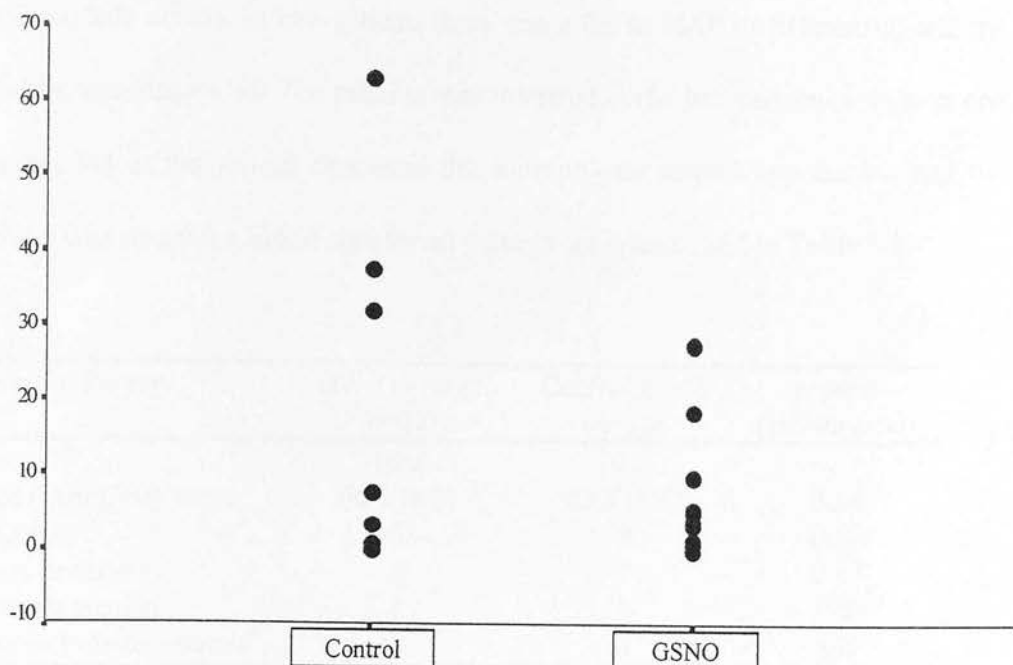


Figure 7.1. Scatterplot of numbers of ES per hour found in each group during dissection phase.

A total of 172 ES were detected in the dissection phase: 115 in the control group versus 57 in the GSNO group. Mean (SD) intensity of ES in the control group was 17.51 dB (8.31) compared with 12.52 dB (4.90) in the GSNO group, $p=0.0001$. However, near half of the ES found in the control group were detected in just 1 patient. In view of the small numbers, further comparison was made using weighted means to smooth the distribution: for controls mean (SD) was 16.01 (6.35) versus 13.21 (4.47) in the treatment group, $p= 0.40$, Mann Whitney U.

7.6.2 Results - Post-Operative Phase

In all patients a one hour recording was made in the 24 hours preceding surgery. All underwent technically successful carotid endarterectomy. Of the 12 patients allocated to receive GSNO, 10 tolerated the full dose with no clinically

apparent side effects. In two patients there was a fall in MAP of >10mmHg, and the infusion was suspended. The infusion was tolerated at the half-maximum dose in one patient, but in the second case even this infusion rate caused hypotension and the GSNO was stopped. Clinical data for all patients are summarised in **Table 7.3**.

Factor	GSNO group (n=12)	Control group (n=12)	p value (chi-squared)
Sex M:F	10:2	10:2	
Age (mean,SD) years	66.3 (8.8)	62.3 (8.6)	0.54
Diabetes	3	0	0.18
Hypertension*	5	7	0.49
Current smoker	1	4	0.2
Hypercholesterolaemia [†]	8	6	0.47

Table 7.3. Patient characteristics - control and GSNO-treated groups - post-operative study group. *Hypertension is defined as systolic pressure of >160mmHg or diastolic >90mmHg or on anti-hypertensive treatment. [†]Hypercholesterolaemia is defined as fasting cholesterol of >6.0mmol/l or treatment. All cases of diabetes were of NIDDM type.

In the control group receiving no GSNO, the median (range) number of ES detected during the 3 hour postoperative recording period was 38.5 (1-219). At 6 hours the median (range) number of ES per hour had fallen to 5.5 (0-105). By 24 hours, median (range) ES per hour had fallen to zero (0-30).

The results for each patient in the study are shown in **Table 7.4**.

On intention to treat analysis, there was a significant reduction in asymptomatic embolisation in the GSNO group during both the initial 3 hour recording period and during hour 6 (**Figure 7.2**). The median (range) number of ES detected in the first 3 hour recording was 7.5 (0-61) (p=0.018 v controls). During hour 6 the median (range) number of embolic signals was zero (0-41)(p=0.014 versus controls). By 24 hours the rate of embolisation in both groups was low and there was

no difference between control and treatment groups: median (range) was zero (0-37) (p=0.74 vs. controls).

Patient	Group	ES pre-op	ES1	ES2	ES3	ES6	ES24
1	Control	0	13	0	38	50	1
2	Control	0	62	69	48	48	30
3	Control	0	46	51	28	7	0
4	Control	0	1	0	1	1	0
5	Control	0	16	4	3	11	0
6	Control	0	5	14	12	1	0
7	Control	0	12	5	3	3	3
8	Control	0	2	0	0	0	0
9	Control	0	3	0	0	0	0
10	Control	0	3	1	4	4	0
11	Control	0	10	6	61	105	1
12	Control	0	26	7	15	13	0
13	GSNO	0	3	5	0	0	0
14	GSNO	0	1	0	0	0	0
15	GSNO	0	0	0	0	0	0
16	GSNO	0	6	13	9	7	1
17	GSNO	0	6	1	3	3	0
18	GSNO	0	1	3	1	0	0
19	GSNO	0	2	3	0	0	0
20	GSNO	0	0	0	0	1	0
21	GSNO	1	1	0	0	0	0
22	GSNO	1	1	3	2	0	3
23	GSNO	2	0	0	0	0	0
24	GSNO	1	11	29	2	41	37

Table 7.4. ES per hour for each patient in the post-operative study - ES1, ES2 and ES3 designate ES detected in the first, second and third hours recordings following skin closure. ES6 and ES24 represent ES detected in the hours recordings taken at 6 and 24 hours post-operatively.

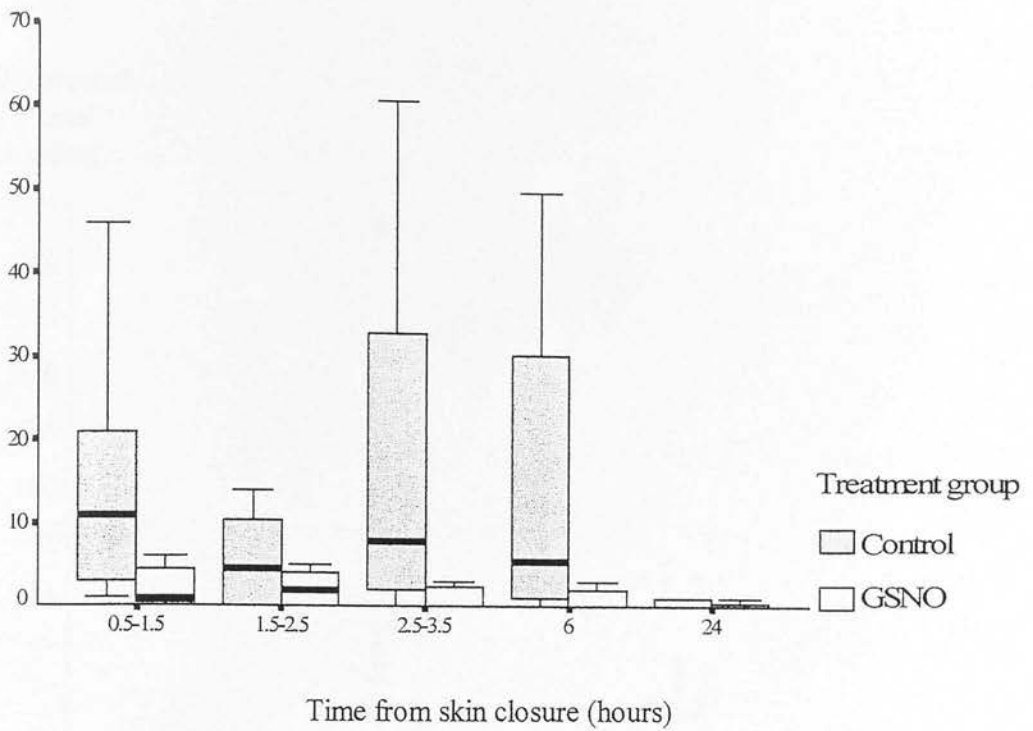


Figure 7.2. Box and whiskers plot - ES per hour against time from skin closure according to treatment group. Median value is shown as solid black line, boxes represent 25th to 75th percentile and lines delineate range.

Individual numbers of embolic signals in the two groups during the first three hours are shown in **Figure 7.3**. The two patients with frequent embolic signals in the GSNO group were the two in whom a full dose of GSNO could not be given due to hypotension. Exclusion of both patients who did not tolerate full dose would increase the difference between the two groups: at 3 hours in the GSNO group median (range) number of embolic signals was 5.5 (0-20)($p=0.005$ versus controls); at 6 hours median was zero (0-7)($p=0.003$) and at 24 hours zero (0-3)($p=0.47$).

ES detected
in 3 hour
recording

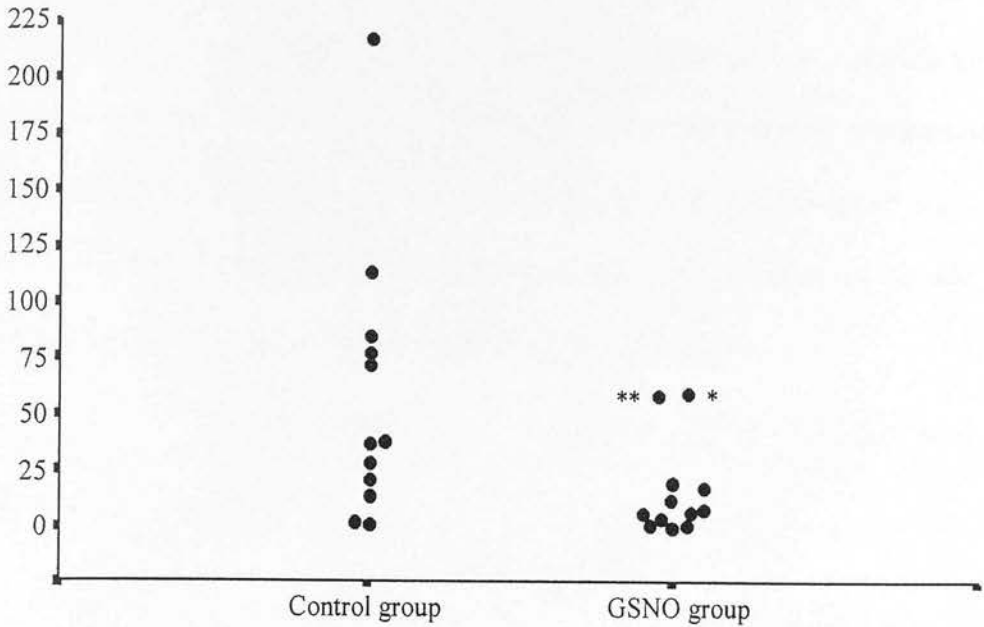


Figure 7.3. Scatter distribution of total embolic signals detected in each patient during 3 hour post-operative recording according to treatment group. *denotes patient in whom infusion was stopped. **denotes patient receiving half-maximum dose.

A total of 715 ES were detected in the initial 3 hour post-operative recording in the control group compared with 197 in the GSNO group. During this period embolic signals in the GSNO group were significantly less intense than those in the control group: mean (SD) 12.30 dB (4.30) versus 14.27 dB (4.71), $p < 0.0001$.

In the control group three patients suffered peri-operative ischaemic events. One patient suffered a stroke at 20 hours post-operation in the ipsilateral internal carotid artery territory with right facial and arm weakness and dysphasia; this recovered fully over three days and CT brain scan showed a cortical infarct. Two further control patients, who both had contralateral carotid occlusion, had strokes in

the contralateral internal carotid artery territory. In one patient aphasia and hemiparesis was noted on recovery from anaesthesia and he was left with a residual deficit; CT scan showed a large area of infarction in the internal carotid artery watershed areas. The second patient developed left hemiparesis and coma on three days post-operation and died; brain CT scan showed an intracerebral haemorrhage. There were no strokes or transient ischaemic attacks in the GSNO group but one patient was noted to have developed internal carotid artery occlusion on the side of the endarterectomy on repeat carotid duplex prior to discharge.

7.6.3 Results - Carotid Angioplasty

The results are shown in table form below:

Factor	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age	72	61	74	63	70
Sex	F	M	F	M	F
Diabetes	Normal	NIDDM (diet)	Normal	Normal	Normal
Hypertension	Normal	Untreated	Treated	Normal	Normal
Smoking	Non	Current	Ex	Ex	Ex
Time since symptoms	41	111	233	2	86
Symptom type	Stroke	Stroke	Stroke	TIA	TIA
Treatment group	Control	Control	Control	Control	GSNO
Treated stenosis (%)	93	85	89	84	79
Residual estimated stenosis (%)	20	60	0	50	40
Balloon inflations	3	3	1	3	3
ES/hour pre-op	0	14	0	1	0
ES1	0	28	23	41	0
ES2	0	8	3	15	0
ES3	0	13	0	20	0
ES6	2	2	0	1	0
ES24	0	0	0	0	0

Table 7.5. Summary clinical data for the 5 studied patients undergoing percutaneous transluminal carotid angioplasty. ES1, ES2, ES3 = number of embolic signal detected in the first, second and third hours of recording post-procedure. ES6 - number detected in the hour 6 hours from catheter withdrawal, ES24 - number in the hour 24 hours from removal of catheter.

The patient receiving GSNO had to have the infusion stopped 30 minutes early secondary to hypotension - though this occurred late, at 135 minutes from commencement. Hypotension in this case persisted despite stopping the infusion, and did not respond well to fluid replacement.

7.7 Discussion

7.7.1 Dissection Phase

GSNO did not reduce embolisation rates in the dissection phase of carotid endarterectomy in this study. In one patient receiving the normal saline control the infusion was stopped on the advice of the anaesthetist due to hypotension, illustrating the fact that operative and anaesthetic factors may confound results. Possible explanations for the lack of efficacy of the agent in this situation may be in part accounted for as follows:

- i) timing of the infusion and agent pharmacokinetics may be such that there is insufficient time for the agent to act within the time window given
- ii) platelet activation at the surface of an intact plaque may act via a different receptor system to cGMP
- iii) the degree of endothelial loss is smaller at an intact plaque - there is therefore endogenous endothelial NO production, the system may be saturated, and addition of further exogenous NO will not affect embolisation
- iv) most importantly, many ES seen during the dissection phase occur in association with handling of the artery²⁰⁹, such as occurs during slinging, and may be of different composition to those seen post-operatively. As their frequency was not influenced by treatment with an anti-platelet agent, this would be consistent with their not comprising platelet aggregates.

The dissection phase of carotid endarterectomy does provide a model of embolisation - i.e. reliably provides sufficient numbers of ES for assessment of therapeutic agents. The association of ES occurrence with handling of the artery may

provide information that can be used in surgical training in endarterectomy technique²⁰⁹.

7.7.2 Post-Operative Phase

GSNO resulted in a highly significant reduction in the frequency of embolisation following carotid endarterectomy. This reduction was maintained at 6 hours post-operatively, even though the infusion was stopped 2 hours post-operatively. In the majority of patients GSNO was well tolerated, but in two individuals it resulted in a drop in blood pressure. In one case it could be continued at half the maximum dose but in the other case it had to be stopped. Our data provide further evidence for the importance of nitric oxide in preventing platelet adhesion and aggregation in-vivo, and illustrate the potential use of platelet specific NO donors which may have relatively less hypotensive effect, for a given anti-platelet effect, than conventional NO donors such as nitroglycerin. GSNO has only been given as an intravenous infusion, but in the future oral analogues, or other platelet specific NO donors, may be effective in preventing thromboembolism. Our results also demonstrate that pre-operative aspirin and intra-operative heparin fail to prevent many embolic events.

The patient numbers were too small, and the study was not designed, to determine whether there was a significant reduction in clinical events. However there were no clinical ischaemic events in the GSNO group. There was one minor stroke in the control group in the ipsilateral internal carotid territory, and two strokes in the contralateral internal carotid artery territory. However the latter were in patients with contralateral occlusion and in one the pathogenesis was probably intraoperative haemodynamic ischaemia, while in the other it was haemorrhage probably due to a

hyperperfusion syndrome. Nevertheless there was certainly no increase of events in the GSNO treated group.

In addition to the lower frequency of ES in the GSNO treated group the mean intensity of the individual ES was lower in the GSNO group. Theoretically embolic signal intensity would be expected to increase with increasing embolic size, and this has been confirmed in experimental models⁸⁷. ES intensity also depends on embolus composition with thrombi resulting in more intense ES than platelet aggregates in experimental models⁸⁷. Assuming embolus composition was similar in the GSNO treated and untreated group our results are consistent with emboli in the GSNO treated group representing smaller platelet aggregates. However there are a number of technical difficulties associated with interpreting ES composition or size from intensity alone⁸⁷.

This study demonstrates that ultrasonic ES detection allows the in-vivo efficacy of anti-platelet agents to be evaluated in relatively small numbers of patients. In the study the ability of GSNO to reduce embolisation from an arterial luminal surface surgically denuded of endothelium was tested.

The use of this model may allow effective initial evaluation of new anti-platelet therapies in small studies prior to their assessment in large and expensive clinical trials, and should also allow dose-response studies to be performed before embarking on such trials.

7.7.3 Carotid Angioplasty

As the numbers studied are small, we cannot draw any statistical inferences from our results.

7.8 Future Potential

It can be seen that combining TCD monitoring with administration of new therapeutic agents following carotid endarterectomy provides us with in-vivo models of embolisation in which new agents can be assessed. It is possible that carotid angioplasty would provide a further suitable model, but this work is still at an early stage, as discussed above.

By combining a knowledge of the molecular action of new anti-platelet agents with TCD monitoring we can gain further information as to the mechanism of production and composition of the embolic material.

This same work may be taken further with assessment of other agents, particularly new anti-platelet agents such as the glycoprotein IIb/IIIa antagonists to reduce embolisation in the same situations.

Chapter 8

Final Conclusion and Discussion

In Chapter 1, the purpose of this thesis was set out - to outline the importance and implications of embolisation in carotid artery stenosis and explore the use of transcranial Doppler ultrasound as a tool in such patients.

Detailed discussion is given at the end of each chapter - here I will present a broad overview and possible future directions for the work contained here.

Areas investigated were:

i.) **Chapter 4 - multi-gated Doppler.** The wider clinical application of TCD is hampered by the lack of an effective on- or off-line analysis programme such that lengthy recordings can be evaluated for ES counts with high specificity and sensitivity without the need for a trained human observer. The possible incorporation of multi-gated Doppler into such a system had been suggested. To address this possibility, we evaluated multi-gated Doppler in both a flow model and in patients. The results suggested that the technique is useful in differentiating ES from artefact - however, it loses sensitivity for low amplitude ES, such as those typically seen in patients with CAS - the patient group in which we are particularly interested.

Future Implications - two factors may be altered to improve the sensitivity of the technique. Firstly, narrower gate separation can be employed - these results influenced our own practice in subsequent studies. To improve the signal:background ratio, a lower frequency probe (e.g. 1MHz) can be employed.

ii) **Chapter 5 - identification of individuals at highest risk of stroke.** Previous studies have shown that embolisation, as assessed using TCD, correlates with a number of clinical markers of stroke risk in CAS, and one small prospective study had suggested an association between presence of ES and future stroke risk.

We have confirmed the association between ES and presence of plaque ulceration as detected angiographically. A significant correlation was shown between time since last symptom attributable to the stenosis and ES. In a series of over 100 CAS patients, we found further evidence of an association between ES and risk of future TIA and stroke both for asymptomatic and symptomatic CAS.

Future Implications - the results lend further support to the importance of ES presence as a predictor of stroke and need to be confirmed in large multi-centre studies. Ongoing trials are assessing risk stratification in asymptomatic CAS - TCD monitoring should be included in such trial protocols.

iii) **Chapter 6, Chapter 3 - recording protocols, variability and generalisability.**

Though much work had been done previously using TCD in CAS evaluation, the sum of the work done was less than all its parts - to be able to combine studies there have to be set basic criteria. Twenty-four hour recordings had shown that over prolonged recording times there was a great degree of variability, both inter- and intra-subject. To address these issues we have studied variability in greater detail, validated our own use of ES identification criteria and from this have generated our own recording protocols.

Future Implications - the work was important in providing recommendations for future work in ES detection in CAS. If utilised in future work by other groups, then

results may be combined and further collaborative work set up. International studies of ES as a possible long-term predictor of stroke will need to employ such generalisable criteria if coherent results are to be produced.

iv) **Chapter 7 - models of embolisation.** Having accepted that ES represent embolic material in the middle cerebral artery, then it is possible to speculate as to the nature of the material by assessing its response to pharmacological intervention. We have shown these ES to be frequent in both carotid endarterectomy and carotid angioplasty, and have assessed a new anti-platelet agent in the context of each of these situations. The work in carotid angioplasty is incomplete, but the data from carotid endarterectomy shows a striking reduction in ES numbers detected following endarterectomy when S-nitrosoglutathione is used. No change in ES numbers is seen when the agent is used during the dissection phase, which allows us to speculate further that the mechanism of embolisation may be different in this situation.

Future Implications - the work with carotid endarterectomy subjects can be taken further in the evaluation of other anti-platelet agents as they emerge - allowing such drugs to be piloted in small numbers of subjects. In the setting of angioplasty, GSNO is being evaluated in further patients. Long-term monitoring of the angioplastied lesion using Doppler ultrasound will also allow conclusions to be drawn regarding effects of treatment on restenosis rates.

In conclusion - we have addressed major issues in the evaluation and development of transcranial Doppler ultrasound. We have shown that the incorporation of multi-depth monitoring into automated ES detection systems may be

advantageous. The predictive value of ES in stroke and TIA risk has been strengthened, and a new in vivo model for the evaluation of anti-platelet and anti-thrombotic agents has been developed. Each of these developments provides a platform for further work in patients with CAS, in working towards improved decision-making in both those with symptomatic and asymptomatic disease.

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Appendix 1

Flow Cytometry

A1.1 Technical Background

Membrane changes accompanying platelet activation have been described in Chapter 5. In 1987, Shattil et al reported the use of dual-colour flow cytometry to develop an assay to detect activated platelets in whole blood, exploiting knowledge of these specific changes.

Flow cytometry is a technique for making measurements on particles or cells as they flow in a fluid stream one by one through a sensing point. Thus measurements are made on each particle within the suspension in turn, and values produced are not just average values for the whole population. The technique depends upon the sample being presented as a suspension of single particles, stained in a specific way, that will pass through the detection system without any disruption of fluid flow or blockage of orifices. It is possible to analyse whole cells, cell organelles or specific clumps of tissue in this way. Platelets are relatively simple to prepare as they exist normally as single particles within a suspension. The cells must be stained by incubation with a fluorescent dye or fluorescent-conjugated antibody or ligand. For accurate interpretation of results it is important that the staining is specific for and proportional to the feature to be measured.

Flow cytometers make measurements based on light as the excitation source. As cells are small and pass rapidly through the detection point, intense illumination is required. Cells are fluorophore-labelled - the light source must be capable of exciting the fluorescent dye by production of specific wavelengths. The scattered fluorescent

light generated by the passage of cells through the illuminating beam is collected by photodetectors which convert the photon pulses into electronic signals.

As the laser hits the particle suspension, the light is scattered, and is collected at both forward (FSC) and side (SSC) collection optics. The forward collection lens gathers scattered light from approximately 1-20 degrees off the laser beam axis. The light collected at this angle is dependent on particle size. The right angle (side collection) lens is designed to measure light over the greatest cone possible, and provides a measure of fluorescence intensity. Particles can be identified from the scatter plot produced as they will appear in a specific region according to their size and intensity.

The mixture of forward and side-scattered light will be of a particular range of wavelengths, depending on the fluorophore used to label the cells or particles. The two most commonly used fluorophores are fluorescein isothiocyanate (FITC, green) and phycoerythrin (PE, red). The absorption and emission spectra for these two fluorophores both overlap to some extent - this is discussed further below, and illustrated in **Figure A1.6**

The results obtained are most commonly displayed as the frequency histogram and the dual-parameter correlated plot or 'dot plot'. The frequency histogram is a direct graphical representation of the number of events occurring. The dot plot is a two-dimensional extension of the frequency histogram - with each detected cell represented by a dot at the coordinates of fluorescence intensity. Typical frequency histograms and dot plots accompany the stepwise description of the technique as used for our studies.

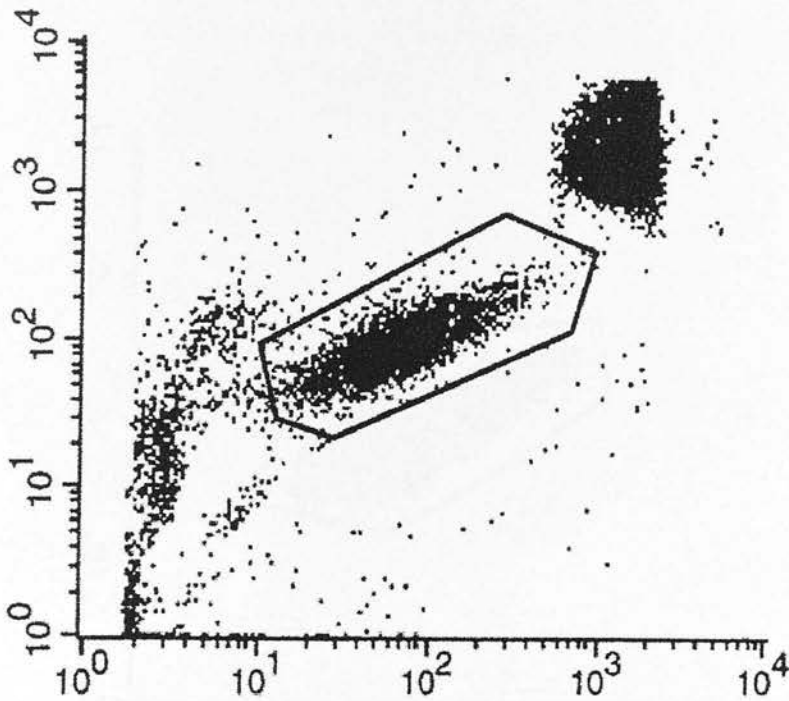


Figure A1.1. Dot-plot from an unstained sample - the platelet population is identified on the basis of forward and side-scatter characteristics alone.

A 'gate' is drawn around the 'region of interest' as shown above - this region can then be selected for the staining studies that follow. The x-axis shows the degree of forward scatter (FSC) while the y-axis represents the degree of side scatter (SSC). For our studies, FSC was set at E00 and SSC at 350.

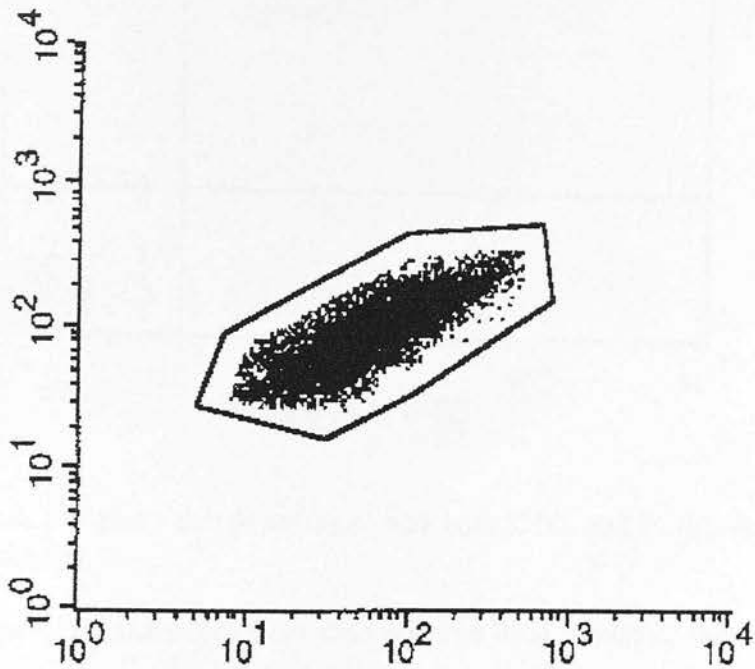


Figure A1.2. Dot-plot - unstained sample.

Having selected the region of interest, all cell counting events outwith this area are excluded from the analysis, leaving the plot as shown above. The cell counter is set to count 10000 events within this area. Axis labelling applies as for **Figure A1.1**.

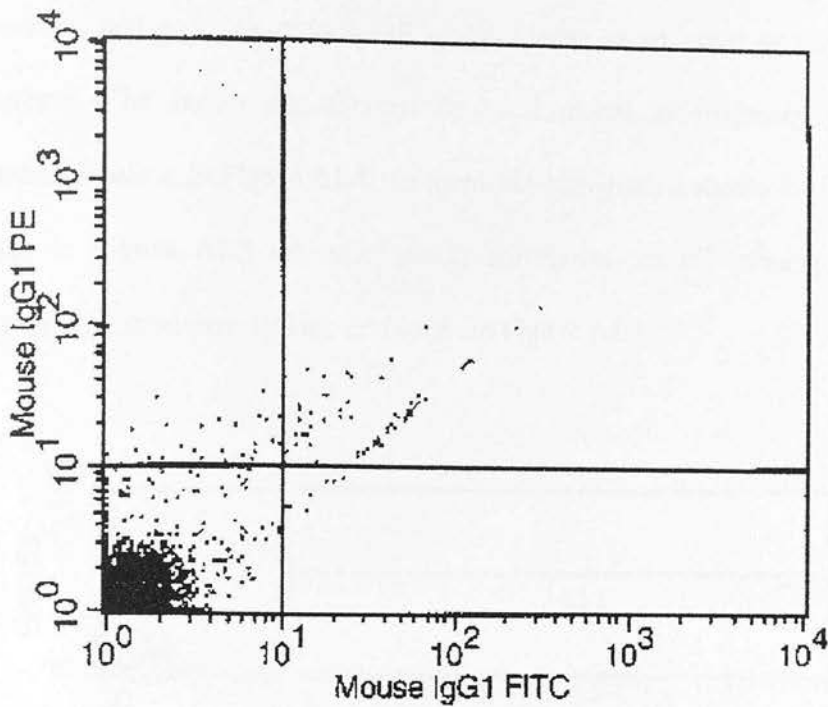


Figure A1.3. Dot-plot - sample incubated with both FITC- and PE-labelled negative control antibodies.

Some of the fluorescence detected will arise from 'unbound' fluorophore, i.e. be non-specific. To minimise the effect of this on results from specific staining, a preliminary sample may be studied using fluorophore bound to a negative control antibody. In the study described here, a sample was incubated with phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)- labelled antibody raised to a rat cell marker. FITC-derived fluorescence is detected at the FL1 detector, with that from PE detected at the FL2 detector. The detector sensitivity was then adjusted such that >98% of the cells within the region of interest were negative for either fluorophore, and hence appear in the lower left quadrant of the plot above. 'Positive' cells are those demonstrating $>10^1$ fluorescence at either the FL1 (FITC) or FL2 (PE) detectors. Therefore a singly positive PE-labelled cell would appear in the upper left

quadrant, a singly positive FITC-labelled cell would appear in the lower right quadrant, and a double-stained cell would appear as an event in the upper right quadrant. The results can alternatively be displayed as frequency histograms - illustrated below. In **Figure A1.4**, the frequency histogram is shown for FITC staining while in **Figure A1.5** the same results are shown for PE staining. Both these histograms accompany and are derived from **Figure A1.3**.

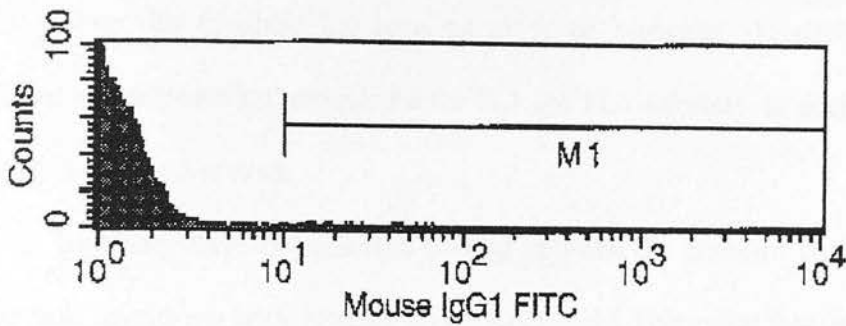


Figure A1.4. Frequency histogram for all FITC staining using the double-negative control. The number of cells 'positive' for FITC is <2% of the total 10 000 events (number of cells lying within the gate M1) as explained above.

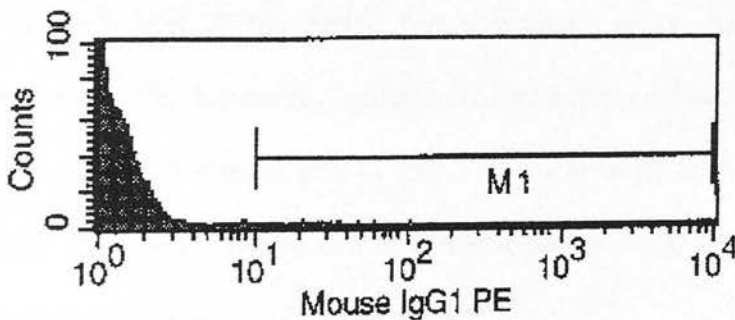


Figure A1.5. Similarly, the above histogram shows that using the PE-stained non-specific antibody <2% of the total cells studied are positive for PE.

Having set the detectors as above, the next step is to define which cells within the region of interest are truly platelet-derived and not other cell particles of similar FSC and SSC characteristics. To do this, a platelet-specific stain may be used - in this study, FITC-labelled anti-CD42b was employed - this being an antibody to platelet glycoprotein Ib (CD42b). The singly-stained FITC cells may then be studied by setting a threshold of $>10^1$ at the FL1 detector to define a platelet - this will be illustrated in **Figure A1.7**.

Once this threshold has been set to define a platelet, the next step is to determine 'compensation' settings for the FL1 and FL2 detectors, to minimise cross-talk between the detectors.

In reality there is significant overlap between the emission and absorption spectra of fluorescein isothiocyanate and phycoerythrin. This means that the emissions from the two fluorochromes will overlap such that some fluorescence from one fluorochrome will pass to the detector set to measure fluorescence from the other and vice versa. The effects of this may be minimised by optical filtration, or application of a bandpass filter. By this method light wavelengths greater than 560nm are reflected towards the PE fluorescence detector, through a filter centred at 578nm while shorter wavelengths 500-560nm pass to the FITC fluorescence detector through a 530nm filter. The spectral overlap may be corrected further during signal processing. This is illustrated in **Figure A1.6**, shown overleaf.

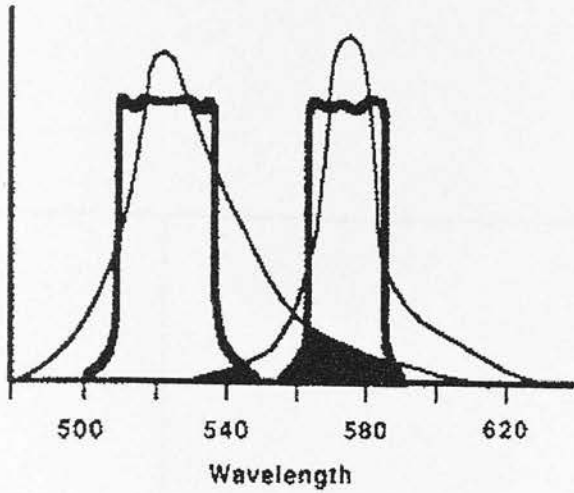


Figure A1.6. The fluorescence emission spectra for FITC (left) and PE (right) are shown above. The bandpass filters applied are outlined with the heavy black lines. It can be seen that some degree of overlap persists even after signal filtration. Wavelength is in nanometres.

Using a single-stained FITC-labelled sample, it is possible to adjust the compensation settings, to minimise the effects of the crosstalk between the two detectors due to the emission spectra overlap. To set compensation of FL2:FL1, the single-stain FITC-labelled antibody to human CD42b was used. The compensation is adjusted such that the majority of cells lie within the lower right quadrant. For our studies this was invariably set at 45%. The typical dot-plot obtained is shown on the following page, **Figure A1.7**.

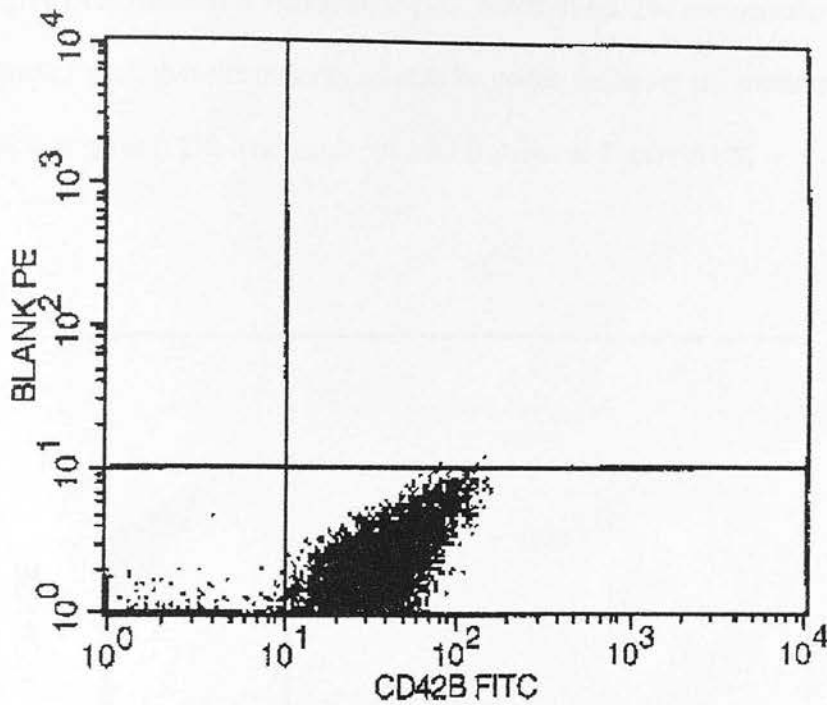


Figure A1.7. Dot-plot from single-stained FITC-labelled CD42b (platelet glycoprotein Ib).

As platelet glycoprotein Ib is not expressed on any other cell line, cells expressing this antigen are identified as platelets. With this knowledge it is possible to set a threshold to look at platelets alone within the region of interest, by only examining cells positive for FITC staining with this antibody. Cells, or 'events', in the lower right quadrant alone would therefore be included in the analysis, and again, 10000 events are counted per sample.

To set compensation settings for FL1:FL2, a single-stained PE-labelled sample is used. For our studies, we used a single-stained PE-labelled monoclonal antibody to CD41, or platelet glycoprotein IIb/IIIa. Again this is expressed on all platelets, but degree of expression is increased on platelet activation. The compensation settings are adjusted such that the majority of cells lie within the upper left quadrant - invariably this was set at 0.2%. The result obtained is shown in **Figure A1.8**.

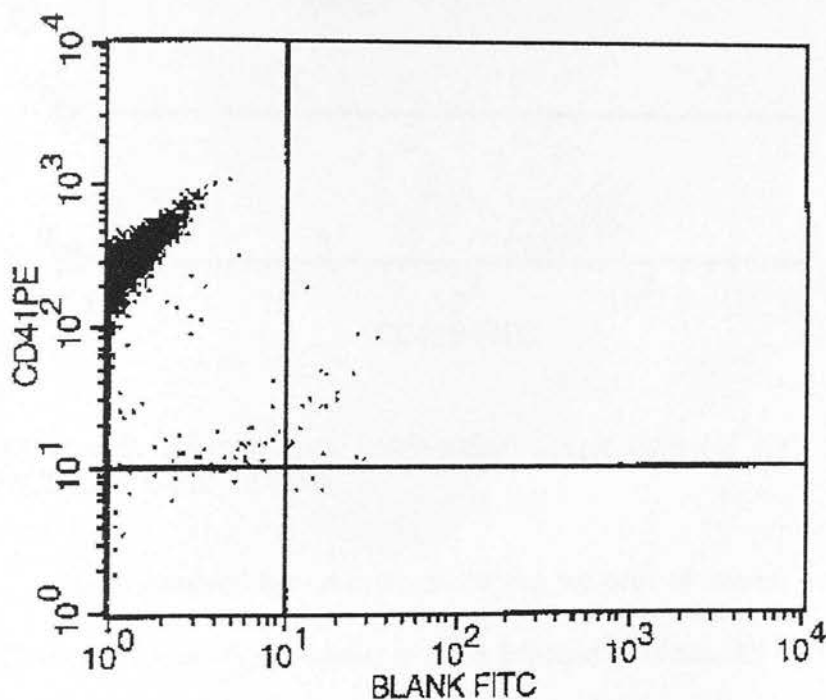


Figure A1.8. Dot-plot from single-stained PE-labelled CD41 (platelet glycoprotein IIb/IIIa)

Having set the threshold and compensation settings, it was then possible to examine the double stained populations to assess degree of platelet activation for each subject. The first double-stained sample examined was the FITC-labelled CD42b and PE-labelled CD41. The dot-plot obtained is shown in **Figure A1.9**.

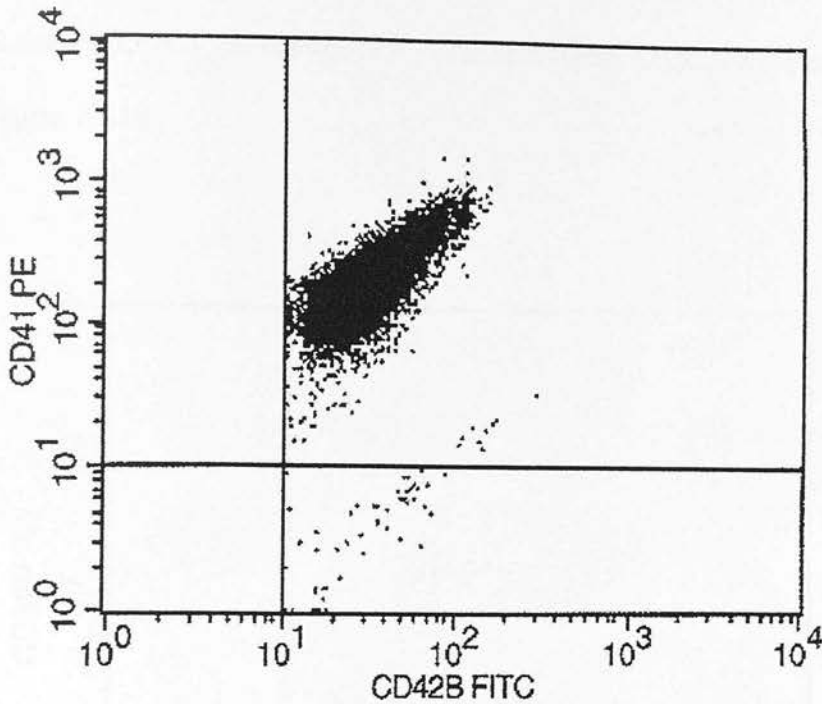


Figure A1.9. Dot-plot from double-stained sample incubated with FITC-labelled CD42b and PE-labelled CD41.

As a threshold has been set, identifying the cells of interest on the basis of FITC-positivity, no events appear in either left-hand quadrant. All cells in the upper right hand quadrant are taken to be positive for both FITC and PE, i.e. to express both CD42b and CD41. As CD41 is ubiquitous in platelets, we cannot take a percentage of cells expressing this marker as being the number activated - instead we must look at the degree to which they express the antigen - the geometric mean intensity (GMI, in arbitrary fluorescence units) is taken. In the result printout this is easily read from the upper right quadrant statistics for each sample run. The higher the GMI, the more activated platelets are present within the sample studied.

The second double-stained sample run uses FITC-labelled CD42b again to identify the platelet population, with the second, PE-labelled antibody used being directed at CD62P (platelet-surface P-selectin). Again, a typical dot-plot is illustrated, **Figure A1.10**.

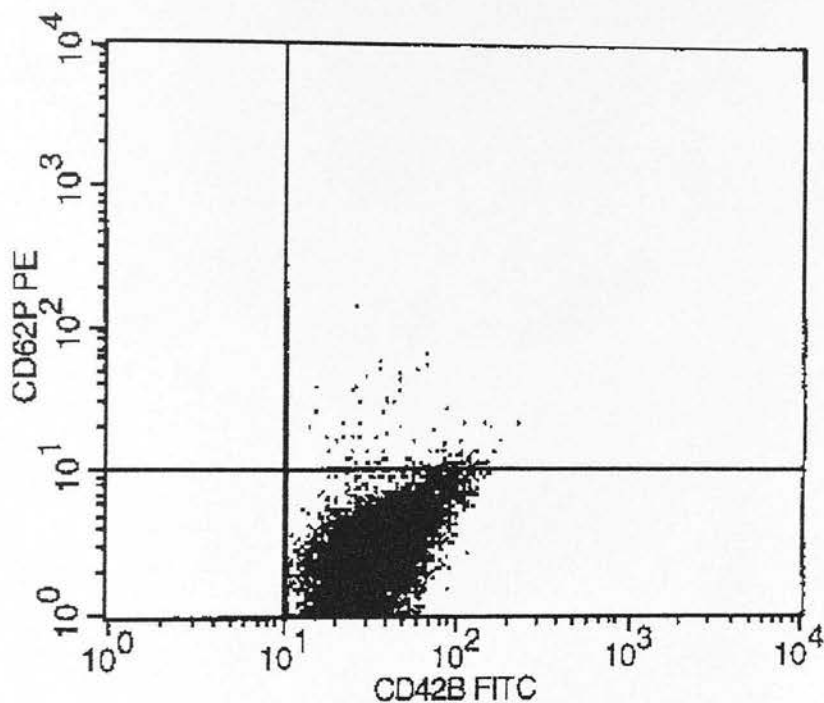


Figure A1.10. Dot-plot from double-stained sample incubated with FITC-labelled CD42b and PE-labelled CD62P.

P-selectin is usually contained within the alpha-granules of platelets, and is only expressed at the cell surface following activation and degranulation. Hence any platelet positive for P-selectin is assumed to be activated and therefore all events appearing in the upper right quadrant (i.e. double positive stained cells) represent activated platelets. The absolute numbers of positive cells can be taken to calculate

'percentage activated' for each sample studied as a percentage of the total of 10000 events.

The results reported in Chapter 5 therefore take GMI PE-CD41 and percentage of PE-CD62P for the samples studied to assess degree of platelet activation.

Appendix 2

Publications Arising Directly from this Work

i) **Papers** - copies of these follow in the order in which they are listed. All papers published by, and copyright permission obtained from, Lippincott Williams and Wilkins, Baltimore, MD.

Molloy J, Martin JF, Baskerville PA, Fraser SCA, Markus HS
S-nitrosoglutathione reduces the rate of embolization in man
Circulation 1998;98:1372-1375

Molloy J, Khan N, Markus HS
Temporal variability of asymptomatic embolization in carotid artery stenosis and optimal recording protocols
Stroke 1998;29:1129-1132

Markus HS, Molloy J
Use of a decibel threshold in detecting Doppler embolic signals.
Stroke 1997;28:692-695

Molloy J, Markus HS
Multigate Doppler ultrasound in the detection of emboli in a flow model, and embolic signals in patients.
Stroke 1996;27:1548-1552

ii) **Abstracts**

Molloy JE, Markus HS
Embolic signals predict risk of stroke and TIA in carotid artery stenosis.
Stroke 1999;30:238(38)(Abstract)

Molloy JE, Markus HS
Determinants of asymptomatic embolisation in carotid artery stenosis
Stroke 1998;29:2234(20)(Abstract)

Molloy J, Martin JF, Baskerville PA, Fraser SCA, Markus HS
Cerebral embolisation following endarterectomy is reduced by GSNO, a targeted nitric oxide donor
Cerebrovascular Diseases 1998;8(S4):17

Molloy J, Markus HS
What determines asymptomatic embolisation in carotid artery stenosis?
Cerebrovascular Diseases 1998;8(S4):36

Smith N, Molloy J, Bath P, Markus HS
Soluble P-selectin levels do not correlate with other markers of stroke risk in carotid artery disease

Cerebrovascular Diseases 1998;8(S4):50

Molloy J, Baskerville PA, Fraser SCA, Martin JF, Markus HS

S-nitrosoglutathione, a targeted NO donor, reduces cerebral embolisation in a new *in vivo* model

Stroke 1998;29(1):286(72)

Molloy J, Khan N, Markus HS

Temporal variability of asymptomatic embolisation, and optimal recording time, in carotid artery stenosis

European J Ultrasound 1997;5(S1):P64

Molloy J, Markus HS

Detection of embolic signals using a multi-gate approach:sensitivity and potential pitfalls in patient recording

*Cerebrovas. Dis.*1996;6 suppl 3:S224

Molloy J, Markus HS

Evaluation of multi-gate Doppler system in a flow model

*Cerebrovas. Dis.*1996;6 suppl 3:S253

S-Nitrosoglutathione Reduces the Rate of Embolization in Humans

Jane Molloy, MRCP; John F. Martin, FRCP; Paul A. Baskerville, FRCS;
Simon C.A. Fraser, FRCS; Hugh S. Markus, DM

Background—Antiplatelet agents presently used in the secondary prevention of cardiovascular disease fail to prevent the majority of cases of recurrent stroke and systemic embolization. An evaluation of the efficacy of new agents is hampered by a lack of *in vivo* models in humans. Asymptomatic cerebral embolic signals (ES) may be detected with the use of transcranial Doppler ultrasonography. These signals are particularly common after carotid endarterectomy, and this provides a situation in which new antiplatelet agents can be evaluated. With this model, we determined the effectiveness of S-nitrosoglutathione (GSNO), a nitric oxide donor with relative platelet specificity, in reducing cerebral embolization.

Methods and Results—Transcranial Doppler ultrasound recordings from the ipsilateral middle cerebral artery were made after carotid endarterectomy in 12 control patients and 12 patients receiving intravenous GSNO from the induction of anesthesia until 2 hours after skin closure. Recording times were 0.5 to 3.5, 6 to 7, and 24 to 25 hours after skin closure. The Doppler signal was recorded onto tape, and analysis for ES was performed, with the investigators blinded to treatment group. All patients received aspirin 300 mg/d before surgery and 5000 IU of heparin during surgery. The median (range) number of ES detected during the initial 3-hour postoperative recording was markedly reduced in the GSNO group compared with the control group: 7.5 (0 to 61) versus 38.5 (1 to 219) ($P=0.018$). This difference persisted until 6 hours after surgery.

Conclusions—Despite the administration of aspirin and heparin, frequent embolization occurred and was markedly reduced after the administration of GSNO. This demonstrates the potential use of platelet-specific nitric oxide donors in the treatment of thromboembolic disease. This model of cerebral embolism may allow determination of the effectiveness of new antiplatelet agents in humans. (*Circulation*. 1998;98:1372-1375.)

Key Words: ultrasonics ■ drugs ■ platelet aggregation inhibitors ■ endothelium-derived factors

Although aspirin is effective in the secondary prevention of thromboembolic disease,¹ many strokes and systemic embolic events occur despite its use, and there is a need for more effective antiplatelet agents. Potential agents include clopidogrel, the new generation of glycoprotein IIb/IIIa antagonists, and nitric oxide (NO) donors. Current methods available for the evaluation of such agents are not ideal; *ex vivo* studies such as platelet aggregation provide an indicator of potential efficacy but may not be completely representative of biological effectiveness *in vivo*.² Presently available animal models³ are not always representative of the situation in humans. Because of the low incidence of outcome events, large, expensive, multi-center clinical trials are required in which as many as 19 000 patients may need to be recruited.⁴ A reliable model in which to evaluate the efficacy of new agents *in vivo* in humans, with small patient numbers, would be useful in bridging the gap between laboratory studies and clinical trials.

Recently, it was demonstrated that circulating cerebral emboli can be detected with the use of transcranial Doppler

ultrasonography.⁵ Emboli appear as high-intensity transient signals with typical acoustic characteristics. This technique has been shown to be highly sensitive and specific in validation studies both *in vitro* and in animal models.^{6,7} Embolic signals (ES) have been reported in a wide variety of patient groups with potential embolic sources such as carotid artery disease, atrial fibrillation, and cardiac valvular disease.⁸ At carotid endarterectomy, endothelial denudation takes place, and the outer layers of the arterial media are exposed, resulting in a potent thrombogenic surface on which platelet adherence and aggregation occur. Asymptomatic ES are frequent after carotid endarterectomy,^{9,10} and recent studies demonstrate that a high frequency of ES during the early postoperative phase correlates with early stroke risk.^{11,12} This situation provides a potential model in which to test the efficacy of new antiplatelet agents. The frequency of ES in this situation may provide sufficient power to allow the evaluation of therapies in relatively small numbers of patients.

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Patient Characteristics in Control and GSNO-Treated Groups

	GSNO Group (n=12)	Control Group (n=12)	P (χ^2)
Sex, M/F	10/2	10/2	
Age, y (mean \pm SD)	66.3 \pm 8.8	62.3 \pm 8.6	0.54
Diabetes, n	3	0	0.18
Hypertension,* n	5	7	0.49
Current smoker, n	1	4	0.2
Hypercholesterolemia,† n	8	6	0.47

*Hypertension is defined as systolic pressure of >160 mm Hg or diastolic pressure of >90 mm Hg or antihypertensive treatment.

†Hypercholesterolemia is defined as a fasting cholesterol level of >6.0 mmol/L or as receiving treatment. All cases of diabetes were non-insulin-dependent diabetes mellitus.

In addition to its effects on vascular tone, NO inhibits platelet aggregation by stimulating soluble guanylate cyclase, thereby increasing cGMP,¹³ which leads to reduced platelet adhesion and aggregation.¹⁴ Organic nitrates, which act through the release of NO, reduce platelet deposition and thrombus formation after angioplasty in pigs but often at doses that cause hypotension.^{15,16} Similarly, in humans, organic nitrates induce hypotension at doses required for an antiplatelet effect.¹⁷ *S*-Nitrosoglutathione (GSNO) is a stable *S*-nitrosothiol from which NO is released by the action of enzymes associated with platelet membranes.¹⁸ In animals and humans, GSNO has significant antiplatelet action at doses that cause less hemodynamic effect than conventional NO donors.¹⁹ In a previous study, we demonstrated that platelet activation occurs after coronary angioplasty and that this activation can be prevented by the administration of GSNO.²⁰ GSNO has also been shown to inhibit platelet activation in the setting of acute myocardial infarction and unstable angina.²¹ In the present study, we examined the hypothesis that GSNO prevents platelet aggregation and adherence and therefore subsequent cerebral thromboembolism, as determined with Doppler ultrasound, immediately after carotid endarterectomy.

Methods

We studied 24 patients who were undergoing carotid endarterectomy for symptomatic internal carotid artery stenosis of $>70\%$ determined angiographically with the European Carotid Surgery Trial method of measurement.²² Their demographic characteristics are summarized in the Table. The study was approved by the King's Healthcare Ethical Committee, and informed consent was obtained from all subjects. All patients were pretreated with aspirin 300 mg/d for ≥ 1 week before surgery and were administered 5000 IU of sodium heparin intravenously during the operation and before carotid clamping. Patients were allocated either to treatment with GSNO (n=12) or to no additional treatment (n=12). In view of the potential side effects of GSNO (eg, hypotension and bleeding), neither the surgeon nor the anesthetist was blinded to treatment, but all data analysis was performed with the investigator blinded to the study group.

GSNO was administered as an intravenous infusion beginning at the induction of anesthesia at a rate of $2.2 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and, if tolerated, increasing to a rate of $4.4 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ at 10 minutes and continuing until 2 hours after skin closure. The criterion for termination of the infusion was a drop in mean arterial pressure (MAP) of ≥ 10 mm Hg. If MAP returned to the pre-GSNO level, an additional test infusion was administered and maintained at the lower

rate. If again there was a drop in MAP of ≥ 10 mm Hg, the infusion was stopped permanently.

A commercially available transcranial Doppler machine (TC 4040; EME/Nicolet Ltd) was used to record from the ipsilateral middle cerebral artery. A sample volume of 5 mm and a mean recording depth of 52.77 mm (range, 48 to 56 mm) were used. Recordings were made to digital audiotape for off-line analysis. In all patients, a 1-hour recording was made in the 24 hours preceding surgery. Postoperatively, recordings were made for 3 hours, beginning 30 minutes from skin closure, and for 1 hour at 6 and 24 hours after skin closure.

Analysis of recordings was performed by an observer (J.M.) who was blinded to the clinical details, time of recording, and study group. ES were identified by their characteristic visual appearance and chirping sound. An intensity threshold of >7 dB was used because previous studies have shown that this improves interobserver reproducibility in the detection of ES.^{23,24} The intensity of the ES was calculated by comparison with the built-in intensity scale on the Doppler as previously described.²⁴ Interobserver reproducibility of ES detection was determined by 2 observers who independently analyzed 4 hours of tape recording from 6 patients with symptomatic carotid artery stenosis. The proportion of specific agreement²³ between observer 1 and observer 2 (H.S.M.) was 0.98 and that between observer 2 and observer 1 was 0.92. For both validation and patient studies, the timing of all ES was noted, and each signal was saved to the hard drive for an analysis of intensity.

The number of ES in each group was not normally distributed, and therefore comparison between the number of ES detected in each group was performed with the Mann-Whitney *U* test for nonparametric data. For comparison of ES intensity, an unpaired *t* test was used.

Results

Details of the patient groups are given in the Table. All patients underwent technically successful carotid endarterectomy. Of the 12 patients allocated to receive GSNO, 10 tolerated the full dose with no clinically apparent side effects. In 2 patients, there was a fall in MAP of >10 mm Hg, and the infusion was suspended. The infusion was tolerated at the half-maximum dose in 1 patient, but in the second patient, even this infusion rate caused hypotension, and GSNO administration was stopped.

In the control group receiving no GSNO, the median (range) number of ES detected during the 3-hour postoperative recording period was 38.5 (1 to 219). At 6 hours, the median (range) number of ES per hour had fallen to 5.5 (0 to 105). By 24 hours, the median (range) number of ES per hour had fallen to 0 (0 to 30).

On an intention-to-treat analysis, there was a significant reduction in asymptomatic embolization in the GSNO group during both the initial 3-hour recording period and hour 6 (Figure 1). The median (range) number of ES detected in the first 3-hour recording was 7.5 (0 to 61) ($P=0.018$ versus controls). During hour 6, the median (range) number of ES was 0 (0 to 41) ($P=0.014$ versus controls). By 24 hours, the rate of embolization in both groups was low, and there was no difference between control and treatment groups: the median (range) was 0 (0 to 37) ($P=0.74$ versus controls).

Individual numbers of ES in the 2 groups during the first 3 hours are given in Figure 2. The 2 patients with frequent ES in the GSNO group were the 2 in whom a full dose of GSNO could not be given because of hypotension. Exclusion of both patients who did not tolerate a full dose increased the difference between the 2 groups: at 3 hours in the GSNO

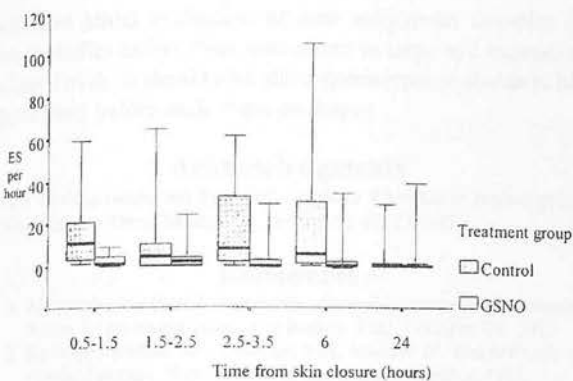


Figure 1. ES per hour versus time from skin closure according to treatment group. Solid line indicates median value; boxes, 25th to 75th percentile; and vertical lines, range.

group, the median (range) number of ES was 5.5 (0 to 20) ($P=0.005$ versus controls); at 6 hours, the median (range) was 0 (0 to 7) ($P=0.003$); and at 24 hours, the median (range) was 0 (0 to 3) ($P=0.47$).

A total of 715 ES were detected in the initial 3-hour postoperative recording in the control group compared with 197 in the GSNO group. During this period, ES in the GSNO group were significantly less intense than those in the control group: the mean (SD) was 12.30 (4.30) versus 14.27 (4.71) dB ($P<0.0001$).

In the control group, three patients experienced perioperative ischemic events. One patient had a stroke 20 hours after surgery in the ipsilateral internal carotid artery territory with right facial and arm weakness and dysphasia; this patient recovered fully over a 3-day period, and a CT brain scan showed a cortical infarct. Two additional control patients, both of whom had contralateral carotid occlusion, had strokes in the contralateral internal carotid artery territory. In 1 patient, aphasia and hemiparesis were noted on recovery from anesthesia, and he was left with a residual deficit; a CT scan showed a large area of infarction in the internal carotid artery watershed areas. The second patient developed left hemiparesis and coma 3 days after surgery and died; a brain CT scan

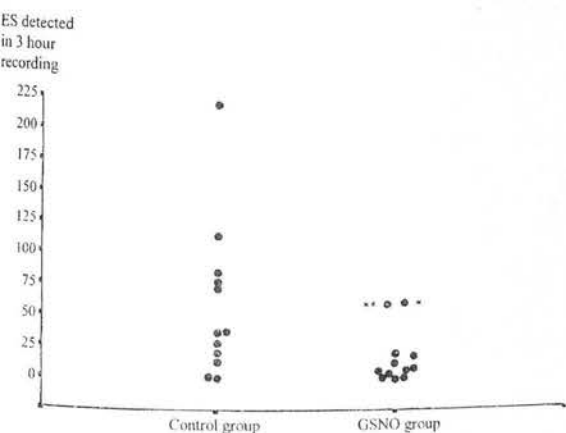


Figure 2. Scatter distribution of total ES detected in each patient during 3-hour postoperative recording according to treatment group. *Patient in whom infusion was stopped. *Patient receiving half-maximum dose.

showed an intracerebral hemorrhage. There were no strokes or transient ischemia attacks in the GSNO group, but 1 patient was noted to have developed internal carotid artery occlusion on the side of the endarterectomy on repeat carotid duplex before discharge.

Discussion

GSNO resulted in a highly significant reduction in the frequency of embolization after carotid endarterectomy. This reduction was maintained at 6 hours after surgery, even though the infusion was stopped 2 hours after surgery. In the majority of patients, GSNO was well tolerated, but in 2 individuals, it resulted in a drop in blood pressure. In 1 patient, it could be continued at half the maximum dose, but in the other, it had to be stopped. Our data provide further evidence for the importance of NO in preventing platelet adhesion and aggregation in vivo and illustrate the potential use of platelet-specific NO donors that may have relatively less hypotensive effect, for a given antiplatelet effect, than conventional NO donors such as nitroglycerin. GSNO has been administered only as an intravenous infusion, but future oral analogs, or other platelet-specific NO donors, may be effective in preventing thromboembolism. Our results also demonstrate that aspirin alone fails to prevent many embolic events.

The patient numbers were too small and the study was not designed to determine whether there was a significant reduction in clinical events. However, there were no clinical ischemic events in the GSNO group. In the control group, there was 1 minor stroke in the ipsilateral internal carotid territory and 2 strokes in the contralateral internal carotid artery territory. The latter occurred in patients with contralateral occlusion; in 1 patient, the pathogenesis was probably intraoperative hemodynamic ischemia, whereas in the other, it was hemorrhage, probably due to a hyperperfusion syndrome. Nevertheless, there certainly was no increase of events in the GSNO-treated group.

In addition to the lower frequency of ES in the GSNO-treated group, the mean intensity of the individual ES was lower in the GSNO group. Theoretically, ES intensity would be expected to increase with increasing embolic size, and this has been confirmed in experimental models.⁶ ES intensity also depends on embolus composition, with thrombi resulting in more intense ES than platelet aggregates in experimental models.⁶ Assuming the embolus compositions were similar in the GSNO-treated and untreated groups, our results are consistent with emboli in the GSNO-treated group representing smaller platelet aggregates. However, there are a number of technical difficulties associated with the interpretation of ES composition or size on the basis of intensity alone.⁶

The present study demonstrates that ultrasonic ES detection allows the in vivo efficacy of antiplatelet agents to be evaluated in relatively small numbers of patients. In this study, we tested the ability of GSNO to reduce embolization from an arterial luminal surface surgically denuded of endothelium. However, the same technology may allow the effectiveness of agents to be tested on embolism resulting from other clinical situations, such as percutaneous transluminal coronary angioplasty. The use of this model may allow

effective initial evaluation of new antiplatelet therapies in small studies before their assessment in large and expensive clinical trials. It should also allow dose-response studies to be performed before such trials are begun.

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Temporal Variability of Asymptomatic Embolization in Carotid Artery Stenosis and Optimal Recording Protocols

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Background and Purpose—Although asymptomatic embolization can be detected in patients with carotid artery stenosis, its temporal variability is unclear. An understanding of this is important in designing optimal recording protocols for future prospective studies of the predictive value of embolic signals (ES). We determined the effect of repeating and extending recording times in patients with symptomatic and asymptomatic carotid stenosis.

Methods—In 20 asymptomatic and 20 symptomatic subjects with >60% carotid stenosis, we used transcranial Doppler ultrasound to record for ES in the ipsilateral middle cerebral artery. Three 1-hour recordings were performed on three separate days, and on one occasion (not necessarily the first) the recording was extended to 2 hours. The recordings were saved onto digital tape for subsequent blinded analysis.

Results—Marked temporal variability was seen in symptomatic patients in whom the cumulative proportion of subjects with ES increased from 10 (50%) after a single hour of recording to 12 (60%) and 15 (75%) after two and three recordings, respectively. Extending the recording to 2 hours increased the yield of ES-positive patients from 6 (30%) to 8 (40%). In symptomatic patients there was excellent agreement between whether patients were positive for ES during each of two consecutive 1-hour recordings ($\kappa=0.78$, $P=0.0003$) but poor agreement between the results of two single-hour recordings performed on different days ($\kappa=0.22$, $P=0.27$). In asymptomatic patients, 4 (20%) were ES positive during the first hour; this increased to 5 (25%) after the recording was repeated once, with no further increase after the third recording. Extending the recording to 2 hours increased the yield from 3 (15%) to 7 (35%). In contrast to symptomatic stenoses, in patients with asymptomatic stenoses there was fair agreement between whether patients were ES positive on two consecutive 1-hour recordings ($\kappa=0.49$, $P=0.01$) or two single-hour recordings performed on different days ($\kappa=0.48$, $P=0.02$). Symptomatic subjects were more likely to have ES (when all 1-hour recordings were considered, 24/60 versus 10/60; $P=0.0046$). ES in symptomatic subjects had a higher relative intensity increase than in asymptomatic subjects ($P=0.01$).

Conclusions—The temporal variability of ES needs to be taken into account in the design of optimal recording protocols and comparisons of results from different studies. Extending the duration of recording beyond an hour in symptomatic stenoses is of less value, but repeating the recording on a different day will often identify additional subjects with ES. In intervention studies in symptomatic patients, the time since last symptoms must be considered. In asymptomatic stenosis, extending the duration of recording beyond an hour will increase the proportion of patients positive for ES. (*Stroke*. 1998;29:1129-1132.)

Key Words: carotid artery diseases ■ cerebral embolism ■ ultrasonography, Doppler

With the use of transcranial Doppler ultrasound (TCD), embolic signals (ES) can be detected in patients with carotid artery stenosis (CAS). Although in this group of patients the value of ES as a predictive factor in stroke is not established, their presence correlates with a number of independent markers of stroke risk. They are more frequent in symptomatic than in asymptomatic subjects,^{1,2} and in symptomatic patients they are more frequently detected soon after the appearance of symptoms.^{3,4} They are more frequent in patients with histologically proven plaque ulceration and thrombosis determined on carotid endarterectomy specimens⁵ and in patients with plaque ulceration demonstrated angiographically.⁶

Previous studies have reported very different proportions of patients with CAS in whom ES can be detected.

One reason for this may be the differing recording times used, which have ranged from 20 minutes to 2 hours.^{1,2,4,7,8} Furthermore, pilot studies have suggested a marked variability in the frequency of ES over time.^{2,9} Before larger prospective studies are performed, it is important to determine an optimum recording protocol. Both recording and subsequent data analysis are time consuming, increasing the importance of using a protocol that will maximize the possibility of ES detection without prolonging recording times unnecessarily.

Therefore, in this study we examined the incidence of ES in asymptomatic and symptomatic patients with CAS. We determined the effect on the proportion of patients in whom ES were detected by both repeating a recording and extending the recording time.

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TABLE 1. Characteristics of the Two Study Groups

Factor	Symptomatic	Asymptomatic
No.	20	20
Mean age (SD, range), y	66.75 (9.73, 42–82)	70.4 (8.41, 43–79)
Male sex	75%	65%
Hypertension	55%	70%
Diabetes	15%	20%
Hyperlipidemia	40%	30%
Ischemic heart disease	25%	25%
Peripheral vascular disease	20%	40%
Smoker	85%	70%
Positive family history	60%	60%
Aspirin treatment*	95%	95%
Warfarin treatment	...	5%

*For symptomatic group on aspirin, doses are as follows: 75 mg, n=9; 150 mg, n=4; 300 mg, n=6. For asymptomatic group: 75 mg, n=13; 150 mg, n=3; 300 mg, n=3.

Subjects and Methods

Twenty symptomatic and 20 asymptomatic patients with >60% CAS as determined by TCD criteria¹¹ were recruited and completed the study. Symptomatic patients were defined as having symptoms (amaurosis fugax, transient ischemic attack, or stroke) in the territory of the stenosed carotid artery within the last year. Patients with potential cardiogenic sources of emboli were excluded. Seven patients were considered but not recruited for the following reasons: 5 had no acoustic window, 1 underwent carotid endarterectomy before completion of the protocol, and 1 had a major stroke while awaiting surgery.

The demographic characteristics and treatment of the 40 patients are summarized in Table 1. For 39 of the 40 patients, antiplatelet and anticoagulant medication was left unaltered. The 40th patient suffered a gastrointestinal bleed, and aspirin was stopped between the first and second recordings. There was no difference in the degree of carotid stenosis between the two groups of patients: symptomatic, 60% to 79%, 5; symptomatic, 80% to 99%, 15; asymptomatic, 60% to 79%, 3; asymptomatic, 80% to 99%, 17 ($\chi^2=0.63$, $P=0.43$).

TCD recordings were made from the middle cerebral artery ipsilateral to the carotid stenosis by the transtemporal route. A commercially available TCD machine (EME Pioneer 4040) was used with a 2-MHz probe held in place with an external fixation device. Each patient was present on three separate occasions for a 1-hour recording, and on one occasion (but not always the first occasion) this was extended to a 2-hour recording. Mean (SD) time between recordings was 11.48 (10.09) days in the symptomatic group and 18.78 (10.09) days in the asymptomatic group. Mean (range) depth of insonation was 52.6 (48 to 56) mm. We aimed for an axial sample volume of 4 mm; when this delivered insufficient power for adequate recording, the sample volume was increased. Median (mean, range) sample volume was 5 mm (5.38, 4 to 12). Sample volume was kept constant for each patient for all three recordings. The Doppler audio signal was recorded onto digital audiotape. It was subsequently played back through the signal processor of the same TCD machine with the use of a 128-point fast Fourier transform and a fast Fourier transform overlap of >50%. All analyses were performed blinded to the clinical information or patient group. ES were identified by their typical visual appearance on the spectral display and their characteristic sound. In addition, an intensity threshold of ≥ 7 dB was used. The intensity was calculated from the color-coded intensity scale on the screen. This can be adjusted so that its intensity can be measured to the nearest decibel. The gain was reduced until the color of the adjacent cardiac cycle reached 0, and the peak intensity of the embolic signal was then determined. Interobserver reproducibility studies were performed for the two observers analyzing the tapes.

TABLE 2. Increase in Proportion of ES-positive Patients Resulting From Increasing the Recording Time From 15 Minutes to 2 Hours for Both Symptomatic and Asymptomatic Patients

Time From Start of Recording, min	No. of Positive Recordings (%)	
	Symptomatic	Asymptomatic
15	3 (15)	2 (10)
30	5 (25)	3 (15)
45	5 (25)	3 (15)
60	6 (30)	3 (15)
75	7 (35)	4 (20)
90	7 (35)	4 (20)
105	8 (40)	4 (20)
120	8 (40)	7 (35)

This was performed on a 2-hour recording comprising six 20-minute recordings from the ipsilateral middle cerebral artery in six patients with symptomatic carotid stenosis. The probability of agreement¹¹ of observer 2 compared with observer 1 was 0.93.

Statistical Analysis

All statistical analyses were performed on a PC with the use of SPSS for Windows and Genstat. The number of ES per tape was recorded, with a positive recording defined as one containing one or more ES. We evaluated the effects of repeating or prolonging recordings in two ways. First, we determined the cumulative yield resulting from extending and repeating the recordings. Second, we determined which recording protocol resulted in the greatest agreement between different recordings. For this analysis we treated the data as if they were two reproducibility studies: one with two consecutive 1-hour recordings (the 2-hour recording) and one with two nonconsecutive recordings (two 1-hour recordings repeated on different days). Kappa statistics¹² were calculated, and their 95% confidence intervals were derived with the use of the approximate standard error of the kappa statistic provided by SPSS. The agreement was considered excellent for $\kappa > 0.75$, fair for $\kappa = 0.4$ to 0.75, and poor for $\kappa < 0.4$.

The distribution of the number of ES or their intensity was not normally distributed, and therefore differences were analyzed with the use of nonparametric statistics. In the symptomatic group, the relationship between time from last symptoms and the number of ES per hour was determined with Spearman's correlation coefficient. To compare the number of ES per hour in the symptomatic and asymptomatic subjects, we attempted to fit a Poisson distribution, but because the variance was much greater than the mean, it was more appropriately fitted by a super Poisson distribution to allow for a dispersion parameter.¹³ We therefore applied the Wald test with the addition of a heterogeneity factor into the model to compensate for the variance.

Results

Effect of Repeating and Extending the Recording on the Cumulative Yield

For the purpose of this analysis, the first hour section of the 2-hour recording is treated as an individual 1-hour record. In this way, each patient effectively attended three separate occasions for 1-hour recordings. In symptomatic subjects, 10 (50%) were ES positive at the first examination. After two and three recordings, the cumulative proportion of ES-positive patients increased to 12 (60%) and 15 (75%), respectively. In asymptomatic subjects, 4 (20%) had a positive first recording. Single repetition of the recording gave a cumulative yield of 5 ES-positive patients (25%). A third recording provided no further increase in yield. As for symptomatic subjects, some subjects

A. SYMPTOMATIC CONSECUTIVE 1 HOUR RECORDINGS Kappa = 0.78, 95% CI 0.50-1.06, p=0.0003				B. ASYMPTOMATIC CONSECUTIVE 1 HOUR RECORDINGS Kappa = 0.49, 95% CI 0.11-0.87, p=0.01			
		2nd hour				2nd hour	
		ES +	ES -			ES +	ES -
1st hour	ES +	6	0	1st hour	ES +	3	0
	ES -	2	12		ES -	4	13
C. SYMPTOMATIC INCONSECUTIVE 1 HOUR RECORDINGS Kappa = 0.22, 95% CI 0.17-0.61, p=0.27				D. ASYMPTOMATIC INCONSECUTIVE 1 HOUR RECORDINGS Kappa = 0.48, 95% CI -0.02-0.98, p=0.02			
		2nd hour				2nd hour	
		ES +	ES -			ES +	ES -
1st hour	ES +	5	6	1st hour	ES +	2	2
	ES -	2	7		ES -	1	15
E. SYMPTOMATIC 2 HOUR RECORDINGS Kappa = 0.15, 95% CI -0.22-0.53, p=0.44				F. ASYMPTOMATIC 2 HOUR RECORDINGS Kappa = 0.53, 95% CI 0.14-0.93, p=0.14			
		inconsecutive				inconsecutive	
		ES +	ES -			ES +	ES -
consecutive	ES +	6	2	consecutive	ES +	4	3
	ES -	7	5		ES -	1	12

Figure 1. Evaluation of the agreement between different periods of recording. For each comparison, the 2x2 table documents whether ES were detected (ES+) or not detected (ES-) during each of the recording periods, which are then compared with the kappa statistic, which is shown with its 95% confidence intervals. Top, Agreement between the first hour and second hour of recordings of the consecutive 2-hour recording is shown for symptomatic (A) and asymptomatic (B) stenoses. Middle, Agreement between two nonconsecutive 1-hour recordings (on different days) is shown for symptomatic (C) and asymptomatic (D) stenoses. Bottom, Agreement between the consecutive 2-hour recording and the nonconsecutive 2-hour recording (made up of two single-hour recordings on different days) is shown (E, F).

who were ES positive on one recording were ES negative on the next recording.

The effect of extending the recording time (by 15-minute increments up to 2 hours) on the yield of ES-positive patients is shown in Table 2. In symptomatic patients during the 2-hour recording, 6 patients (30%) were ES positive by 1 hour; extending the recording for a further hour increased the yield to 8 (40%). In asymptomatic patients during the 2-hour recording, 3 patients (15%) were ES positive by 1 hour; extending the recording for an additional hour increased the yield to 7 (35%). The values for the yield in the first hour of the 2-hour recording are not necessarily the same as those for the first single-hour recording because the 2-hour recording was not always performed on the first occasion (see "Subjects and Methods").

Comparison Between the Different Recording Strategies

This analysis was performed by treating the data as a "reproducibility" study. The comparison between different recording strategies is presented in Figure 1 and assessed by the kappa statistic. The greater the κ value, the greater is the agreement between the two recordings, and therefore the less informative is the second recording. There was an excellent level of agreement between 2 consecutive hours of recording

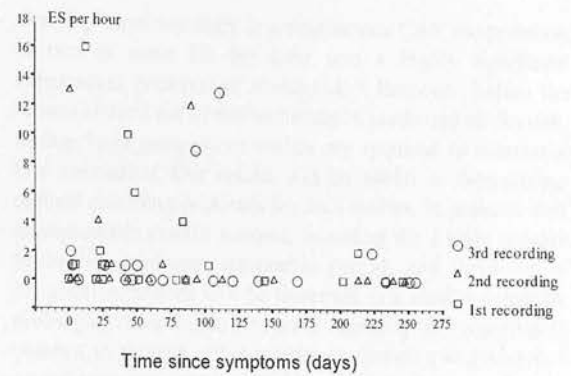


Figure 2. Relationship between time since last symptoms and number of ES per hour for the sixty 1-hour recordings in symptomatic patients. Data include the two 1-hour recordings and the first hour of the 2-hour recording.

in symptomatic subjects ($\kappa=0.78$, $P=0.0003$). In contrast, there was no significant agreement between two hour-long recordings performed on different days in symptomatic patients ($\kappa=0.22$, $P=0.27$). In contrast, in asymptomatic patients there was a fair level of agreement between two consecutive 1-hour recordings ($\kappa=0.49$, $P=0.01$) and between two hour-long recordings performed on different days ($\kappa=0.48$, $P=0.02$).

Using a similar method of analysis, we determined whether it is more useful to perform a single 2-hour recording or two 1-hour recordings on separate days. We compared which patients were ES positive during the consecutive 2-hour recording with those detected during the summed 2-hour recording made up of the two single-hour recordings performed on different days (Figure 1). For asymptomatic stenosis there was a fair level of agreement between the two methods of recording ($\kappa=0.53$, $P=0.01$), whereas for symptomatic recording there was poor agreement ($\kappa=0.15$, $P=0.44$).

Comparison Between Symptomatic and Asymptomatic Patients

When we considered all 1-hour recordings, a higher proportion of recordings with ES was found among symptomatic recordings (24/60 versus 10/60; $\chi^2=8.04$, $P=0.0046$). However, when we considered only those recordings in which ES were detected, there was no difference in the mean (variance) of the total number of ES in the two groups: symptomatic, 7.1 (136.3); asymptomatic, 3.6 (93.8) ($b=0$ to 0.09, $P<0.1$). However, there was a significant difference in the intensity of ES between the two groups, with median (mean, range) values as follows: symptomatic, 12 (13.73, 7 to 30) dB; asymptomatic, 11 (11.86, 7 to 30) dB ($P=0.01$, Mann-Whitney U test).

The relationship between the time since last symptoms and the number of ES per hour is shown in Figure 2. Recordings made on each of the first, second, and third occasions are shown separately. For the 2-hour recording, only the results from the first hour are shown. There was a negative relationship between time since symptoms and the number of ES per hour, which reached significance for two of the three recording periods: first recording, $\rho=-0.25$, $P=0.1$; second recording, $\rho=-0.42$, $P=0.03$; third recording, $\rho=-0.37$, $P=0.05$.

Discussion

This study demonstrates that the recording protocol is crucial in determining the proportion of patients in whom ES can be detected. In common with previous pilot studies,^{2,9} our results confirm the variability of asymptomatic embolization over time. By repeating recordings on three separate occasions, the proportion of ES-positive patients increased from 50% to 75% in symptomatic patients and 20% to 25% in asymptomatic patients.

If ES were detected in symptomatic stenosis patients during a 1-hour period, they were usually detectable in the same subjects during a second consecutive 1-hour recording, as reflected by excellent agreement between the two recordings ($\kappa=0.78$). Therefore, extending the recording from 1 to 2 hours in symptomatic patients is of limited benefit. In contrast, there was no significant agreement between which patients were positive for ES on 1 hour of recording compared with a nonconsecutive second hour separated by a number of days; the mean time between recordings was 11 days in the symptomatic group. These results demonstrate the variability of asymptomatic embolization in patients with symptomatic stenosis; they also demonstrate that although within a 2-hour period the process may be relatively constant, a few days later embolization status may well have changed.

In asymptomatic patients there was fair agreement between which patients were positive for ES during 1-hour recordings compared with those positive during a second consecutive hour ($\kappa=0.49$ versus $\kappa=0.78$ for symptomatic stenosis). This was a level of agreement similar to that seen between two nonconsecutive hours of recording separated by a few days ($\kappa=0.48$). This reflects in part the lower frequency of patients with asymptomatic stenosis in whom ES can be detected and demonstrates the usefulness of either increasing the recording time or repeating recordings in patients with asymptomatic stenosis.

We found a significantly higher proportion of recordings with ES in symptomatic than in asymptomatic patients, as reported previously.^{1,2} When we considered only those recordings in which ES were detected, there was no difference in the frequency of ES between asymptomatic and symptomatic patients. However, there was a highly significant difference between the intensity increase of ES in the two groups, with those in symptomatic recordings having a higher relative intensity. Although there are many technical difficulties in deriving information on embolus size from the intensity of the ES,¹⁴ this difference is consistent with emboli in symptomatic patients being larger or possibly of more echogenic material. In addition, our results confirm a significant inverse relationship between the number of ES per hour and time since last symptoms.^{3,4} It is important that this relationship be taken into account in any studies determining the predictive value of ES in patients with asymptomatic stenosis or the effect of any therapeutic intervention in this group of patients. Patients would need to be matched for time since last symptom.

Substantial indirect evidence suggests that ES in patients with carotid stenosis may be an important predictor of disease risk. They correlate with clinical parameters, as described in our study, and are also associated with plaque ulceration and degree of stenosis, which are both markers of increased stroke risk.^{5,6} In individual case reports they have responded to treatment with antiplatelet or anticoagulant therapy, while in

a small prospective study in asymptomatic CAS, the presence of two or more ES per hour was a highly significant independent predictor of stroke risk.¹⁵ However, before the routine clinical use of this technique in predicting stroke risk, further large prospective studies are required to determine this association. Our results will be useful in determining optimal recording protocols for such studies. In patients with asymptomatic carotid stenosis, recording for 1 hour appears to be the minimum reasonable period, and detection of ES-positive patients will be increased to a similar extent by prolonging recording to 2 hours or repeating the recording. In contrast, in patients with symptomatic disease a single hour of recording at one time is probably sufficient, but repeating the recording on a second occasion will identify additional subjects in whom embolization is occurring.

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Use of a Decibel Threshold in Detecting Doppler Embolic Signals

Hugh S. Markus, DM; Jane Molloy, MRCP

Background and Purpose To improve reproducibility and reliability in the identification of embolic signals detected with the use of Doppler ultrasound, many studies have used an intensity threshold. However, variable thresholds between 3 and 12 dB have been used, and often the method of measurement of intensity is not stated. Potentially different methods of measurement could result in different intensity measurements for the same embolic signal. We determined the effect of these differences using commercial transcranial Doppler systems.

Methods We analyzed 81 embolic signals recorded from the middle cerebral arteries of patients with carotid artery disease using three different methods of measuring intensity that had been previously used in research studies. In method 1 individual time frames of the frequency spectra were analyzed, in method 2 a color-coded intensity scale was used, and in method 3 automated software was used.

Results There was a highly significant correlation between measurements made by the different techniques (method 1 versus method 2: $r = .68$, $P < .0001$; method 1 versus method 3: $r = .66$, $P < .0001$; method 2 versus method 3: $r = .70$, $P < .0001$). However, the absolute values of intensity for the same embolic

signals varied markedly for the different methods. For example, a 4-dB threshold according to method 1 was equivalent to an approximately 7-dB threshold measured by method 2. These differences had major effects on the proportion of embolic signals detected with the use of the same decibel threshold but with intensity measured in the different ways. For example, using a threshold of 7 dB would result in only 4.9% of signals being missed by method 2 but 42.2% and 51.4% being missed by methods 1 and 3, respectively.

Conclusions Our results demonstrate that the intensities of the same embolic signals, recorded with the same parameters, are markedly different when analyzed in the different ways used in previous studies. This has important implications when a decibel threshold is used and emphasizes that criteria developed by one investigator on one machine cannot be used by another investigator without initial reevaluation. This could account for some of the differences in frequencies of embolic signals reported in previous clinical studies. (*Stroke*. 1997;28:692-695.)

Key Words • carotid stenosis • cerebral embolism • ultrasonics

Cerebral embolus detection with the use of Doppler ultrasound has many potential applications in the management of patients with cerebrovascular disease. In certain conditions, such as carotid artery stenosis, the presence of embolic signals appears to correlate with indicators of disease activity.^{1,2} However, initial studies have produced widely varying proportions of patients in whom embolic signals can be detected. For symptomatic carotid stenosis this has varied between 20% and more than 90%.³⁻⁷ Reproducibility studies within one center or between two centers have shown that good interobserver reproducibility can be obtained but there may be disagreement, particularly for signals of low intensity,⁷⁻⁹ and studies between larger groups of observers have resulted in less agreement. Many researchers in this field have found that often patients with clear embolic signals also exhibit very small increases in relative intensity accompanied by a characteristic sound, and deciding which to count as definite embolic signals can be difficult. The use of a decibel threshold is one way of resolving this problem. Only signals above a certain intensity will then be recorded as

embolic signals. This results in greater interobserver agreement since disagreement usually occurs for low-intensity signals.⁸ Many recent studies have included the use of a decibel threshold, which has varied from 3 to 12 dB.^{7,9-17} However, few of these state how the intensity was calculated. A number of studies have been performed in which an intensity threshold suggested by one investigator on one machine with certain settings is applied to either a different machine or to the same machine with different settings, without any reevaluation.

Intensity is calculated from the logarithm of the ratio of the power of the embolic signal to that of the Doppler spectrum in the absence of any embolic signal. However, each of these two parameters can be measured in a number of ways. We determined whether three different methods employed in clinical studies in which commercially available transcranial Doppler equipment was used resulted in significant differences in the analysis of embolic signals from patients with carotid artery disease. We specifically chose this patient group because the embolic signals in patients with carotid stenosis are of lower intensity than those detected in patients with prosthetic heart valves¹⁸ or during cardiopulmonary bypass and present greater diagnostic difficulty.

Subjects and Methods

Recordings were made from the middle cerebral artery ipsilateral to a symptomatic carotid stenosis in eight patients. Recordings were made on an EME TC 2000 transcranial Doppler machine with a 2-MHz transducer, a sample volume of 10 mm, and a depth of 45 to 52 mm. The recordings had

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previously been blindly evaluated by four observers from two centers in an interobserver reproducibility study.⁸ Eighty-one embolic signals that had been detected by all four observers were used for the subsequent analysis. One method (method 3 below) of determining the relative intensity increase of an embolic signal required that the embolic signal was detected with the use of an automated detection system. Even when a low detection threshold was used, 9 embolic signals were not detected with this method; therefore, only 72 are included in analyses in which this method is used.

The Doppler audio signal was recorded on digital audiotape before any fast Fourier transform processing and subsequently played back into two transcranial Doppler systems: (1) the same EME TC2000 and (2) an EME Pioneer 4/40 system. The intensity of each Doppler embolic signal was determined by three methods used in previously published studies, as described below.

Method 1

Individual time frames of the fast Fourier transform were analyzed on the TC2000 according to a previously described method.¹⁹ The maximum relative power amplitude (RPA) associated with the embolic signal was recorded. The background RPA in the absence of an embolic signal was measured from a Doppler spectrum of the previous or next cardiac cycle, at the same point in the cycle and at the same velocity. A mean of three background readings was taken. Intensity was then calculated from the following equation: Intensity Increase = $10 \log (\text{Maximum RPA of Embolic Signal/RPA in Absence of Embolic Signal})$.

Method 2

With the use of the EME Pioneer, the intensity was calculated from the color-coded intensity scale on the screen. This can be adjusted so that its intensity can be measured to the nearest decibel.²⁰ The gain was reduced until the color of the adjacent cardiac cycle reached 0, and the intensity of the embolic signal was then determined.

Method 3

With the use of the EME Pioneer, the intensity of each embolic signal was calculated with the automatic embolus software supplied with the machine. The algorithm determines the power of the embolic signal over the whole spectral line; similarly, the background power is calculated over the whole spectral line using a running average of background intensity over the preceding spectral lines.

Previously it has been suggested that an appropriate intensity threshold may be determined by measuring the intensity increase occurring with random episodes of Doppler "speckle"; these variations in intensity occur in the normal Doppler spectra and result from a number of factors, including nonuniformity of the ultrasound field and nonuniformity of the distribution of red blood cell scatterers. For this reason, the intensity increases associated with 200 episodes of random Doppler speckle using recordings from normal volunteers, made at the same depth, sample volume, and gain, were calculated with intensity measured as in methods 1 and 2. It was not possible to perform this analysis with the automated embolic signal detection software used in method 3.

Results

The relationship between embolic signal intensity measured by the different methods is demonstrated in Fig 1. There was a highly significant correlation between measurements made by the different techniques (method 1 versus method 2: $r = .68, P < .0001$; method 1 versus method 3: $r = .66, P < .0001$; method 2 versus method 3: $r = .70, P < .0001$). However, as can be seen in Fig 1, the absolute values of intensity for the same embolic signals

varied markedly for the different methods, with intensity usually being higher for method 2. A 4-dB threshold according to method 1 was equivalent to an approximately 7-dB threshold measured by method 2. We estimated the effect of these differences on the proportion of embolic signals detected using the same decibel threshold but with intensity measured in different ways (Table). The percentage of embolic signals detected varied markedly; for example, using a threshold of 7 dB would result in only 4.9% of signals being missed by method 2 but 42.2% and 51.4% being missed by methods 1 and 3, respectively. A similar difference was apparent when an appropriate threshold was determined from analysis of the relative intensity increase associated with episodes of Doppler speckle. The use of an upper limit of 2 SD above the mean resulted in a threshold of greater than 4 dB for method 1 and greater than 7 dB for method 2 (Fig 2).

Discussion

Our results demonstrate that the intensity of the same embolic signals, recorded with the use of the same parameters, can be markedly different when analyzed in different ways. This has important implications when a decibel threshold is used and emphasizes that criteria developed by one investigator on one machine cannot be used by another investigator without initial reevaluation. Such use can lead to great differences in the number of detected embolic signals. For example, the use of a 7-dB threshold measured by method 2 resulted in 95% detection of embolic signals, whereas the use of the same threshold with intensity measured with method 1 or 3 would result in approximately half of the embolic signals being missed. The figures may be even more discrepant if very-low-intensity embolic signals are used. We only studied embolic signals that four observers had agreed were present, and this by definition introduced a detection threshold. Furthermore, using an inappropriately low threshold for the method of measurement of intensity may lead to many episodes of Doppler speckle being inappropriately counted as embolic signals. This may account for the detection of "embolic signals" in 100% of patients in a recent study when a low-intensity threshold was used.²¹

The differences between the results obtained by the three methods may have a number of explanations. The power increase associated with the embolic signal can be measured in a number of ways. These include the peak increase at one velocity, the area under the power increase measured both across velocities and across time, or the power increase along the whole spectral line, which will include the power increase of the embolic signal and also of the background Doppler spectrum at other velocities. Similarly, the background power may be measured in a number of ways. The background power can be measured at the same velocity or at all velocities, at the same point of the cardiac cycle or averaging across the whole cardiac cycle, and only within the Doppler spectrum or along the whole spectral line. For example, the background intensity is higher in diastole than in systole, and therefore the position of the cardiac cycle will alter measurements. Similarly, if the whole spectral line is used, for technically poor recordings with artifactual extraspectral noise, the background

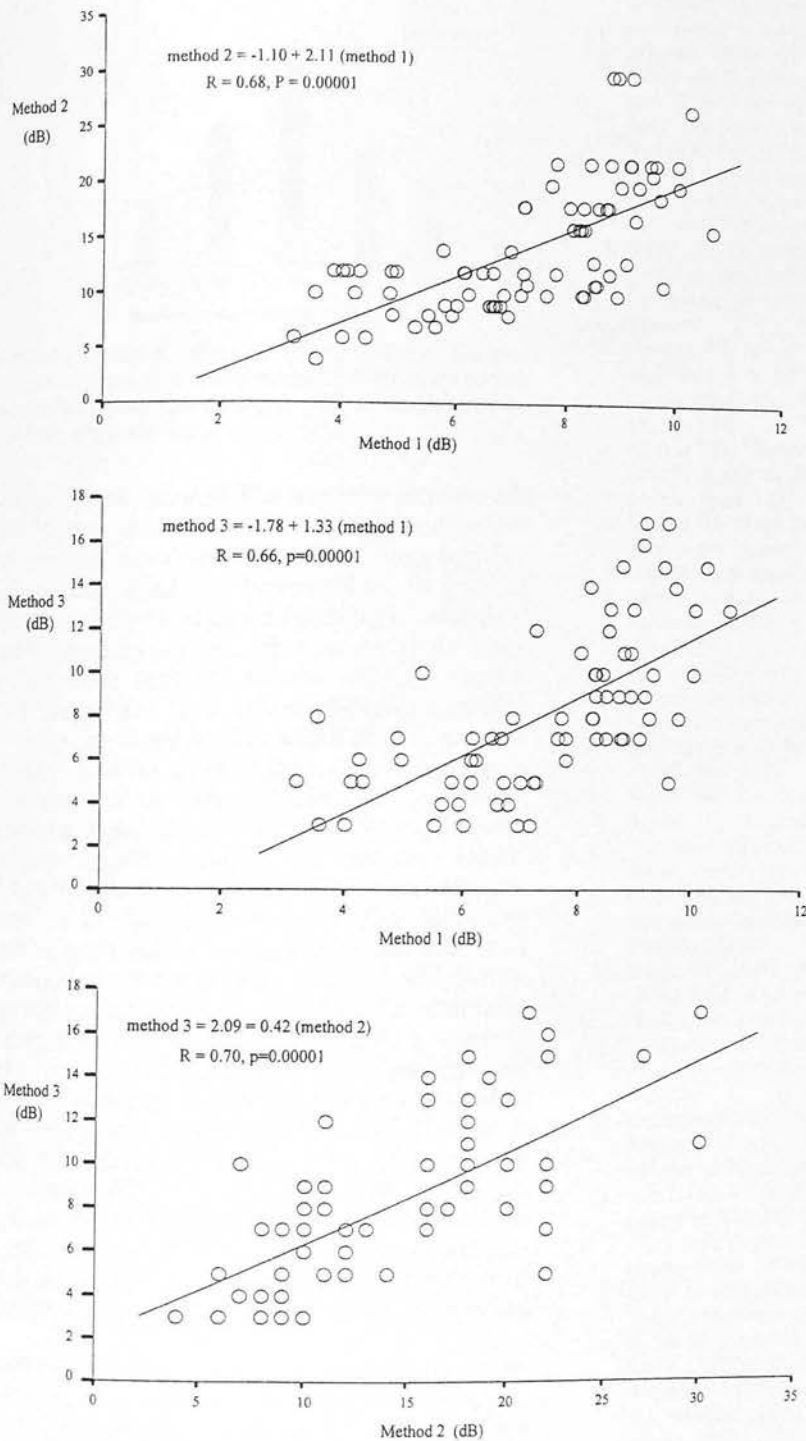


FIG 1. Correlation between the three different methods of measuring relative intensity increase.

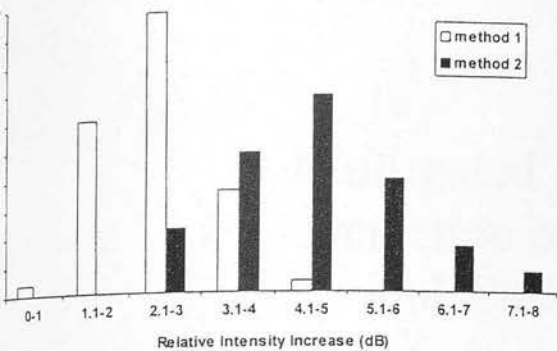
power will appear higher, resulting in a lower intensity increase of the embolic signal.

In this study we used the same recordings to measure intensity in three ways. In practice there are additional

problems with comparing intensity measurements from one study to another study. The intensity of an embolic signal will be highly dependent on the recording parameters used. For example, the shorter the length of the sample volume, the greater will be the ratio of the power of the embolic signal to the background spectra and therefore the higher the intensity of the embolic signal. The intensity may vary if the depth of insonation is varied because of differences in sample volume width for focused ultrasound beams at different depths. Differences may arise even with the same apparatus and settings if, for example, the middle cerebral artery lies in the center of the sample volume in one patient, but if it is not centrally insonated in another patient. Additional problems may arise if the degree of fast Fourier trans-

Effect of Method of Measurement of Intensity on Proportion of Embolic Signals Detected With Use of an Intensity Threshold

Method of Analysis	Proportion of Embolic Signals Detected Using Each Threshold, %			
	4 dB	5 dB	6 dB	7 dB
1	95.1	81.5	74.1	57.8
2	100	98.8	98.8	95.1
3	91.7	86.1	72.2	48.6



Relative intensity increase of 200 episodes of Doppler signals from middle cerebral artery recordings of normal control subjects measured by methods 1 and 2. The results obtained by method 2 are significantly higher.

overlap is inadequate.²² The intensity increase will be greater if the embolic signal is processed in the middle of one time window rather than falling between two adjacent time windows. Nevertheless, in practice these differences can be reduced by using standardized settings and recording parameters, as in this study. Some of these difficulties suggest that the use of a decibel threshold is inappropriate. However, different investigators have different thresholds at which they interpret a relative intensity increase as an embolic signal, and therefore we believe that an intensity threshold may aid in comparing data from different studies. We suggest that an approximate threshold can be determined by analyzing intensity increases resulting from random Doppler signals, as in this study, but that this should be determined by each center on its equipment and at its own settings, and the equipment setting should then be constant for subsequent recordings. An additional difficulty of ensuring the use of an appropriate intensity threshold is to analyze mixed data from patients and normal control subjects while blinded to the source of an individual recording. Embolic signal detection depends on features other than intensity increase alone, such as unidirectional velocity of the signal and the characteristic sound. Further technological advances such as multigate technique^{23,24} may allow more unambiguous detection of embolic signals and determination of whether low-intensity signals result from circulating emboli.

The marked difference in intensity measurements with the use of commercially available transcranial Doppler techniques has important implications for clinical studies in embolic signal detection. If an intensity threshold is to be established, this should be determined by each center on its own equipment. Wherever possible, all recordings should then be made at a similar depth with a similar sample volume and settings. These findings also have important implications if intensity measurements are to be used to gain information about the composition of the underlying embolic material.

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Multigated Doppler Ultrasound in the Detection of Emboli in a Flow Model and Embolic Signals in Patients

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Background and Purpose The ability to detect asymptomatic circulating cerebral emboli may contribute to the management of patients with stroke, but its clinical usefulness will depend on effective systems for automatically detecting embolic signals (ES) and differentiating them from artifact. A new method involves the use of a multidepth probe that allows recording from both distal and proximal sample volumes along the same vessel. Theoretically, an embolus should appear sequentially, with a time delay, between the two channels, whereas an artifact should appear simultaneously in the two channels.

Methods We evaluated this method in an in vitro flow model and in patients. In an in vitro model, with a flow pattern mimicking intracerebral flow, 181 air bubbles and 193 thrombus emboli were compared with the signals resulting from 368 episodes of artifact; a sample volume of 5 mm and a channel separation of 10 mm were used. ES from two groups of patients—those with carotid artery stenosis (141 ES) and those with mechanical prosthetic cardiac valves (125 ES)—were studied and compared with 222 episodes of artifact produced in the same patients.

Results In the model the mean (SD) time delay was 17.32 (9.94) ms for air emboli and 17.78 (10.66) ms for thrombus emboli compared with -0.01 (0.39) ms for artifact (air and thrombus emboli versus artifact, $P < .0001$). A sensitivity of 100% and specificity of 100% were obtained when a cutoff of >2 ms was used for an embolus. The method allowed equally good detection of those air emboli that resulted in receiver overload and aliasing.

In patients the mean (SD) time delay was 29.6 (28.2) ms for valve ES and 14.9 (15.42) for carotid ES compared with 0.00 (0.46) for artifact (carotid and valve ES versus artifact, $P < .0001$). Considering only those signals that were visible in both Doppler time domains resulted in a sensitivity for valve ES of 98.9% and for carotid ES of 94.0%, with a specificity of 99.0%. However, in one patient in the valve group some ES were visible only in the proximal channel, possibly because of passage of emboli down branch vessels between the two sample volumes. In addition, for the less intense carotid ES some signals were unclear or absent in one or both of the time domain signals at either depth, although visible in the post-fast Fourier transform spectra. Including those ES visible in only one channel reduced the sensitivity to 75.2% for valve ES and 92.6% for carotid ES.

Conclusions The multigated technique offers a new method of detecting ES and differentiating them from artifact and is the first reliable method for differentiating intense ES resulting in receiver overload from artifact. Occasionally its sensitivity is reduced when ES do not appear in the distal channel, probably because they pass down a side branch; this may be reduced by reducing gate separation. Some less intense carotid ES can be difficult to detect if the amplitude increase is small compared with the amplitude of the background Doppler signal. (*Stroke*. 1996;27:1548-1552.)

Key Words • carotid artery diseases • cerebral embolism • heart valve prosthesis • ultrasonics

Stroke is the third leading cause of death and a major cause of disability. Cerebral embolism is the underlying pathogenic mechanism in many cases of stroke. Emboli may arise from the heart, carotid plaques, aortic plaques, intracranial atherosclerotic stenoses, or from systemic venous thrombosis in the presence of a venous to arterial shunt. The ability to detect asymptomatic circulating cerebral emboli offers important potential advances in the localization of an actively embolizing source, the selection of high-risk patients for appropriate treatment, monitoring of the effectiveness of anticoagulant and antiplatelet therapy, and perioperative monitoring. Embolic signals (ES) appear as a unidirectional frequency-focused intensity increase, usually within the background spectral pattern and occurring at random within the cardiac cycle, accompanied by a characteristic harmonic sound. The technique has been demonstrated to

be highly sensitive and specific in both in vitro¹ and animal^{2,3} models. ES have been detected in patients with a variety of embolic sources, including carotid stenosis, atrial fibrillation, and prosthetic cardiac valves,^{4,6} and during and after operative procedures such as carotid endarterectomy⁷ and carotid angioplasty.⁸ One of the major factors hindering the wider clinical application of the technique is the time taken to analyze off-line recordings from patients. Although the optimal recording time has not yet been defined for each separate patient subgroup, recordings are normally made for a minimum of 30 minutes, with much longer periods being used by some groups. If embolic detection with transcranial Doppler ultrasonography is to become clinically useful, a reliable automated detection system is required. Such a technique should be both highly specific and sensitive for ES and be able to differentiate these from both patient and probe artifact. Previous investigators have used a computer algorithm to identify the characteristic bell-shaped relative intensity increase occurring with an ES and to differentiate this from the characteristic bidirectional intensity increase seen with an artifact. High sensitivity and specificity were achieved in an off-line system,⁹ but results with on-line systems have not yet been as good.¹⁰ An alternative approach is to train a neural network; such a

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system has been shown to identify ES on-line with a high specificity, but its sensitivity is only in the order of 70%.¹¹ Unfortunately, artifact may also occasionally produce embolus-like signals and therefore cannot always be differentiated on the basis of intensity, duration, and directionality of the signal alone. Furthermore, in cases in which ES cause receiver overload, aliasing occurs and bidirectional signals may be produced; these cannot be differentiated from artifact on the basis of previous embolus-defining criteria.

A very promising method that involves the use of multigated Doppler ultrasound has recently been described by Aaslid. Since an embolus is in motion in the direction of blood flow within the vessel being studied, if recordings are made at two depths along the vessel length, there should be a time delay between the ES seen at the distal depth and that seen more proximally. In contrast, signals produced by external interference would be expected to be seen simultaneously in both channels. Results with such a system have been recently reported¹²; however, this study used the post-fast Fourier transform (FFT) spectra from which to calculate the time delay, and even better results would be expected when the time domain data with their much higher temporal resolution are used. Furthermore, the *in vitro* validation in this study¹² used air emboli, and there has been no validation when solid or formed emboli, such as thrombus, are used, which result in less intense signals and have been more difficult to detect in previous automated systems.

In this study we initially evaluated a multigated Doppler ultrasound system in an *in vitro* flow model using both thrombus and air emboli, and we then applied it to a group of patients with potential embolic sources.

Subjects and Methods

In all studies the same transcranial pulsed Doppler machine was used (Pioneer 4040, EME Ltd) with a multidepth 2-MHz transducer. The Doppler signal was saved with software that allows the pre-FFT time domain Doppler signal, the post-FFT spectra, and the audio signal to be stored on the computer hard disk and replayed. The initial increase in amplitude at the time of arrival of an embolus or artifact in each of the two channels was measured from the time domain data, allowing a time resolution of 1 ms or greater.

In Vitro Studies

We constructed a flow circuit using polyethylene infusion tubing (4 mm ID, Codan Limited), driven by a programmable flow pump (University Developmental Cooperation Flow System) and filled with a proprietary blood analogue, a suspension of nylon filaments in machine oil that has been previously validated to have scatter properties similar to those of blood (Elf Atochem).¹³ A waveform similar to that normally obtained from the middle cerebral artery (MCA) with relative preservation of diastolic flow was obtained; mean systolic flow velocity was 40 cm/s (peak, 95 cm/s; diastolic, 30 cm/s). Air was removed from the circuit through a built-in reservoir tank. A length of the tubing was fixed in a water bath and resonated at an incident angle of 35°, such that there was a comparable waveform with equal gain settings at each depth. Channel 1 was set at a depth of 52 mm with the following parameters: sample size, 5 mm; power, 25% (100% power is equivalent to 675 mW/m²); and gain, 8. Channel 2 was set at a depth of 42 mm at the same settings of sample size, power, and gain.

We introduced emboli of two types through a transparent side-arm device, allowing visualization of any accidentally introduced air: 181 air bubbles were introduced with a 1-mL syringe to produce bubbles of 0.01 mL in volume (diameter, 0.64 mm); 193

thrombus emboli were prepared from fresh human blood that had been allowed to clot, cut in cuboid shapes with a maximum dimension of 0.5, and then suspended in normal saline. In addition, 368 episodes of artifact were produced; 187 of these were produced by tapping the probe and 181 by tapping the tubing to mimic patient artifact.

Patient Studies

The aim of the patient studies was twofold: (1) to determine in what proportion of patients we could successfully insonate the MCA with satisfactory gate separation and (2) to determine the sensitivity and specificity of the method in detecting ES and differentiating them from artifact. ES in patients with mechanical prosthetic cardiac valves are more intense than those in patients with carotid stenosis¹⁴ and have been easier to detect by previous automated systems; therefore, we evaluated the method in both patient groups separately. In the carotid group we included ES recorded during the recovery phases after carotid endarterectomy and carotid angioplasty; these ES have an intensity similar to those recorded in symptomatic carotid stenosis.⁸

Insonation of 35 MCAs in 24 patients was attempted (16 men, 8 women; mean [SD, range] age, 66.5 years [10.39, 50 to 86]). Of these patients, 14 had a known potential source of emboli. Six patients with metallic prosthetic cardiac valves were monitored bilaterally for 20 minutes. Eight patients with symptomatic carotid stenosis (mean stenosis, 84.4%; range, 60% to 95%) were monitored ipsilateral to the stenosis for 1 hour. Four patients later underwent carotid endarterectomy, and 1 patient underwent carotid angioplasty. In these patients more extensive postoperative recordings were made, for a total of 300 minutes per patient. All these recordings were made following the procedure, either after catheter removal for percutaneous transluminal angioplasty or in the recovery room for endarterectomy, and no recordings were made during the procedures when ES could represent air bubbles. In the additional 10 subjects recruited from hospital inpatients, monitoring was continued only for the time it took to obtain a satisfactory signal; this was to determine whether adequate gate separation could be obtained.

Insonation was achieved by the transtemporal route, with the use of a 2-MHz probe held in place with an external fixation device. Once the MCA was identified, the depth in the two channels was adjusted, and the axial sample volume width was reduced while satisfactory visualization of the Doppler spectra within the two samples was maintained. A standard protocol was used in siting the position of the two sample volumes; we aimed for a sample volume of 5 mm and a distance between the center points of the sample volumes (ie, between the two depths) of 10 mm. Episodes of artifact were also recorded for off-line analysis as above. These were produced by requesting that the patient cough, speak, or swallow and by tapping or moving the probe, probe holder, or headgear.

Signal Analysis

For all ES, maximum relative intensity increase was determined. The intensity was taken from the intensity color-coded spectral display. The background intensity was calculated from a similar point in the preceding or following cardiac cycle. The pre-FFT time domain data from the two channels were used to determine whether the intensity increase was present in both channels; if so, the time delay in the onset of the intensity increase in the two channels was measured. Comparisons between groups were made with the use of Student's *t* test for unpaired data.

Results

In Vitro Studies

All 374 emboli were detected as high-intensity signals. Air emboli resulted in more intense signals than thrombus emboli (mean [SD, range] for air emboli, 31.5 dB [11.8, 11 to 39] versus 22.5 dB [4.8, 11 to 39] for thrombus emboli; $P < .0001$). Forty-three air emboli but no thrombus emboli resulted in receiver overload and a bidirectional signal, as previously reported; these were included in the analysis. All

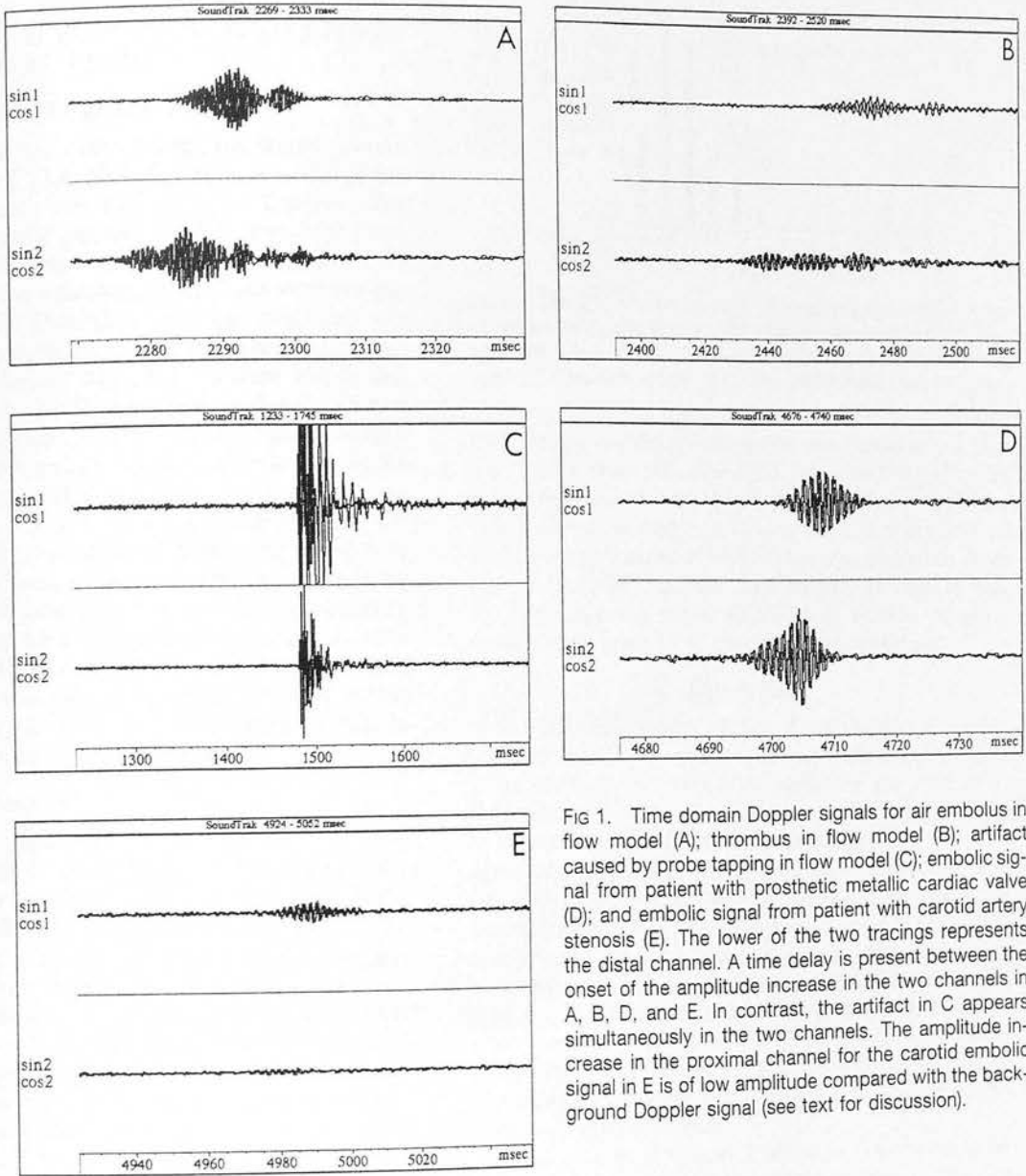


Fig 1. Time domain Doppler signals for air embolus in flow model (A); thrombus in flow model (B); artifact caused by probe tapping in flow model (C); embolic signal from patient with prosthetic metallic cardiac valve stenosis (D); and embolic signal from patient with carotid artery stenosis (E). The lower of the two tracings represents the distal channel. A time delay is present between the onset of the amplitude increase in the two channels in A, B, D, and E. In contrast, the artifact in C appears simultaneously in the two channels. The amplitude increase in the proximal channel for the carotid embolic signal in E is of low amplitude compared with the background Doppler signal (see text for discussion).

emboli were detected in both channels (Fig 1A and 1B), in all cases first in the more proximal channel, with a mean (SD, range) time delay between the two channels of 17.56 ms (10.31, 3 to 53). The mean (SD, range) delay was 17.32 ms (9.94, 3 to 53) for air emboli and 17.78 ms (10.66, 4 to 50) for thrombus emboli. In contrast, artifact appeared simultaneously or near simultaneously in the two channels (Fig 1C),

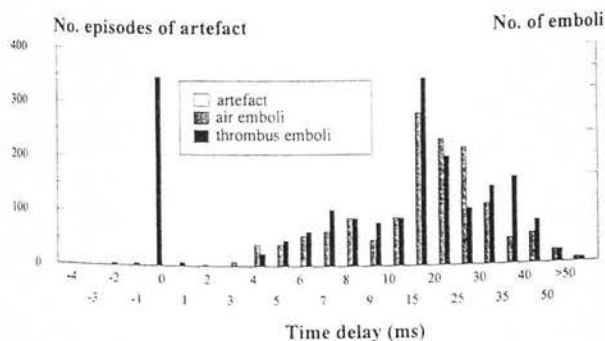


Fig 2. Frequency histogram of the time delay between the onset of the amplitude increase in the two channels for air and thrombus emboli and artifact in the flow model.

with a mean (SD, range) time delay of -0.01 ms (0.39, -4 to 2; $P < .0001$ versus emboli, t test).

Time delays for air and thrombus emboli and artifact are shown in Fig 2. When we specified a cutoff time delay of more than 2 ms to define a signal as an embolus, the method could detect emboli and differentiate them from artifact with a sensitivity of 100% and a specificity of 100%; this was similarly good when either air emboli alone or thrombus emboli alone were considered. These values include the 43 air emboli that resulted in receiver overload and aliasing; for these cases analyzed as a separate group, mean (SD, range) time delay between gates was 19.81 ms (9.61, 9 to 53).

There was a highly significant relationship between the velocity at which the ES intensity increase occurred and the time delay between the two channels: air emboli, $r = -.77$, $P < .0001$; thrombus, $r = -.74$, $P < .0001$.

Patient Studies

Successful insonation of the MCA was possible in 33 of 35 arteries (94.3%). In the two failed cases this was due to unilateral absence of an acoustic temporal window. In all MCAs that could be insonated, it was possible to record

at two depths, with a mean (SD, range) sample volume of 4.96 mm (0.20, 4 to 5) and mean (SD, range) gate separation of 4.71 mm (1.85, 1 to 9).

Prosthetic Mechanical Cardiac Valves

We recorded 125 ES, with a mean (SD, range) relative intensity increase of 31.1 dB (7.7, 10 to 55). The mean (SD, range) time delay was 29.6 ms (28.2, 2 to 122). For 30 ES, all in the same patient, the ES were heard and detected in both the time domain data and the post-FFT spectral display in the proximal channel but were not audible or visible in the distal channel, suggesting that some emboli may have passed into a branching artery between the two sample volumes. All other ES were visible and audible in both channels. The mean time delay for ES was significantly longer than that for the 222 episodes of artifact, which had a mean (SD, range) time delay of 0.0 ms (0.46, -2 to 3; $P < .0001$). Time delays for the ES and artifact are shown in Fig 3. When we included only the ES detected in both channels and used a threshold of a time delay of 2 ms between the two depths as the defining criterion for ES, the sensitivity was 98.9% and specificity 99.0%; however, if the ES visible in only one channel were also included, the sensitivity fell to 75.2%. On the whole, the ES in patients with cardiac valves were clearly visible as a large amplitude increase in the time domain data, in contrast to some of the carotid ES (Fig 1D).

Carotid Artery Stenosis

We recorded 141 ES, with a mean (SD, range) relative intensity increase of 19.3 dB (5.9, 5 to 33). No ES were visible and audible in the proximal channel but not in the distal channel, unlike the valve ES. However, two ES audible and visible in the post-FFT spectra in both channels were identifiable in the time domain data of only one channel; one ES was detected only in the proximal but not the distal time domain data, while one ES was clearly audible and visible in the proximal channel post-FFT spectral display but not visible in the time domain data at that depth. This appeared to be a reflection of the fact that the amplitude increase in the time domain data was small for many ES and frequently difficult to distinguish from the background Doppler signal; a typical example is shown in Fig 1E. The ratio of the maximum amplitude increase to the background Doppler signal amplitude in the time domain data was small for many carotid ES, and the mean value was significantly smaller than that for valve ES (5.1 [2.5] versus 13.2 [5.7]; $P < .0001$); individual values are shown in Fig 4. For ES identifiable in both channels, the mean

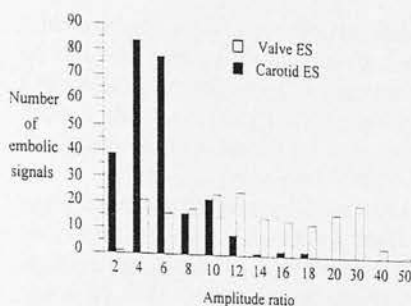


FIG 4. The ratio of the amplitude increase in the pre-fast Fourier transform time domain signal for embolic signals (ES) compared with that due to the background Doppler signal in the absence of an ES. Channels at both recording depths have been included.

(SD, range) time delay between the two channels was 14.9 ms (15.42, 0 to 90). The time delay was significantly longer than that of the 222 episodes of artifact ($P < .0001$). When the same 2-ms cutoff was used, a sensitivity of 94.0% and specificity of 99.0% were obtained when those signals identifiable in both channels were considered (Fig 3). The sensitivity fell to 92.6% when all ES, including those visible in only one channel, were considered.

Discussion

In our in vitro model, the use of a multigated technique allowed the identification of ES and their discrimination from artifact with a very high sensitivity and specificity. Of particular note, it allowed detection of ES that resulted in receiver overload with a similar high sensitivity and specificity; this is the first method to be able to identify such ES, which cannot be identified from the frequency spectral data alone either visually or with the use of a neural network¹¹ or computer algorithm.¹⁰ This application may be particularly useful when it is used during operative procedures, when both echogenic air emboli and artifact may be common. In addition, the multigate data may allow the unambiguous identification of low-velocity ES, which can sometimes be difficult to separate from artifact on frequency spectral data alone.

In all patients in whom an acoustic window could be obtained, it was technically possible to record using the multidepth probe with adequate gate separation. We identified the sensitivity and specificity of the multigated system using ES identified according to conventional criteria and compared them with known episodes of artifact. The results in patients were encouraging but not as impressive as the in vitro data for a number of reasons. This was primarily because in some patients the ES did not appear in both channels. In one patient in the valve group, 30 ES (approximately 40% of the signals in this patient) were detected proximally but were not audible or visible distally. This is likely because between the two sample volumes a proportion of the emboli pass down a branch vessel. Our protocol aimed for a gate separation, between the centers of each sample volume, of 10 mm, and sometimes the distal gate was a fairly shallow depth of 42 mm; the problem may be reduced with the use of a deeper distal gate, thereby lowering the chance that this gate may be sited beyond the branching of the MCA. Further studies are required to examine whether reduced gate separation allows such good separation between emboli and artifact. On the other hand, gate separation that is too narrow will reduce the time interval between detection of emboli in the

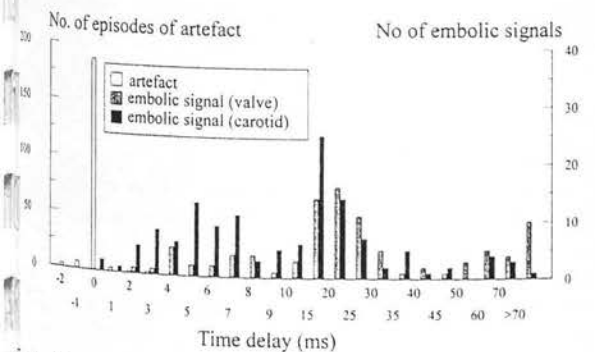


FIG 3. Frequency histogram of the time delay between the onset of the amplitude increase in the two channels for embolic signals in patients with carotid artery stenosis and mechanical cardiac valves and artifact in the same patients.

proximal and distal gates and will reduce the specificity in differentiating emboli from artifact. Apart from this difficulty in a single patient, the use of a multigated probe allowed detection of emboli and differentiation from artifact with a very high sensitivity, similar to that seen in the in vitro model. This is largely a reflection of the higher intensity of the more echogenic mechanical valve ES, as previously reported. This resulted in a large and clear amplitude increase in the time domain signal (Fig 1).

In contrast, for the less intense carotid ES, some signals were identifiable audibly and in the post-FFT spectral display but not in the time domain data. This occurred for low-intensity emboli, in which the power or intensity increase was small and lost in the background Doppler signal. An additional possible explanation is that the total cross-sectional area of the MCA is not fully covered by the sample volume at both depths. This will be less of a problem for more echogenic emboli, which have a larger effective sample volume.¹⁵ Separating the intensity increase at different frequencies by an FFT analysis allowed identification of the ES since the intensity increase of an ES is maximal at one frequency. Although only two carotid ES were not detectable in the time domain data, a much larger number resulted in an amplitude increase only slightly greater than that of the background Doppler signal. This is illustrated by the relatively low ratio of the maximum amplitude to background amplitude for many carotid ES in the time domain data (Figs 1E and 4). With the use of an off-line analysis in combination with the post-FFT spectral data, it is possible to determine the time delay for most carotid ES. However, it may prove difficult in automated on-line systems based on the time domain data alone to distinguish the small amplitude increase occurring with some carotid ES from amplitude fluctuations in the normal background Doppler signal. Further work is required to improve the sensitivity of the multigated technique for small-amplitude ES.

The predominant factor determining the time taken to travel between the two channels was the velocity at which the ES was maximal, which presumably reflects the speed at which the embolus is traveling. However, in the model there was a much greater range of time taken between detection in each channels than would be expected by simple mathematical principles. The theoretical distance traveled by an embolus as detected by the probe is 5 mm (ie, distance between the edges of the axial sample volumes). If we correct for angle of insonation, an embolus traveling at the mean velocity (40 cm/s) would be expected to take 15.3 ms to cross between the two axial sample volumes. Any embolus traveling at the peak velocity for the system (95 cm/s) would be expected to give a time delay of 6.4 ms between the two depths. It is noteworthy that 46 of the emboli we produced traversed the distance in 6 ms or less. This reflects the fact that rather than being cylindrical in shape, with a sharp cutoff of the ultrasound beam at each end of the sample, there is a gradual weakening of the beam at each end. It follows that the effective sample volume will be greater for more echogenic emboli such as air emboli.¹⁵ In patient studies some ES took much longer to travel between the two sample volumes, and this was particularly so for some valve ES. This may be due to their passage being slowed by turbulence, nonlaminar flow, and momentary adhesion to the vessel wall.

Our results demonstrate that a multigated approach can detect ES and differentiate them from artifact, but as with

other methods, it has some inherent problems that need to be resolved before it is suitable for routine clinical use. Our results are similar to those reported previously¹² but show a lower sensitivity for low-amplitude carotid ES. In this previous study, carotid ES, a minority of the ES, were not separated from those from patients with heart valve replacements or left ventricular assist devices, and no analysis of the intensity of the ES was made. Our results demonstrate that in patients with the more intense mechanical valve ES, high sensitivity and specificity are likely to be obtained; the only major difficulty appeared to be passage of emboli down a branching vessel between the two sample volumes, and this may be improved by reduced gate separation. In patients with carotid ES, the less intense ES may be unclear in one or both channels. For these ES, combining the multigated method with a method analyzing the post-FFT spectral data may improve sensitivity; use of the frequency spectra may provide greater resolution because the intensity increase associated with an ES is usually centered on a narrow frequency band.

Acknowledgments

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