THE ROLE OF HEPARIN IN THROMBOEMBOLIC COMPLICATIONS FOLLOWING CAROTID ENDARTERECTOMY

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by

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Abstract

The importance of platelets emerged from a local research programme, which aimed to reduce the stroke risk associated with carotid endarterectomy (CEA). It had been demonstrated that intra-operative heparinisation induced a transient reversal of aspirin inhibition; platelets were able to aggregate in response to arachidonic acid (AA).

It was hypothesized that intra-operative anticoagulation with intravenous low molecular weight heparin (LMWH) instead of unfractionated heparin (UFH) might be associated with a reduction in pleiotropic platelet effects, and that this would result in a reduction of post-CEA embolization, a surrogate marker for stroke risk.

A randomized controlled trial recruited 183 patients; 91 randomized to receive standard intra-operative anticoagulation with 5000IU UFH, and; 92 who received 2500IU LMWH intravenously. Studies conducted in sub-populations aimed to investigate the platelet aggregatory responses to AA and adenosine diphosphate (ADP) and the platelet pathways that were active (plasma and serum were assayed for the stable products of platelet metabolism; thromboxane (TXB_2) from the cyclo-oxygenase-1 (COX-1) pathway and 12-hydroxyeicosatretraenoic acid (12-HETE) from the 12-lipoxygenase (12-LOX) pathway). To determine how heparin might interact with the platelet, lipase activity, the presence of heparin antibodies and anti-factorXa (FXa) activity were studied.

Increases in platelet aggregation to AA and ADP were observed 3 minutes after heparinisation. In response to AA, these increases were similar for both UFH and LMWH, but patients who received UFH demonstrated significantly greater aggregation in response to ADP. Whilst there was no increase in the production of TXB_2, there was a significant increase in the generation of 12-HETE. The increase in platelet response was associated with anti-FXa activity, but not with lipase or heparin antibody activity. The intra-operative substitution of LMWH for UFH was associated with a significant reduction in the risk of patients experiencing high-rate embolization post-operatively, and there is an argument for the re-evaluation of anticoagulation during CEA.

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Prizes, Papers & Presentations

Prizes

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Papers

McMahon GS, Webster SE, Hayes PD, Jones CI, Goodall AH, Naylor AR. Low molecular weight heparin significantly reduces embolization after carotid endarterectomy – a randomised controlled trial. *European Journal of Vascular and Endovascular Surgery*. 2009;37:633-9.

Presentations

Sheehan NR, McMahon GS, Jones CI, Goodall AH. The anti-platelet effect of aspirin is transiently reversed after administration of unfractionated heparin. 8th UK Platelet Meeting, November 2006.

McMahon GS, Webster SE, Hayes PD, Jones CI, Goodall AH, Naylor AR. Low molecular weight heparin significantly reduces embolization after carotid endarterectomy – a randomised controlled trial. Annual Meeting of the Vascular Society of Great Britain and Ireland, November 2006.

Sheehan NR, McMahon GS, Chambers C, James TE, Jones CI, Goodall AH. Transient reversal of the anti-platelet effect of aspirin after administration of unfractionated heparin in healthy subjects. 21st Congress of the International Society on Thrombosis and Haemostasis, July 2007.

McMahon GS, Webster SE, Hayes PD, Jones CI, Goodall AH, Naylor AR. Low molecular weight heparin significantly reduces embolization after carotid endarterectomy – a randomised controlled trial. 35th Annual Meeting of the Japanes Society of Vascular Surgery, May 2007.

McMahon GS, Jones CI, Hayes PD, Goodall AH, Naylor AR. Heparin activates platelet 12-LOX: "transient aspirin resistance" explained? Annual Meeting of the Vascular Society of Great Britain and Ireland, November 2009.

McMahon GS, Goodall AH, Naylor AR. Platelet aggregation to ADP may influence symptoms associated with carotid artery disease. "E-Posters of distinction", International Congress of the Association of Surgeons of Great Britain and Ireland, May 2011.

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Introduction

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Carotid Endarterectomy

1.1 Stroke

The World Health Organisation defines stroke as the clinical syndrome of rapid onset of focal (or global, as in patients in deep coma or with subarachnoid haemorrhage) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin.[1] Worldwide, it is the third commonest cause of death, and is a major public health burden,[2-4] accounting for around 5% of health care budgets.[5-7]

The incidence of first-ever stroke is about 200 per 100,000 per year, rising steeply with age.[8-10] The prevalence of stroke is between 5 and 12 per 1000 population,[11] with the annual mortality varying internationally between 20 and 250 per 100,000 population.[12] There is a case fatality rate of 12% at 7 days, through to 20% at 30 days with the direct effects of the brain injury itself being primarily responsible for these early deaths. Subsequent deaths are most commonly due to recurrent cerebro- and cardiovascular events.[13,14] Having suffered a stroke, the risk of being dependent at 1 year is about 20%-30%.[15]

1.1.1 Types of stroke

Stroke can be classified into three pathological types (*Figure 1*). The majority (80%) are secondary to an ischaemic event, whilst primary intra-cerebral haemorrhage accounts for about 15% and subarachnoid haemorrhage for the remaining 5%. Of the ischaemic strokes, about 50% result from atherothromboembolism, with the remainder mainly being due to intra-cranial small vessel disease and cardiac sources of embolism (*Figure 2*).[16]

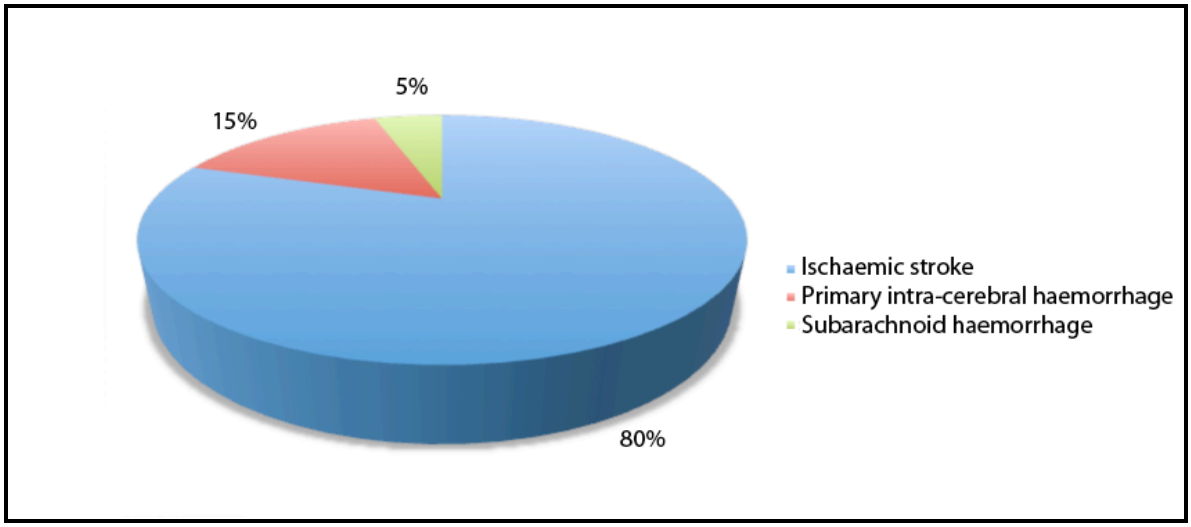

Figure 1 Approximate frequency of three main pathological types of stroke

1.1.2 Pathogenesis of atherothromboembolic stroke

Carotid artery stenosis increases stroke risk by two mechanisms. Firstly, the atherosclerotic narrowing of the lumen causes reduction and turbulence in blood flow, with the severity of stenosis proportional to the risk of stroke.[17] Secondly, rupture of the atherosclerotic plaque itself exposes thrombogenic material, leading to thrombosis and either subsequent vessel occlusion or embolization.[18]

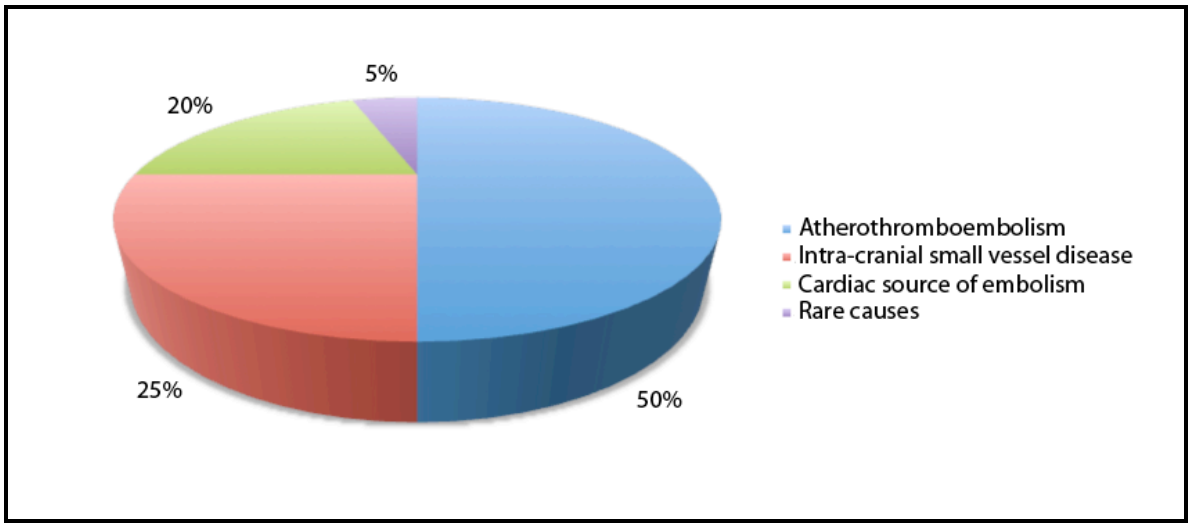

Figure 2 The main subtypes of ischaemic stroke

1.1.2.1 The arterial intima

The intima of the arterial wall extends from, and incorporates, the luminal endothelial surface to the luminal margin of the arterial media.[19] The intima is principally composed of endothelial and smooth muscle cells, with isolated macrophages also always present.

1.1.2.1.1 Intimal matrix

Around 60% of the volume of the intima is comprised of the arterial intimal extracellular matrix. Nutrients are transported through this medium and there is accumulation of the products of the various intimal cells.[19] It consists of proteoglycans, collagens, elastin, fibronectin, laminin and plasma components. The large proteoglycan molecules have functions in arterial permeability, filtration, ion exchange, transport and deposition of plasma materials and regulation of cellular metabolism. Collagen is involved in the

attachment of endothelial cells to the subendothelial matrix.[19] Collagen types I and II are the major collagens in the arterial wall, and with aging there is a shift in the intimal ratio in favour of type I.[20,21]

1.1.2.1.2 Intimal endothelial cells

At the surface of the lumen, the endothelial cells are coated by a glycocalyx consisting of free polysaccharides and glycosaminoglycans plus glycoprotein and glycolipid side chains emanating from the plasma membrane.[22] Endothelial cells secrete a number of extra-cellular matrix components as well as exhibiting several plasma membrane receptors such as those for low density lipoprotein (LDL),[23] insulin,[24] and histamine.[25]

All plasma proteins can permeate through the endothelium. The rates of transport are dependent on several factors, including the plasma concentration of the protein itself, the location of the artery, the age, blood pressure and vascular tone.[26,27] Under normal physiological conditions platelets do not adhere to the endothelium and thrombosis does not occur, which may be related to the cell membrane content of thrombomodulin and its ability to bind thrombin.[28] This complex activates protein C, which binds with protein S to inactivate coagulation factor Va.[29] Endothelial cells can also metabolize a range of platelet agonists, including adenosine diphosphate (ADP), serotonin, angiotensin and prostaglandin F_1, as well as being able to synthesize and secrete plasminogen activator[30] and prostacyclin.[31]

1.1.2.1.3 Intimal smooth muscle cells

Generally recognised as a normal component of the intima, smooth muscle cells are involved in a variety of functions of the vessel wall, including the contractile response to mediators of vascular tone,[32] synthesis and secretion of connective tissues (collagen, elastin and proteoglycan)[33,34] and removal of deposited lipoproteins via the expression of LDL receptors and phagocytosis.[35,36]

1.1.2.1.4 Macrophages

The precise role played by the macrophage in the intima is unclear, but it is thought that these isolated and irregularly placed cells are involved in the remodelling of the intima via the synthesis and secretion of collagenase[37] and elastase.[38] They may well also play inflammatory and immune roles,[39] with receptor-mediated uptake of native and modified lipoproteins[40] and phagocytosis of dead cells and immune complexes. They are probably also involved in fibrinolysis and the removal of thrombi and other deposited plasma proteins.[41]

1.1.2.1.5 Physiological intimal wall thickening

Thickening of the arterial intima occurs in regions of altered mechanical stress,[42] probably as a physiological adaptive remodelling to maintain normal conditions of flow and/or wall tension. In areas of arterial branching, such as the carotid bifurcation, this thickening tends to be eccentric, as a reflection of the non-uniformity of flows and tensile wall strengths.

Areas of intimal adaptive thickening are functionally distinct from adjacent, thinner regions of arterial wall. The turnover of endothelial[43] and smooth muscle cells is increased,[44] as are the concentrations of low density lipoproteins and other plasma components;[45] changes which are physiological, rather than pathological.

1.1.2.2 From intimal thickening to "pre-atheroma"

The regions of physiological intimal thickening are where atherosclerosis develops initially. In humans, the distribution of eccentric intimal thickening correlates with advanced atherosclerosis – the coronary arteries,[46] renal arteries,[47] internal carotid arteries,[42] and abdominal aorta.[48]

The development of advanced atherosclerosis is preceded by a spectrum of morphologically characteristic changes, which can be divided into three sub-types.[49]

1.1.2.2.1 Type 1 lesions

These early lesions are characterised by the first microscopically and chemically detectable lipid deposits in the intima, along with the associated cell reactions. Small, isolated groups of macrophages containing lipid droplets (macrophage foam cells) form.[46,50]

1.1.2.2.2 Type II lesions

Fatty streaks, which are visible as yellow deposits on the intimal surface of the vessel, are included here, although not all type II lesions are fatty streaks. The classification is made

on the lesion's microscopic composition rather than its macroscopic appearance. Type II lesions consist of macrophage foam cells stratified in layers rather than being present only in isolated groups. Intimal smooth muscle cells now also contain lipid droplets and there is thinly dispersed lipid in the extracellular matrix. There are isolated mast cells which can influence plaque progression through the secretion of various vasoactive substances.[51,52]

1.1.2.2.3 Type III lesions

This lesion is characterized histologically by extracellular lipid droplets. The lipid lies deep to the layers of macrophages and macrophage foam cells, replacing intercellular proteoglycans and fibres and driving smooth muscle cells apart. Similar to type II lesions, smooth muscle cells may contain lipid droplets. There tends to be more free cholesterol, fatty acid and triglyceride than in type II lesions.[53]

1.1.2.3 Advanced atherosclerotic lesions

It is the type IV and type V lesions from which atherosclerotic morbidity and mortality largely arises. There is disruption of the lesion surface, haematoma, haemorrhage and thrombotic deposits.

1.1.2.3.1 Type IV lesions

Also known as atheroma, there is a dense accumulation of extracellular lipid occupying an extensive region of the intima, known as the lipid core. However, there is still no fibrous tissue increase, and complications such as defects of the lesion surface and thrombosis do

not occur. The tissue between the lipid core and the endothelial surface is still largely the intima that preceded lesion development.

The lipid core develops from an increase and coalescence of the isolated pools of extracellular lipid of the type III lesion.[46] There is dispersal and replacement of the intimal smooth muscle cells and the intercellular matrix by extracellular lipid. The dispersed cells have attenuated and elongated bodies and may have unusually thick basement membranes. The organelles of the smooth muscle cells can become calcified, and calcium can often be found in the lipid core. The lipid core becomes bordered by capillaries, particularly at the lateral margins and facing the lumen.

At first, type IV lesions are found in the same locations as the physiological eccentric adaptive intimal thickenings, and thus atheroma is initially a similarly eccentric phenomenon.[54] Although lipid cores thicken the artery wall, this often fails to cause luminal stenosis. Instead, the thickening leads to enlargement of the external boundary of the vessel.[55]

1.1.2.3.2 Type V lesions

Reparative fibrous connective tissue forms in and around regions of the intima in which lipid cores have disrupted the normal cell and intercellular matrix structure. There are substantial increases in collagen and smooth muscle cells, generally giving rise to narrowing of the lumen. Type V lesions are prone to the development of fissures, haematoma and/or thrombus (then becoming type VI lesions).

There may be larger and more numerous capillaries at the periphery of the lipid core, frequently associated with lymphocytes, macrophages and plasma cells. Micro-haemorrhages may be present around the capillaries.

1.1.2.3.3 Type VI lesions

Atheromatous lesions (types IV and V) are especially prone to disruptions of the lesion surface,[56,57] and type VI lesions are essentially complicated type IV or type V lesions. The type VI lesion may be further classified depending on the exact nature of the complication (type VIa, disruption of the surface; type VIb, haematoma or haemorrhage; type VIc, thrombosis).

There is a spectrum of lesion surface disruption, from isolated loss of a part of the endothelial cell layer, to deep ulceration exposing and releasing lipid from the lipid core. Fissures may occur more frequently in regions of lesions with more macrophage foam cells.[58] There is probably a degree of cyclic fissuring and re-sealing, incorporating haematomas and thrombi into the lesion,[57] with small thrombi reforming many times. The thrombus is eventually covered by endothelial cells at the lumen.[54] Thus, the arterial lumen is gradually narrowed by repeated incorporation of small recurrent haematomas and thrombi into a lesion over time.

Some thrombi continue to propagate and occlude the lumen within hours or days. Systemic factors play a vital role in determining whether a thrombus will expand, independent of the degree of surface disruption or the haemodynamic susceptibility of the location. For

example, high plasma fibrinogen levels have been found in individuals with clinical ischaemic episodes,[59,60] and high levels of LDL may also promote thrombosis through an adverse effect on platelet function.[61] Furthermore, the platelets of patients with primary hypercholesterolaemia show increased platelet activation.[62]

1.1.2.4 Correlation of lesion types with clinical syndromes

The extent of lesion disruption determines the ensuing clinical state. With deep disruption, such as with fissuring, a transient (lasting minutes) thrombotic occlusion may occur, which may be repetitive.[57] If ulceration exposes the lipid core, a relatively persistent (lasting hours) thrombotic occlusion may result in acute infarction of the under-perfused distal tissue.[63] At the carotid bifurcation, luminal surface disruption may cause lesion instability, acting as a source of embolization, resulting in transient cerebral symptoms. Occlusion can also occur, with varying clinical consequences.

1.2 The origins of carotid surgery

Although the relationship between carotid artery disease and neurological function had been appreciated for over 2000 years,[64] the concept that aside from complete occlusion, stenosis of the vessel could also precipitate symptoms, was probably not fully recognised until the early twentieth century.

DeBakey is credited as having performed the first successful carotid endarterectomy (CEA) on August 7th 1953. His patient had a two-year history of transient ischaemic attacks

(TIA), and exploration of the left carotid bifurcation revealed a localised, severely stenotic, atheromatous plaque, together with a partially organized fresh clot impeding the lumen of the common carotid artery. These were successfully removed, and the continuity of the artery was restored. The patient survived a further 19 years free from cerebrovascular complications.[65,66] The following year, Eastcott, Pickering and Rob encountered a patient with almost identical symptoms. They successfully resected the stenotic carotid bifurcation, ligated the external carotid artery, and anastamosed the proximal common carotid directly to the distal internal carotid artery stump.[67] These four surgeons were central to the development of carotid surgery, and as the end of the twentieth century approached, well over 100,000 CEAs a year were taking place worldwide.[68]

1.3 The randomised controlled trials of CEA

Although CEA is the most frequently performed surgical method of stroke prevention,[69] the inherent paradox is that the operation itself is associated with a small but important risk of disabling stroke or death.[70] In order to justify exposing an individual to the risk of iatrogenic death or serious disability, the net benefits must be clearly greater than the immediate risk, a balance that for CEA was for some time the subject of controversy. As the procedure was gaining popularity, two negative randomised trials were reported,[71,72] resonated by reports of unacceptably high rates of complications, and ultimately, questioning of the merit of CEA.[70,73-78]

Thus, in order to define the role of CEA in the management of stroke disease, a series of large-scale randomised trials were established. Three large, multi-centre, randomised trials

of CEA for recently symptomatic carotid stenosis have been performed to assess its role in the management of symptomatic patients.[79-81] There have also been three multi-centre trials investigating CEA in patients with asymptomatic stenosis.[17,82,83] Subsequent combination analyses have been performed, taking into account differences in trial protocols and stenosis measurement (*Figure 8* & *Figure 9*).

1.3.1 The MRC European Carotid Surgery Trial (ECST)[79]

From 1981 to 1994, the multicentre ECST randomised 3024 symptomatic patients to the management policies of either "immediate surgery" (1811 patients, 60%) or "no immediate surgery" (1213 patients, 40%). All the patients demonstrated some degree of carotid stenosis, and had, within the previous six months, experienced at least one transient or permanent symptomatic ipsilateral carotid territory episode that had not resulted in severe disability. The primary objective of the trial was to estimate the range of stenoses within which CEA would confer benefit, by assessing stroke-free life expectancy. Stroke was characterised by symptoms and/or signs of focal and at times global loss of cerebral function lasting longer than 24 hours or leading to death, with no apparent cause other than that of vascular origin. To this definition, "major stroke" added symptoms lasting longer than 7 days, with "disabling stroke" defined as a stroke that after 6 months was associated with disability as recorded on the modified Rankin scale of 3, 4 or 5.[84] After a disabling stroke, a patient was classified as permanently disabled. Follow up was at 4, 12 months and annually thereafter.

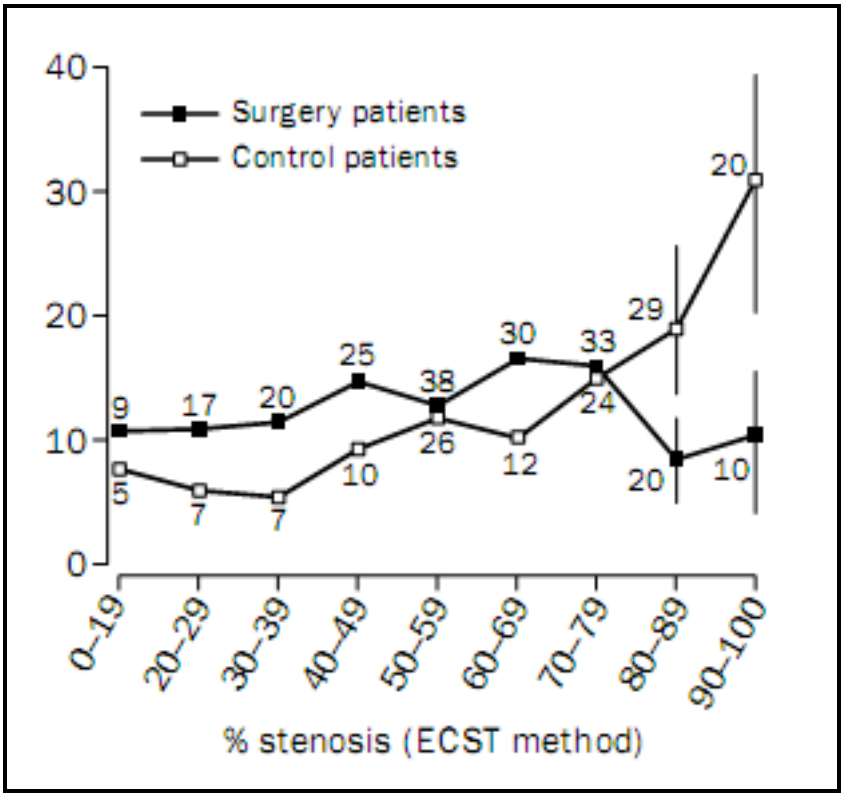

Figure 3 Predicted risk of any major stroke at 3 years and actual risk of a surgical event by treatment group and severity of symptomatic carotid stenosis. Reproduced and adapted from ECST Lancet 1998;351:1379-87

All patients received "best medical therapy", combining smoking cessation advice, hypertension treatment and anti-platelet therapy. Mean follow-up was 6.1 years, and after accounting for patients lost to follow-up, 3018 patients were included in the trial analysis (1807 in the surgery group, 1211 in the control group).

Among the 1745 patients (62 patients allocated surgery did not undergo CEA within a year of randomisation) who were allocated and received surgery, there were 122 non-fatal major strokes or deaths (7%). Of these, 61 had non-disabling major strokes, 40 non-fatal disabling major strokes, 15 fatal strokes (10 of whom died within 30 days of trial surgery

and so were counted as "surgical" deaths) and 7 non-stroke deaths. The overall surgical risk among the patients allocated control treatment that crossed over and underwent CEA was 4.8% (2 of 42 patients).

In the "no immediate surgery" group the risk of major stroke was related to the severity of carotid stenosis, within the first 2-3 years after randomisation, but thereafter, there was no relation between stroke risk and severity of stenosis. The authors of ECST proposed stroke-free life expectancy a more appropriate measure of CEA-benefit, since due to the short-term stroke-risk associated with surgery, CEA patients tended to have strokes earlier than those allocated control. They used a Cox proportional-hazards model to estimate stroke-free life expectancy. At 3 years, for patients with symptomatic carotid stenosis of 80-99%, the estimated "any major stroke or death" rate was 14.9% for the surgery patients, and 26.5% for the controls, an absolute difference of 11.6% (p=0.001, *Figure 3* & *Figure 4*); 116 major strokes or deaths from any cause might be avoided per 1000 patients treated.

So the trial showed how treatment effect at 3 years varied with stenosis, the contribution from the short-term risk of surgery and the long-term prevention of stroke after surgery, and also the size of benefit that might be achieved if the treatment decision was based on a stenosis of 80% or above. Time to death or major stroke was analysed using not only treatment allocation and stenosis severity, but also age and sex. These variables were included because ageing is associated with an increased risk of stroke after TIA, and female sex with an increased risk of stroke complicating CEA.

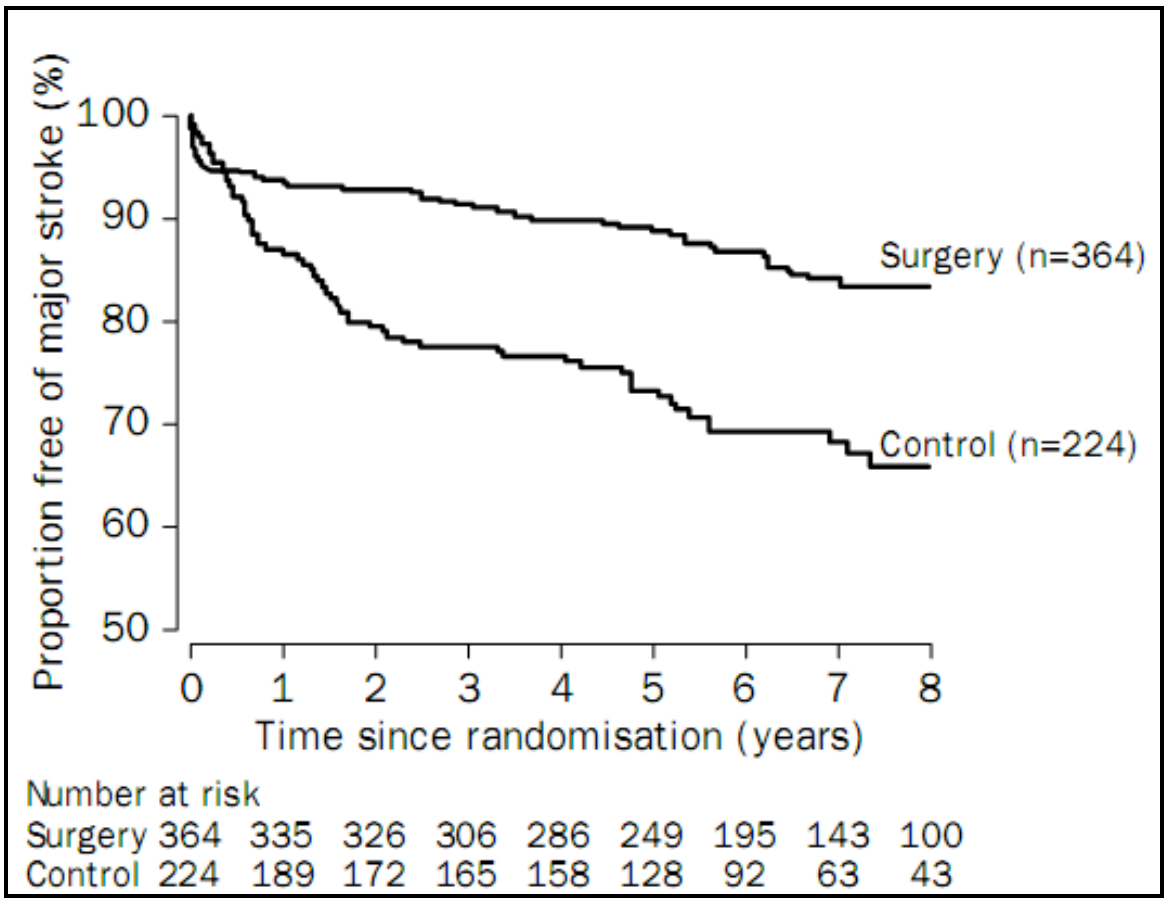

Figure 4 Kaplan-Meier survival curves to show survival free of major stroke (with non-stroke deaths occurring more than 30 days after surgery removed) in surgery and control patients with 80-99% stenosis of symptomatic carotid artery. Reproduced from ECST Lancet 1998;351:1379-87

Age and sex had a highly significant effect on major stroke or death, similar for control patients and surgery patients beyond the 5-day surgical risk period. Post-surgery risk was greatly increased in a manner dependent on a function of stenosis and was also higher in women than in men. Men derived rather more benefit from surgery than did women, there was more benefit with increasing severity of stenosis, and younger patients showed definite benefit over a narrower range of severe stenosis than did older patients.

1.3.2 The North American Symptomatic Carotid Endarterectomy Trial (NASCET)[80]

This multi-centre randomized trial was conducted at 106 centres throughout the USA and Canada, where 2885 patients were recruited between 1987 and 1996. Stratification was performed according to the degree of carotid stenosis. Patients had stenosis either in the range 30% to 69% ("moderate stenosis"), or between 70% and 99% ("severe stenosis"). Follow-up was with neurologists at 1, 3, 6, 9 and 12 months, and then at 4 monthly intervals thereafter. The average follow-up for all patients was 5 years, and all 1818 surviving patients (911 in the medical group and 907 in the surgical group) underwent final assessment in 1997. Strokes were considered disabling if patients had a Rankin score of 3 or more at 90 days. The primary intention-to-treat analysis compared medical and surgical patients in terms of the time to treatment failure (defined as a fatal or non-fatal ipsilateral stroke).

In February 1991, after 659 patients with ≥70% carotid stenosis had been randomised, the "severe stenosis" arm of the study was halted.

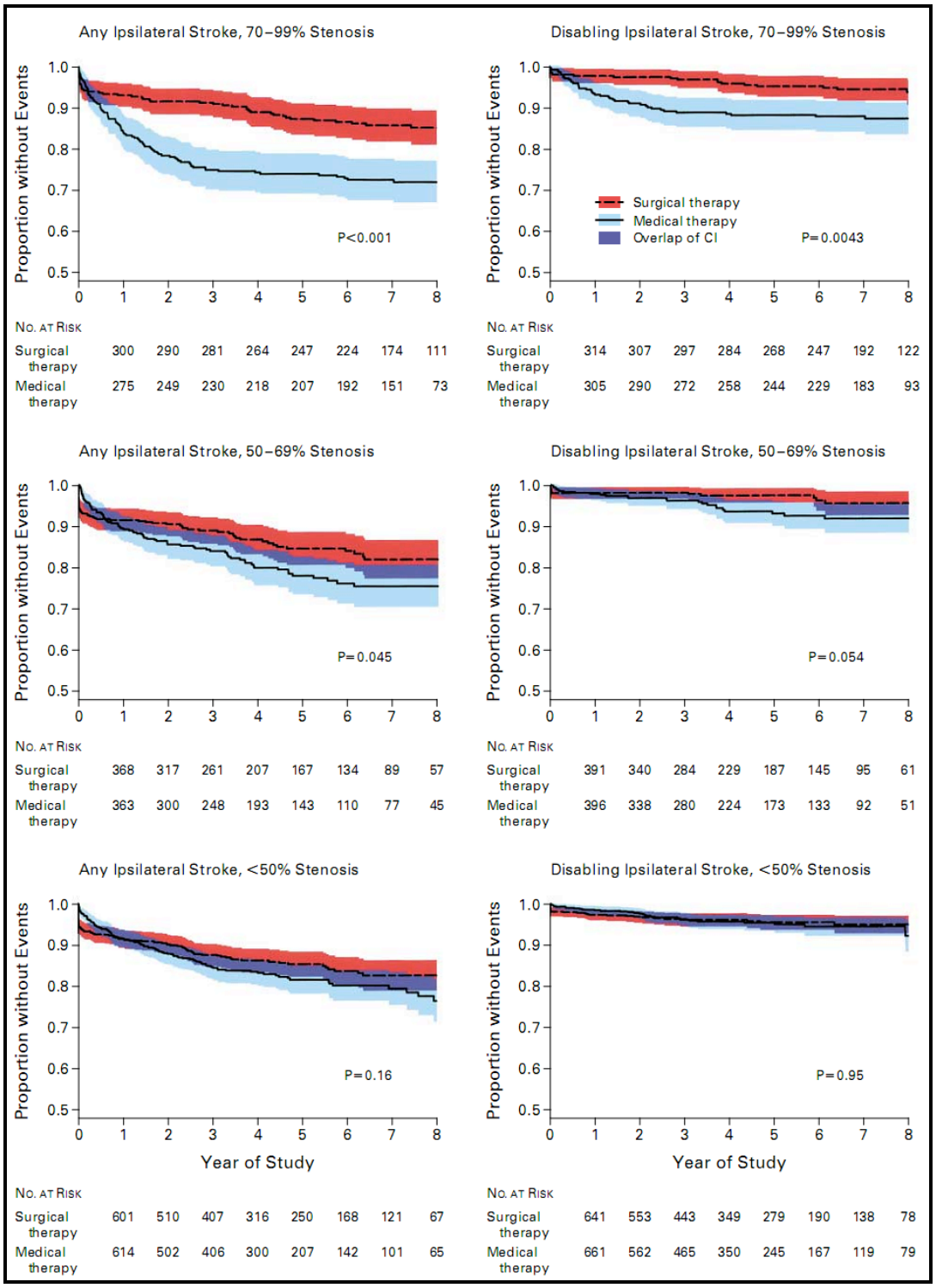

Figure 5 Kaplan-Meier curves for event-free survival among patients with severe and moderate stenosis. Reproduced from Barnett et al. N Engl J Med 1998;339:1415-25

Life-table estimates of the cumulative risk of any stroke at 2 years were 26% in the 331 medical patients and 9% in the 328 surgical patients, an absolute reduction of 17% in the risk of all ipsilateral strokes. Furthermore, there was an absolute reduction of 10.6% in the risk of major or fatal ipsilateral stroke, from 13.1% in the medical group to 2.5% in the surgical group.[85] Consequently, CEA was recommended for the patients with severe stenosis who had been randomly assigned to best medical therapy.

Patients with moderate stenosis continued to be enrolled. They were eligible if they had symptoms of focal cerebral ischaemia ipsilateral to a stenosis of less than 70% in the internal carotid artery (on selective angiography) persisting less than 24 hours or producing non-disabling stroke, within 180 days. There were 2226 eligible patients with stenoses of <70%; 1108 (50%) were allocated to the CEA group, with 1118 (50%) patients designated to receive best medical therapy. In the surgery group, 21 of the 1108 received only medical therapy (12 withdrew consent, 6 had medical complications and the surgeons elected not to perform CEA in 3), leaving 1087 patients undergoing surgery.

The overall 30-day stroke or death rate after CEA, was 6.7% (73 of 1087 patients). For patients with carotid stenoses between 50% and 69%, the five-year rate of any ipsilateral stroke was 15.7% among those treated surgically and 22.2% among those treated medically, corresponding to a relative risk reduction of 29%. For the patients with <50% stenosis, there was no significant difference between those treated with CEA and the medically treated group (14.9% vs, 18.7%). Patients with 50 to 69% stenosis were at greater risk when treated medically, and obtained a greater benefit from surgery than patients with stenosis of <50%.

Figure 5 shows the Kaplan-Meier curves for event-free survival, with the curves showing the probability of avoiding an ipsilateral stroke of any degree of severity (left-hand side) and a disabling ipsilateral stroke (right-hand side) among patients with carotid stenosis of 50 to 69% (centre), and less than 50% (bottom) who were randomly assigned to undergo CEA or to receive best medical therapy.

For the patients 70 to 99% (top), enrolled who had stenosis of 70% or more, the 95% confidence intervals for the curves remain separate at all times. Among the patients with 50 to 69% stenosis, the confidence intervals overlap slightly at all times. The overlap is greater for disabling stroke than for any stroke. The confidence intervals totally overlapped among the patients with stenosis of <50%.

Among patients treated surgically, the risk of ipsilateral stroke dropped within 10 days after CEA to about 2% per year (*Figure 6*); for medically treated patients, the risk of ipsilateral stroke, which was highest immediately after the initial ischaemic event, dropped more gradually to about 3% per year within 2 or 3 years. This was true both for patients with moderate stenosis (50 to 69%) and for those with severe stenosis (70 to 99%). Among medically treated patients, the risk of ipsilateral stroke dropped to an annual level similar to that among surgically treated patients. This suggested that in the absence of recurring symptoms, there was probably little to gain from CEA after 2 to 3 years.

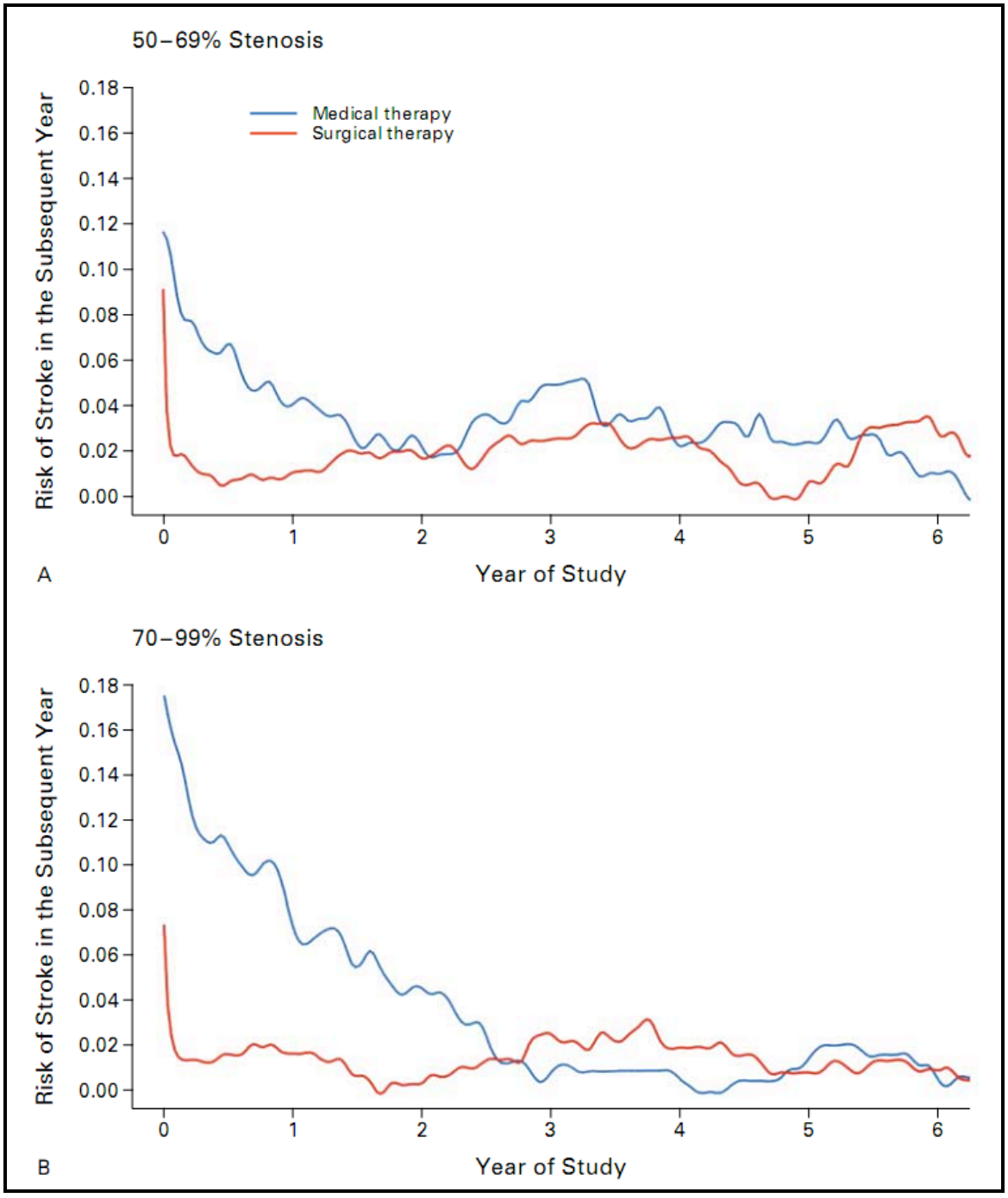

Figure 6 Change in the risk of ipsilateral stroke over time, according to severity of stenosis and treatment group. The curves show the risk of an ipsilateral stroke over the next year among patients who had not had an ipsilateral stroke since randomisation. Reproduced from Barnett et al. N Engl J Med 1998;339:1415-25

Further analysis identified that male sex, a recent stroke, recent hemispheric symptoms and taking 650mg or more of aspirin per day were all associated with greater long-term benefit of surgery.

The 326 patients with symptomatic stenosis of 70% or more who underwent CEA were followed for an average of 8 years. Complete data on outcome events were available for 98.8%. The Kaplan-Meier survival curves (*Figure 7*) show the risk of disabling ipsilateral stroke and stroke of any severity in these patients from 30 days to 8 years.

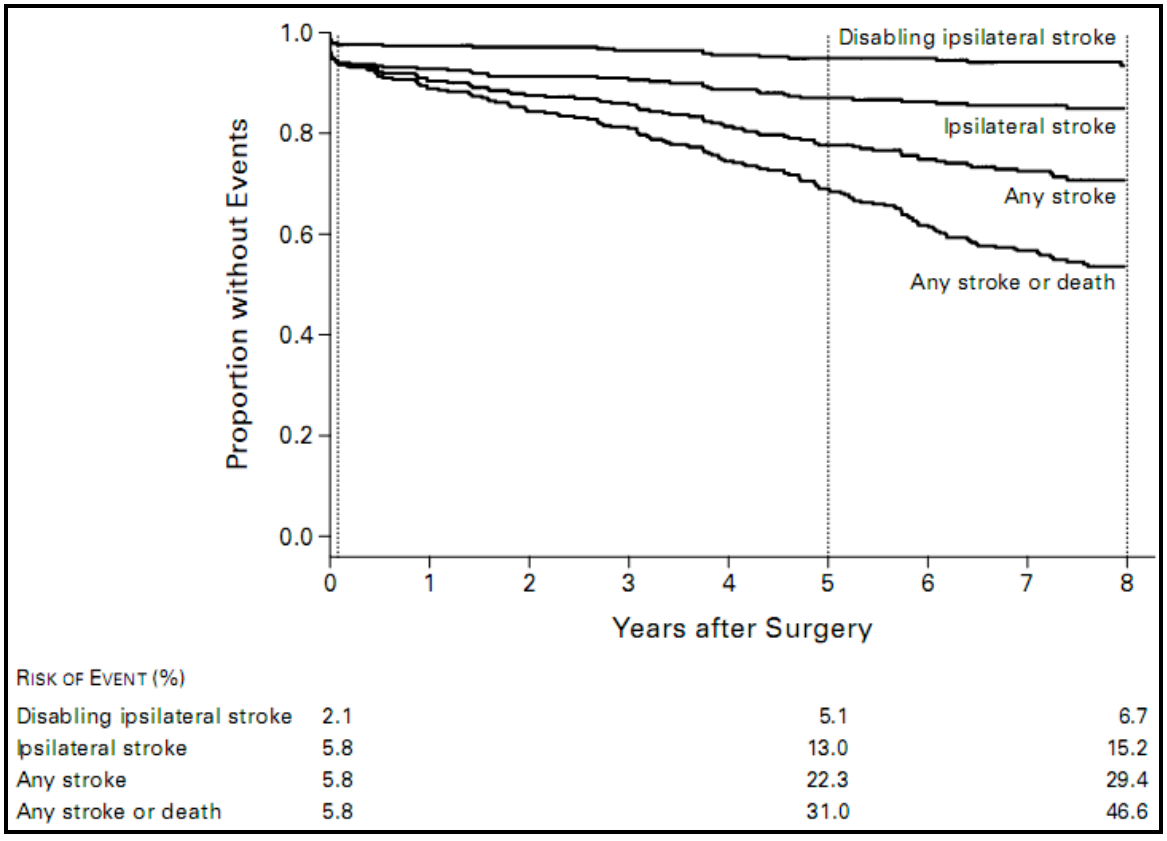

Figure 7 Kaplan-Meier curves for event-free survival after CEA among 326 patients with severe stenosis. Reproduced from Barnett et al. N Engl J Med 1998:339:1415-25

1.3.3 The Veterans Affairs Cooperative Studies Program 309 (VA309)[81]

This prospective, randomised, multicentre trial within the Veterans Affairs health care system, was designed to examine the efficacy of CEA combined with best medical care, versus best medical care alone. Data demonstrating benefit for CEA from the ECST[86] and NASCET[87] trials prompted early termination and analysis prior to the planned completion date.

Participants were all men, who presented to VA centres within 120 days of onset of symptoms that were consistent with TIA, amaurosis fugax or recent small completed strokes. There was an extensive list of concurrent illnesses which warranted exclusion, including poorly controlled diabetes, severe hypertension, renal failure, chronic obstructive airways disease, malignancy estimated to restrict life to 3 years, atrial fibrillation, myocardial infarction within 6 months and congestive cardiac failure. Concurrent use of oral anticoagulants was also excluded. Taking all exclusion criteria into account, fewer than 1 in 27 patients who were referred for evaluation were eligible.

Patients were enrolled if their stenosis was 50% or greater by NASCET angiography (*Figure 8*). The primary end-points were cerebral or retinal infarction in the ipsilateral carotid territory persisting for more than 24 hours and crescendo TIA (increased frequency, duration or severity of transient cerebral or retinal ischaemia) in the distribution of the ipsilateral carotid artery.

One hundred and ninety three men aged 35 to 82 (mean 65 years) were randomised to either surgical (92) or non-surgical (101) treatment groups. Four patients were retrospectively excluded from the trial after central review of their angiograms suggested them to have stenosis less than 50%. CEA was performed in 90 of 91 patients randomized to surgical treatment. At the mean follow-up point of 11.9 months, there was a significant reduction in stroke or crescendo TIA in patients who received CEA (7.7%) compared with non-surgical patients (19.4%, absolute risk reduction 11.7%; p=0.01). Among patients with internal carotid artery stenosis of greater than 70%, the benefit of surgery was more pronounced (7.9% vs. 25.6%, absolute risk reduction 17.7%; p=0.004). For 58 patients with stenosis in the range 50-69%, primary end-points were not different for surgical patients (7.1%) compared with non-surgical patients (6.7%, p=0.556).

1.3.4 The Carotid Endarterectomy Trialists' Collaboration (CETC) analysis of the symptomatic trials[88]

Methodological differences between ECST and NASCET made comparison complicated. Differing methods of stenosis quantification meant that any given carotid narrowing would warrant a higher value of stenosis in ECST than in NASCET and VA309 (*Figure 8 & Figure* 9).[89,90] The outcome measures also differed between the trials. The CETC Group pooled the individual patient data and re-assessed all of the original pre-randomisation angiograms, applying a uniform method of stenosis measurement and re-analysing the results with the same outcome endpoints.

The angiograms from the ECST, NASCET and VA Trials were re-measured and the degree of stenosis recalculated using the NASCET method. This technique is based on measurement of the minimal residual lumen at the point of maximum stenosis and the diameter of normal internal carotid artery distal to the carotid bulb. In the ECST measurements, the denominator was an estimate of the diameter of the carotid bulb at the level of the stenosis. As a result, the ECST system tends to report higher values for any given NASCET stenosis (*Figure 8 & Figure* 9).

In NASCET and VA309, a stroke was defined as a cerebrovascular event with symptoms lasting longer than 24 hours. In ECST, all such events were recorded, but analysis was restricted to events with symptoms that lasted for at least 7 days. In NASCET and VA309, retinal infarcts were included as stroke outcomes.

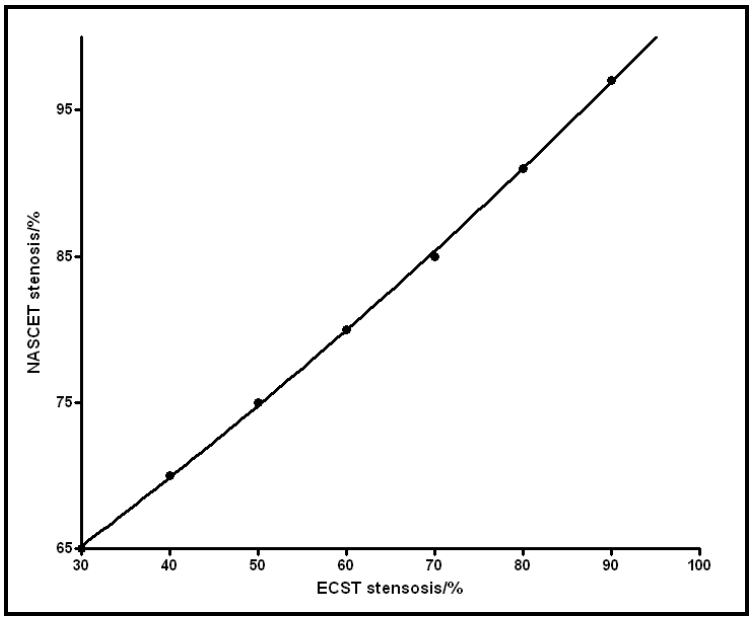

Figure 8 Comparison of stenosis quantification between NASCET and ECST

Again, in ECST these were recorded, but they were not included in the analysis. For the purposes of the CETC analysis, stroke was defined as any cerebral or retinal event with symptoms lasting longer than 24 hours. ECST and NASCET defined stroke outcomes using the modified Rankin scale, whereas in VA309 an equivalent scale was used. Disability was defined 3 months after stroke in the NASCET, at 6 months in ECST, and at the next routine follow-up assessment in VA309. For the combined analysis of CETC, disabling stroke was defined as a stroke that resulted in a Rankin score of 3 or more, or equivalent, at these points of follow-up.

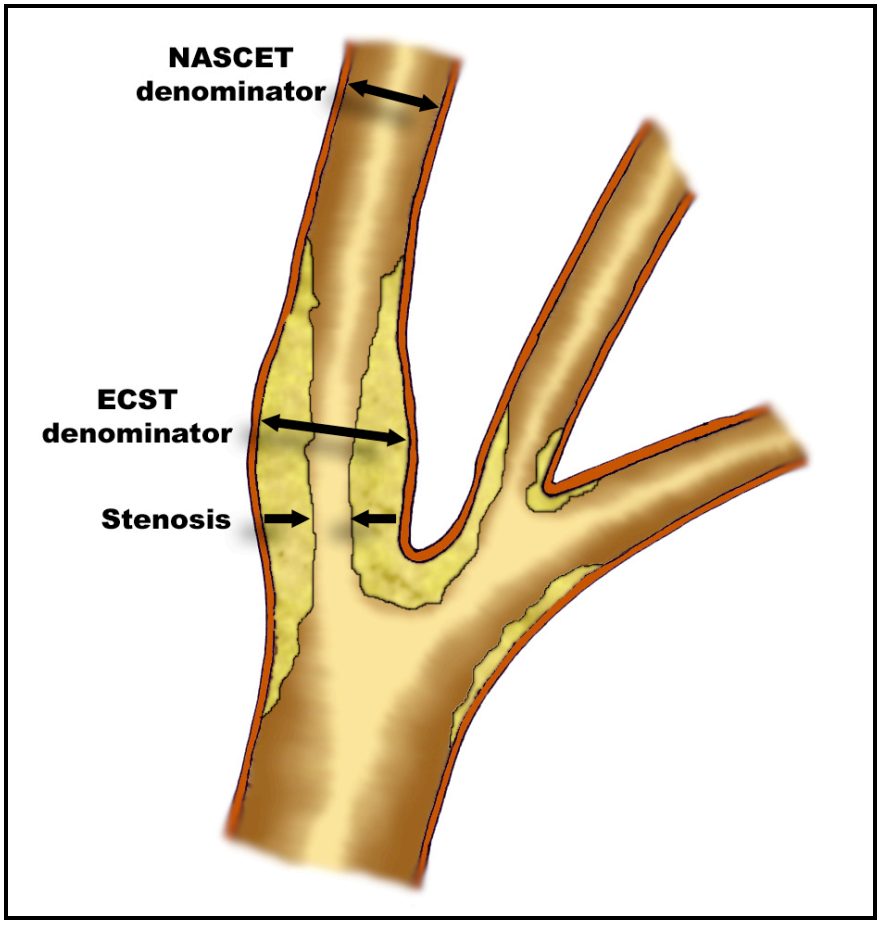

Figure 9 Different methods of stenosis measurement

Analyses were stratified according to the stenosis groups that were used in NASCET (<30%, 30-49%, 50-69%, ≥70%), with "sub-occlusions" analysed separately. Outcomes defined for analysis of the effectiveness of surgery were: (1) time to any first stroke or operative death; (2) time to first ipsilateral ischaemic stroke in the territory of the symptomatic carotid artery, and any stroke or death that occurred within 30 days of trial a surgery; and (3) time to first ipsilateral disabling or fatal ischaemic stroke in the territory of the symptomatic carotid artery, and any disabling stroke or death that occurred within 30 days of trial surgery. Operative risk was defined as any stroke or death that occurred within 30 days of trial surgery. Surgical death included all deaths within 30 days of trial surgery.

This CETC re-analysis included 6081 (99.8%) of patients from the original three trials. Mean follow-up was 65 months (range 1 day to 167 months), giving a total of 35000 patient-years of follow-up, with 1711 stroke outcomes in 1265 patients.

Of the 3334 patients who were randomised to surgery, 3248 (98%) actually underwent trial surgery. Overall, the 30-day stroke or death rate was 7.1%, and operative risk did not differ between stenosis sub-groups. The 30-day risk of death for CEA was 1.1% (35 of 3248), and the 30-day case-fatality for operative strokes was 9.6% (20 of 209). Surgery tended to be harmful in patients with less than 30% stenosis. In patients with 30-49% stenosis, the risks for all the main outcomes were higher in the surgery group for the first 2 years follow-up. Thereafter, the risks were similar in both treatment groups, with no significant benefit from surgery for any of the main outcomes.

In patients with 50-69% stenosis, surgery was also associated with a higher risk of all the main outcomes for the first 2 years of follow-up, but this trend reversed during subsequent follow-up, resulting in significant benefit from surgery for any stroke or operative death and ipsilateral carotid territory ischaemic stroke and operative stroke or death.

In patients with 70% stenosis or greater without sub-occlusion, there was a highly significant reduction in the surgery group in the risks of all the main outcomes. Benefit was apparent during the first year of follow-up, reached a maximum by 3 years, and was still present at 8 years.

CETC Stenosis/%	30-day surgical risk/%	5-year surgical risk/%	5-year medical risk/%	Absolute risk reduction/ %	Relative risk reduction/%	Strokes prevented/ 1000 CEA at 5 years
<30		18.4	15.7	-2.6	not applicable	0
30-49	6.7	22.8	25.5	2.6	10	26
50-69	8.4	20.0	27.8	7.8	28	78
70-99	6.2	17.1	32.7	15.6	48	156
sub-occlusion	5.4	22.4	22.3	-0.1	not applicable	0

Table 1 Overview of CETC data from ECST, NASCET and VA.
Adapted from Naylor, J Cardiovasc Surg 2009;50:773-82

For each of the main outcomes, benefit from surgery increased steadily from 50-59% stenosis to 90% stenosis or greater (without sub-occlusion). In patients with 50-59% and 60-69% stenosis, the benefit was small at 3 years' follow-up, but increased with time. In

patients with 60-69% stenoses, benefit was similar to that in patients with 70-79% stenosis by 8 years. However, benefit from surgery in respect of disabling or fatal ipsilateral ischaemic stroke or operative stroke and operative death was only seen in patients with 80-89% and 90% or greater (without sub-occlusion). The results of the CETC analysis are summarized in Table 1.

1.3.5 The Veterans Affairs Cooperative Study Group – efficacy of CEA for asymptomatic carotid stenosis[82]

The primary objective of this randomized clinical trial was to compare the incidence of transient ischaemic attack, amaurosis fugax and stroke in patients with asymptomatic carotid stenosis randomly assigned to CEA with best medical therapy (the "surgical group"), as compared with best medical therapy alone (the "medical group").

Enrolment began in 1983 and follow up ended in 1991. After non-invasive screening with ocular pneumoplethysmography or duplex ultrasonography, the percentage stenosis was confirmed arteriographically in all patients.

Patients were randomly assigned based on the arteriographic presence of a substantial asymptomatic carotid stenosis, defined as 50% using the NASCET criteria (*Figure 9*). Patients so randomised, underwent CEA within 10 days. All the patients received an initial dose of aspirin (650mg twice daily), which was reduced to 325mg daily for patients who could not tolerate the larger dose during the subsequent clinical follow-up. All the patients

were scheduled for clinical follow up every 13 weeks during the first year and every 26 weeks thereafter.

Of 444 patients, 233 were randomised to the medical group and 211 were randomised to surgery. The 30-day operative mortality was 1.9% (4 of 211), with 3 deaths from myocardial infarction and 1 from myocardial infarction followed by stroke.

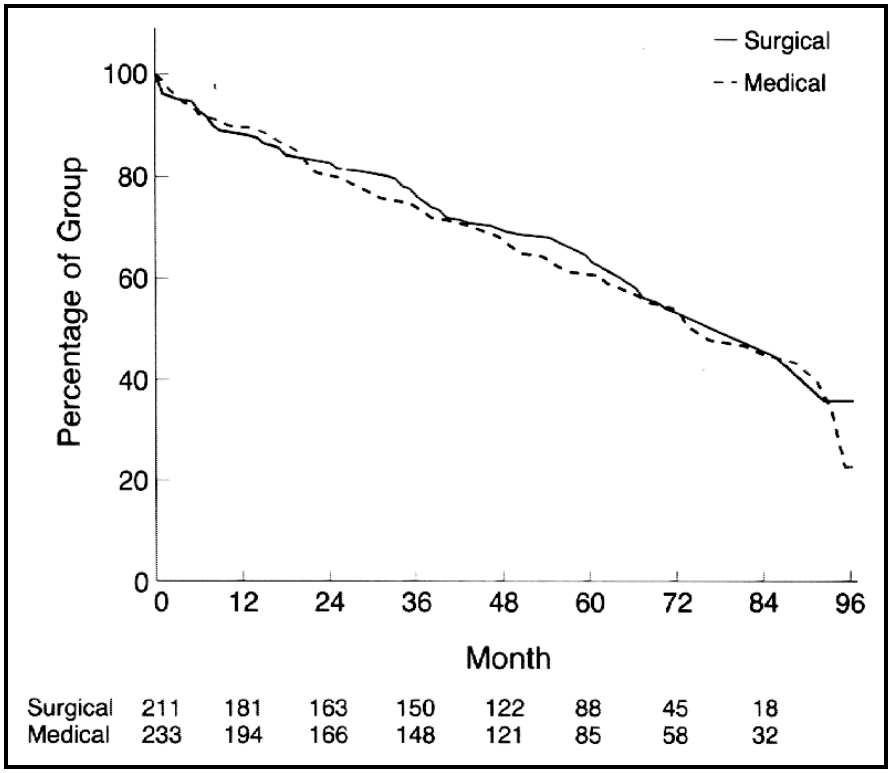

Figure 10 Kaplan-Meier curves for event-free rates of stroke and death in the surgical and medical groups in the VA Asymptomatic Trial. Reproduced from Hobson et al. N Engl J Med 1993:328:221-7

There were 5 post-operative non-fatal strokes, giving an incidence of 2.4% (5 of 211), and TIA was observed in 0.9% (2 of 211). Of 714 patients who underwent initial arteriography, 3 (0.4%) suffered non-fatal strokes as a direct result of the investigation. The rate of

permanent stroke and death within 30 days of randomisation was 4.7% in the surgical group (including strokes related to diagnostic angiography) and 4.3% if angiogram related strokes were excluded.

There were 84 ipsilateral and contralateral neurological events observed during the course of follow-up (*Table 2*). In the surgical group there were 27 (12.8%), with 57 (24.5%) in the medical group. This represented an absolute risk reduction of 11.6% and a relative risk reduction (for the surgical group vs the medical group) of 0.51.

The differences in the incidence of stroke alone suggested a preference for surgical management ($p<0.06$). However, when the 4 peri-operative deaths (1.9%) and 3 strokes associated with arteriography (0.4%) were assigned to the surgical group, there was no significant difference seen between surgical and medical treatment for stroke alone.

End Point	Surgical Group (n=211)	Medical Group (n=233)
Transient Ischaemic Attack	9 (4.3%)	17 (7.3%)
Amaurosis fugax	1 (0.5%)	12 (5.2%)
Stroke (non-fatal and fatal)	17 (8.1%)	28 (12%)
All	27 (12.8%)	57 (24.5%)

Table 2 Incidence of neurological end points ipsilateral and contralateral events after 4 years' follow-up. Reproduced from Hobson et al. N Engl J Med 1993:328:221-7

Figure 10 shows the Kaplan-Meier curves for the two groups in terms of stroke and death. There were no significant differences between the two groups.

1.3.6 The Asymptomatic Carotid Atherosclerosis Study (ACAS)[83]

In 1987 the Asymptomatic Carotid Atherosclerosis Study (ACAS) was started in North America. This was a randomized trial designed to test whether CEA should be a component of management for selected patients with asymptomatic stenosis. The specific hypothesis was that CEA, in addition to aggressive reduction of modifiable risk factors and administration of aspirin, would reduce the 5-year risk of ipsilateral cerebral infarction in individuals with asymptomatic haemodynamically significant carotid artery stenosis. Secondary objectives were to determine the surgical success in lesion removal and the incidence of recurrent carotid stenosis, the rate of progression or regression of carotid atherosclerosis in the medically treated comparison group, and the incidence of all other vascular events, such as TIA, myocardial infarction, and death related to vascular disease during follow up. ACAS defined haemodynamically significant carotid stenosis by at least one of three criteria: arteriography within the previous 60 days indicating stenosis of at least 60% reduction in diameter; Doppler ultrasonography within the preceding 60 days showing a frequency or velocity greater than the instrument-specific cut point with 95% positive predictive value; or Doppler examination showing a frequency or velocity greater than the instrument-specific 90% PPV cut point confirmed by ocular pneumoplethysmographic (OPG-Gee) examination performed within the previous 60 days. Patients randomized to CEA on the basis of Doppler or Doppler with OPG-Gee were required to have an arteriogram prior to surgery.

Stenosis was measured in a similar way to the NASCET method (*Figure 9*).[80] All patients received 325mg aspirin daily, and stroke risk factors and their modification were reviewed with all patients at the time of randomization and again during subsequent interviews. This included discussion of blood pressure control, diabetes, lipidaemia, alcohol consumption and smoking. In addition to these measures, patients randomised to the surgery arm were scheduled to undergo CEA within 2 weeks.

Follow up was conducted at 24 hours for the surgical patients, then at 1 month and thereafter every 3 months for all participants. A TIA was defined as a focal ischaemic neurological deficit of abrupt onset lasting at least 30 seconds and resolving completely within 24 hours. Deficits persisting longer than 24 hours were classified as stroke. All strokes occurring within 30 days after randomization in the surgical and 42 days in the medical groups were included as end points to reflect the operative morbidity and mortality. The difference in times reflected an average 12-day interval between randomization and surgery. Secondary analysis considered any stroke and peri-operative death; any stroke and any death; and any ipsilateral TIA and stroke and any peri-operative TIA, stroke or death.

Since the Veterans Affairs trial demonstrated that CEA was superior to medical management alone for preventing TIA in asymptomatic carotid stenosis, and NASCET demonstrated that infarction following TIA was better managed surgically, the primary end-points of ACAS were subsequently restricted to stroke and peri-operative complications or death.

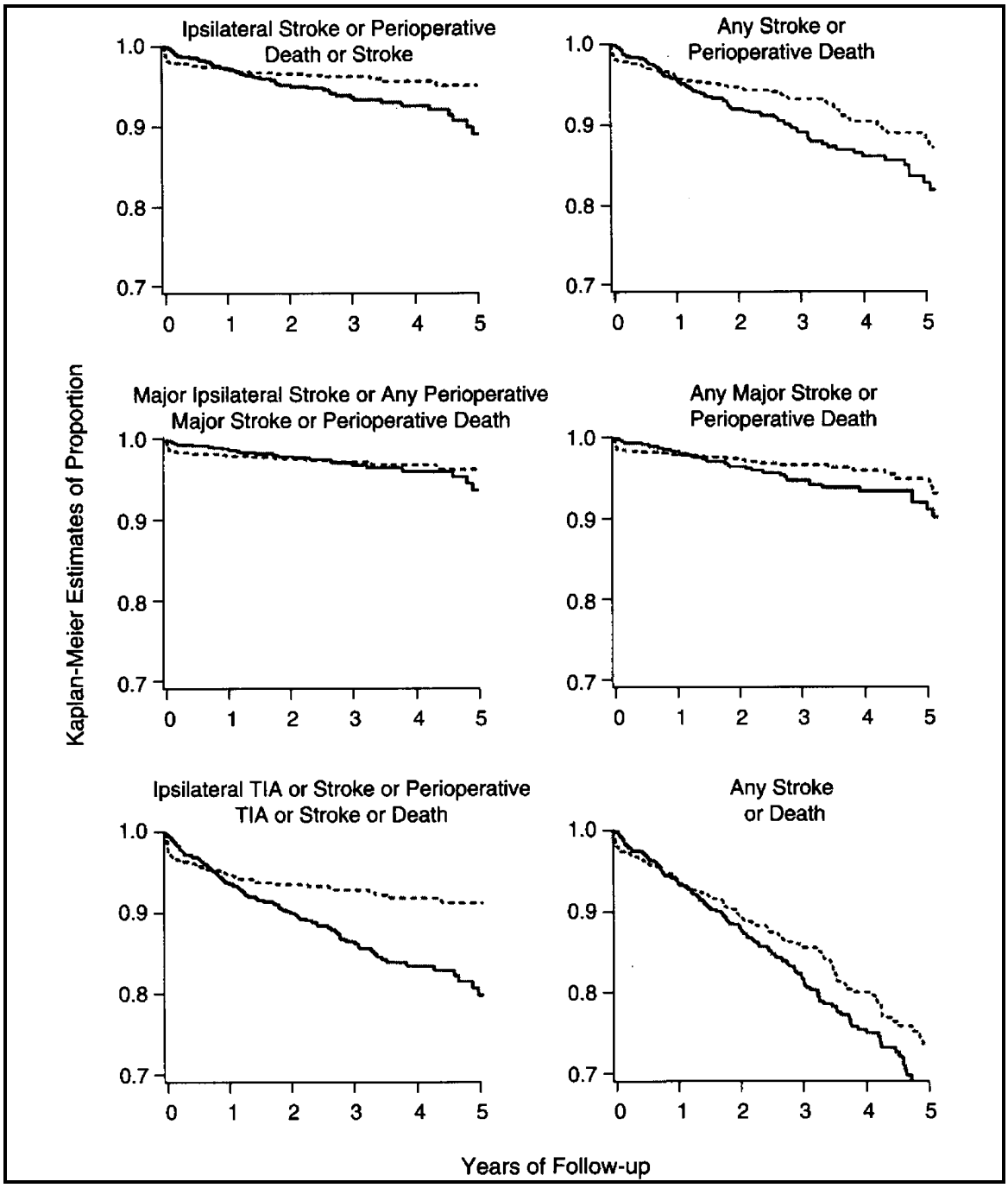

Figure 11 Proportion of patients without end point at a given time during follow-up, by treatment group. Solid line, medical patients, broken line, surgical patients. Reproduced from ACAS, JAMA 1995;273: 1421-8

There were 1662 asymptomatic patients with carotid artery stenosis ≥60% randomized to either surgery (825 patients, 50%) or best medical therapy (834 patients, 50%), with 6 patients excluded from analysis.

One hundred and forty six (9%) patients did not receive the assigned treatment. The stroke or death rate in the peri-operative period was 2.3% (19 patients) in the surgical group, with 5 patients suffering cerebral infarction as a result of arteriography, one of whom died. In the medical group, the risk in the same period was 0.4%.

The study achieved its significance boundary after a median of 2.7 years of follow-up, and the estimated 5-year risk of ipsilateral stroke (including peri-operative stroke) was 11% for the medical group and 5.1% for the surgical group. The reduction in 5-year ipsilateral stroke risk in the surgical group was 53% of the estimated 5-year risk in the medical group. For the primary end-point of ipsilateral stroke and any peri-operative stroke or death, the survival curves cross near 10 months and become significantly reduced in the surgical group by 3 years (*Figure 11*).

In men, CEA reduced the 5-year event rate by 66%; in women the event rate was reduced by 17%, although this difference between genders was not statistically significant. The proportion of women with peri-operative complications was 3.6%, compared with 1.7% for men. However, among patients who had no peri-operative event, 5-year risk was reduced by 56% for women, compared with a reduction of 79% for men.

1.3.7 The MRC Asymptomatic Carotid Surgery Trial (ACST)[17]

The ACST was an international, multicentre study established in 1993 with the aim of being large enough and having long enough follow up to assess the long-term effects of CEA on overall stroke risk and on fatal or disabling stroke among patients with asymptomatic carotid artery stenosis. The trial compared immediate CEA with deferral of any CEA until a definitive indication was thought to have arisen. All other aspects of the management of the patients were left to the discretion of the clinician, and usually included anti-platelet, antihypertensive and lipid-lowering therapies.

Patients were eligible if they had unilateral or bilateral carotid artery stenosis ≥ 60% on ultrasound that had been asymptomatic in the past 6 months, and had no known circumstances likely to preclude long term follow up. The degree of carotid artery stenosis recorded at randomisation was generally rounded to 60%, 70%, 80% or 90%.

Patients who underwent CEA were assessed neurologically prior to discharge, then at 4 months, 12 months, and yearly thereafter. The main trial outcomes were peri-operative mortality and morbidity (stroke and myocardial infarction) and the incidence of non-peri-operative stroke (particularly in the ipsilateral carotid territory). The peri-operative period was considered as the 30 days post-CEA.

In the ten years between 1993 and 2003, 3120 patients were randomised in 126 centres in 30 countries, with 1560 allocated to immediate CEA and 1560 to deferral of any CEA. Among those allocated to immediate CEA, half had ipsilateral surgery by 1 month after

randomisation, 88% by 1 year, and 91% by 5 years. The main reason for not undergoing surgery was patient choice. The median time from randomisation to surgery was 1 month, so most patients were still neurologically asymptomatic at the time of surgery. In this group, 1348 patients underwent a total of 1405 procedures, and the risk per CEA of peri-operative stroke or death was 2.8%. Among those allocated deferral of any CEA, 229 patients underwent a total of 245 CEAs within 5 years of randomisation (half of which were because of the occurrence of neurological symptoms after randomisation), and the risk per CEA of peri-operative stroke or death was 4.5%. These risks were not significantly different, and overall the risk per CEA of peri-operative stroke or death was 3.1%.

Figure 12 shows the main 5-year results for all strokes (including peri-operative deaths) and for non-peri-operative strokes, subdivided by the severity of the stroke. For any type of stroke or peri-operative death, the risk was 6.4% in those patients randomized to immediate CEA, versus 11.8% for those in the "deferred surgery" group. In Figure 12A the early hazards of being allocated immediate surgery are clearly seen, as are the subsequent benefits of successful surgery. Most of these surgical hazards occurred within the first few months (when most of the immediate surgery takes place), after which over the next 5 years the annual stroke rate is much lower among those allocated immediate CEA. At 2 years the lines in Figure 12A cross over, and at 5 years the absolute difference between them is highly significant.

Overall, including both the surgical hazards and the later benefits, there was a highly significant net reduction in the 5-year risk of stroke or peri-operative death in those allocated immediate surgery. The difference in the net 5-year risk of fatal or disabling

stroke or peri-operative death in Figure 12B was significant. For peri-operative death or fatal stroke, the 5-year risk was 2.1% vs. 4.2% (net gain 2.1%).

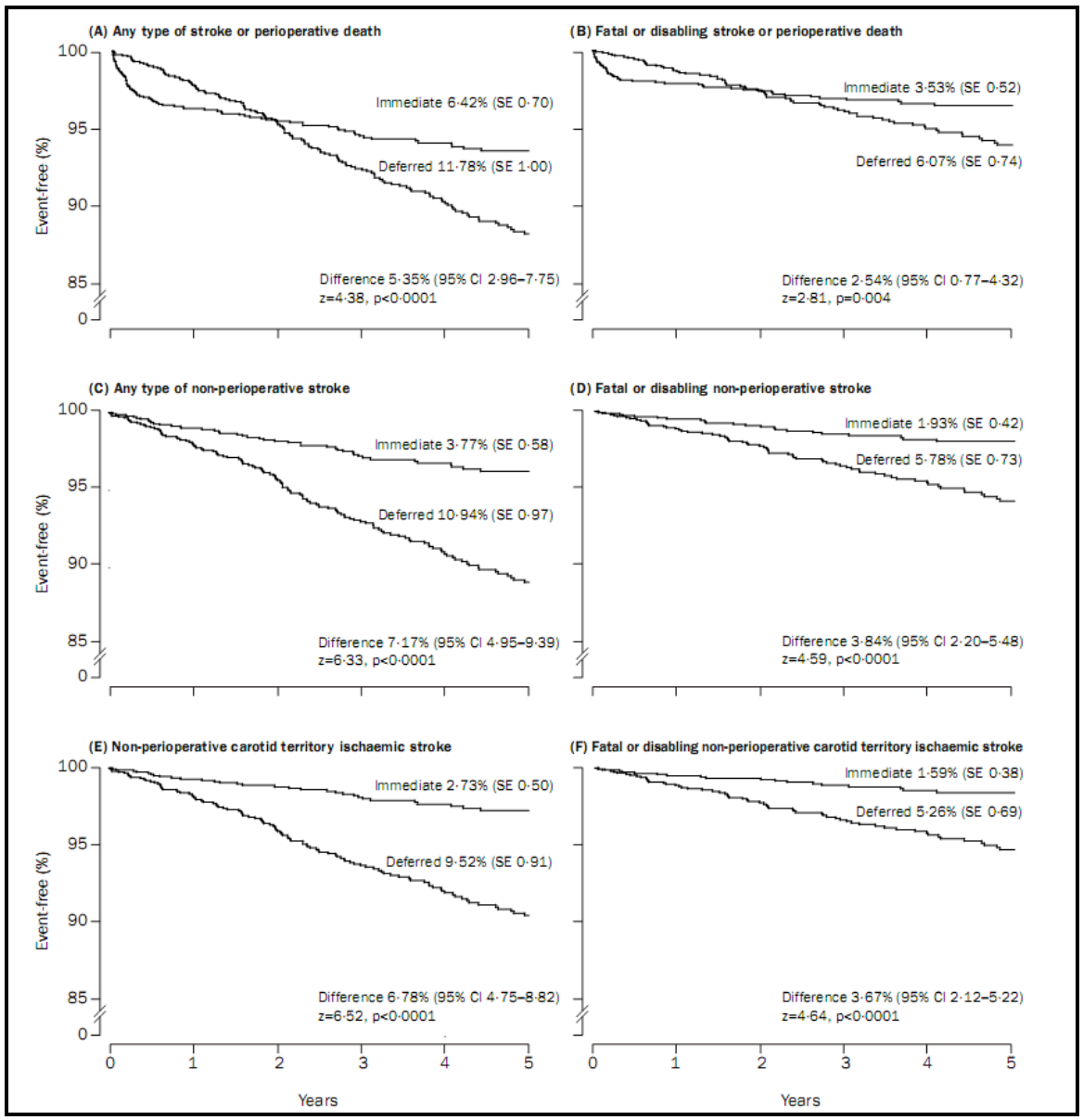

Figure 12 5-year risks of various types of stroke. Reproduced from ACST, Lancet 2004;363: 1491-502

1.3.8 Interpretation of the trials

The results of the multi-centre randomized trials are summarized in Table 3. A consensus was proposed following the reporting of these trials, and the American Academy of Neurology made a statement in 2005. They concluded that CEA was effective for recently symptomatic (within the previous 6 months) patients with 70% to 99% ICA stenosis, providing the peri-operative stroke/death rate was <6%. CEA should not be considered for symptomatic patients with less than 50% stenosis. It was reasonable to consider CEA for patients aged between 40 and 75 years, with asymptomatic stenosis of 60% to 99%, only if the surgical stroke or death frequency could be reliably documented to be <3%. They recommended that any patient being considered for surgery should have at least a five-year life expectancy, since peri-operative strokes pose an "up-front" risk to the patient and the benefit from CEA emerges only after a number of years.[69]

Despite the beneficial role for CEA demonstrated by the randomised trials, debate persists about the application of the results in routine clinical practice. Strictly, the results are only applicable to those surgeons who took part[91] – a pertinent consideration since the long-term benefit of CEA is inextricably linked to the initial surgical risk. In the studies of symptomatic patients, the surgeons who participated were chosen because of their high level of expertise and their low complication rates.[80] NASCET noted that if the risk of disabling stroke and death associated with CEA exceeded the levels they reported (2.0%), the small benefit of surgery in patients with stenoses of 50% to 69% is eliminated.

ACAS only accepted surgeons with an excellent safety record, rejecting 40% of initial applicants and subsequently barring from further participation some surgeons who had adverse operative outcomes during the trial.[92]

Stenosis/%	Surgical risk/%	Medical risk/%	Absolute risk reduction/%	Relative risk reduction/%	Strokes prevented/1000 CEA
ECST					
<30	9.8 at 5 years	3.9 at 5 years	-5.9	not applicable	not applicable
30-49	10.2 at 5 years	8.2 at 5 years	-2.0	not applicable	not applicable
50-69	15.0 at 5 years	12.1 at 5 years	-2.9	not applicable	not applicable
70-99	10.5 at 5 years	19.0 at 5 years	8.5	45	83 at 5 years
NASCET					
30-49	14.9 at 5 years	18.7 at 5 years	3.8	20	38 at 3 years
50-69	15.7 at 3 years	22.2 at 3 years	6.5	29	67 at 3 years
70-99	8.9 at 3 years	28.3 at 3 years	19.4	69	200 at 3 years
ACAS					
60-99	5.1 at 5 years	11.0 at 5 years	5.9	53	59 at 5 years
ACST					
60-99	6.4 at 5 years	11.8 at 5 years	5.4	46	54 at 5 years

Table 3 Long-term risk of ipsilateral stroke, including peri-operative stroke or death. Adapted from Naylor et al. Eur J Vasc Endovasc Surg 2003;26:115-29

Likewise, ACST also stipulated that contributing surgeons must display a 30-day stroke or death rate below 6%. However, in the US, >93% of CEAs are performed in "non-NASCET" centres,[93] and this clearly has implications regarding the application of the international trial results. In an audit carried out in parallel with NASCET, the operative mortality was almost 5 times that reported in the trial.[94]

With the risks of surgery at the levels described in the trials, CEA confers a 45% relative reduction in the long-term risk of stroke in symptomatic patients with a severe (>70%) stenosis, as compared to best medical therapy alone. But what if the operative risk could be reduced to zero? CEA would then confer an overall 75% relative risk reduction in stroke,[95] and understanding the causes of peri-operative stroke is perhaps the key to lowering the rate of CEA-related stroke.

1.4 The aetiology of peri-operative stroke

Intracerebral haemorrhage accounts for a minority of peri-operative strokes (0.4-1% of all operations).[96] Although several mechanisms have been proposed to explain post-CEA stroke, the majority are ischaemic and follow one of two pathological events – thromboembolism or haemodynamic failure.[97] In addition, it is important to differentiate between strokes apparent upon recovery from anaesthesia (the intra-operative stroke) and those occurring sometime after recovery from anaesthesia (the post-operative stroke); they have different aetiologies.[98]

1.4.1 Intra-operative stroke

A number of ischaemic insults can occur intra-operatively which have the potential to cause a stroke. Although ipsilateral haemodynamic insufficiency can be precipitated by global hypotension, and during the carotid clamping phase of surgery,[99,100] this probably accounts for less than 20% of all intra-operative strokes.[97] Thus, the majority of intra-operative strokes are thromboembolic in origin.[101] Cerebral embolization can occur at several intra-operative stages, from inadvertent technical error during dissection of the atheroma-bearing artery; as a complication of shunt deployment; from disruption of the atheroma by the arterial clamp or following on table thrombosis.[102,103]

1.4.2 Early post-operative stroke

The important distinction is that the patient awakes from surgery neurologically intact, with the subsequent development of a sensorimotor deficit that is usually progressive. Despite this period of neurologically normal recovery, the early post-operative stroke results from a dynamic process, initiated during the procedure itself. The most common cause of early post-operative stroke is thrombosis developing and propagating at the site of the endarterectomy[104] or in association with a technical error.[105,106] In addition to the technical errors implicated in intra-operative stroke, thrombus formation can also be initiated at, or near, the operative area by other technical complications of endarterectomy, including: intimal flap; a kink caused by additional elongation after endarterectomy; intramural haematoma; or an irregular/constricting suture line.[98]

1.4.2.1 The cause of peri-operative stroke after CEA[100]

Riles et al. reviewed all patients who sustained a stroke in the New York University Medical Center within 30 days of primary CEA from 1965 to 1991, with emphasis on the cause of peri-operative stroke.[100]

During this period 3062 primary CEAs were performed on 2365 patients. The procedure was mostly performed under local anaesthetic with selective shunting. The determination of the cause of intra-operative stroke was made by correlation of the onset of neurological symptoms with specific operative events in the awake patient, such as carotid artery dissection, clamping and de-clamping. For those patients with post-operative strokes, an effort was made to establish the cause of the stroke either by rapid re-exploration of the carotid artery, arteriography, CT head scanning, non-invasive testing or a combination of these methods.

The peri-operative stroke rate was 2.2% (66 patients) over the entire series, with the specific or most probable cause determined in 63 cases. More than 20 different mechanisms were found to be responsible for peri-operative strokes, but most could be grouped into broad categories: ischaemia during carotid artery clamping (n=10); post-operative thrombosis and embolization (n=25); intra-cerebral haemorrhage (n=12); strokes from other mechanisms (n=8) and; stroke unrelated to the reconstructed artery (n=8).

Post-operative thrombosis and embolization was the mechanism responsible for 25 of the strokes after CEA, and this group further divided: immediate post-operative internal carotid

artery thrombosis with embolization and; post-operative embolization without evidence of arterial occlusion. There was a common mechanism - a technical imperfection at the endarterectomy zone.

Thrombosis accounted for 15 peri-operative strokes, 9 of which occurred while the patient was still in the recovery area, and 4 others within 10 hours of the operation, with only 2 occurring more than 24 hours post-operatively. The fact that almost all of these patients had tolerated carotid artery clamping intra-operatively, suggested that the stroke was not due to the obstruction of blood flow by the thrombus *per se*, but rather from embolization of evolving thrombus prior to the occlusion. These patients all underwent emergent return to theatre, and on re-exploration the technical defect was usually identified at the point of platelet aggregation. Identifiable defects included clamp injuries, kinks in redundant vessels, ledges at the end of the endarterectomy, stenosis at the closure of the arteriotomy, and rough endarterectomy surfaces.

1.5 Intra-operative monitoring methods of cerebral perfusion

The prevention of CEA-related stroke is clearly preferable to its subsequent treatment, and a number of methods have been developed for monitoring cerebral perfusion during the procedure. However, although generally the aim is the identification of new neurological impairment sufficiently early to allow prompt correction of the cause,[107,108] monitoring has sometimes been viewed as simply a means for determining the need for shunt placement.[109] Clamping the carotid artery gives rise to focal or regional hypoperfusion, with the potential for ischaemic stroke. Animal studies have demonstrated that a cerebral perfusion of

$\geq$20mlmin^{-1} per 100g of brain tissue maintains normal function, that 10 to 15mlmin^{-1} per 100g of brain tissue causes neuronal dysfunction, and that <10mlmin^{-1} per 100g of brain tissue results in irreversible damage.[110,111] Modalities for assessing the integrity of cerebral perfusion during CEA can be described in terms of how closely the variables measured actually relate to cerebral blood flow. Most of the available methods offer indirect assessment.

1.5.1 Indirect assessment of cerebral blood flow

1.5.1.1 Awake testing

The neurological evaluation of conscious patients is regarded as a robust method for assessing cerebral tolerance to carotid occlusion during CEA, but is clearly precluded for patients under general anaesthetic. The hypothesis that local anaesthesia is better than general anaesthesia for CEA is based on the idea that it is associated with more appropriate and less frequent shunt use, fewer cardiorespiratory complications and preserved cerebrovascular autoregulation.[112] One potential drawback of awake testing is that patient responses can be delayed in comparison to the underlying haemodynamic fluctuation.[113] Conversely, there is probably a reduction in the damaging effect of cerebral ischaemia induced by the lowered cerebral metabolic rate associated with inhalational anaesthetic agents.[109] This method of intra-operative monitoring has been viewed by some as the gold standard.[114,115]

1.5.1.1.1 General anaesthesia versus local anaesthesia for carotid surgery (GALA)[116]

This was a two-arm, parallel group, multi-centre randomised controlled trial of general anaesthesia versus local anaesthesia for CEA, which recruited 3526 patients from 95 centres in 24 countries. The outcomes assessed at 1 month were: stroke (including retinal infarction), myocardial infarction, death (and cause), TIA and other complications after surgery, between randomisation and 30 days after anaesthesia.

Primary outcome data were available from 1752 of 1753 patients allocated to general anaesthesia and 1771 of 1773 patients allocated to local anaesthesia. Primary outcomes occurred in 84 of 1752 (4.8%) patients allocated to general anaesthesia and 80 of 1771 (4.5%) patients allocated to local anaesthesia, a difference that was not statistically significant. The conclusion from this study was that both methods of anaesthesia were equally acceptable for the conduct of CEA, and the choice should be guided by local expertise and preference.

1.5.1.2 Electroencephalogram (EEG)

EEG changes are associated with cerebral perfusion of $<20 ml min^{-1}$ per 100g of brain tissue.[117] However, in studies of CEA with local/regional anaesthesia and simultaneous EEG monitoring, it has been shown that EEG changes can be absent while the patient is displaying neurological impairment, with the converse also true.[118]

1.5.1.3 Somatosensory evoked potentials (SSEP)

Median nerve-generated SSEP monitoring has been developed as a replacement, or adjunct to, EEG. Prolongation of the central conduction time, a measure that reflects intracranial conduction between the foramen magnum and the somatosensory cortex, or reduction in amplitude of the primary sensory cortical component of the evoked potential are both features indicative of cerebral ischaemia.[109]

1.5.1.4 Stump pressure measurement

Carotid stump pressure is the mean arterial back pressure in the distal portion of the clamped internal carotid artery. It is influenced both by the systemic mean arterial pressure, and the flow contribution from ipsilateral and contralateral collateral vessels.[119] Studies have reported an association between the stump pressure and EEG changes, and although there may be a correlation with peri-operative morbidity or mortality,[119,120] the information provided by the technique tends primarily to be used in making the decision to selectively deploy a carotid shunt.[121]

1.5.1.5 Continuous jugular venous oxygen saturation

In this invasive method, a fibre-optic catheter transmitting infra-red light is placed in the jugular vein. The absorption of the light is measured, and a processor calculates the saturation of the jugular venous blood.[112] Assuming the haematocrit and oxygen dissociation curve are stable, changes in the saturation of the jugular venous blood reflect variation in the cerebral oxygen supply to oxygen consumption ratio.

1.5.1.6 Near infra-red spectroscopy

This system provides a non-invasive parallel to jugular venous oxygen saturation, and exploits the fact that the scalp and skull are relatively transparent to near infra-red light. The concentrations of three cerebral chromophores are directly influenced by changes in cerebral oxygen supply, and a probe incorporating an emitter and detector can be applied to the forehead and used to calculate the cerebral oxygen supply.[112]

1.5.2 Direct assessment of cerebral blood flow

1.5.2.1 Transcranial Doppler (TCD) Ultrasound

The application of the Doppler-shift principle to cerebral blood flow assessment was described in the early 1980's.[122,123] A 2MHz pulsed signal is focused to a depth of 4.5-6.0cm on the middle cerebral artery through the temporal bone. Solid components of the blood (primarily erythrocytes) reflect the signal, and distort it according to the Doppler-shift principle, and the resulting reflected waveform gives information about systolic, diastolic and mean blood flow velocity. But, in addition to haemodynamic information, TCD also has the advantage of being able to provide information concerning embolic phenomena. Gaseous or solid microemboli within the middle cerebral artery are detected as high intensity, transient, or "cerebral microembolic", signals, which are unidirectional and randomly occurring within the cardiac cycle.[124] Several reports have shown that the introduction of standardised TCD monitoring during CEA results in a decrease in the intra-operative stroke rate.[125,126] In terms of intra-operative cerebral perfusion monitoring, this is the only modality that can provide direct information about microemboli,[108,127] which has

important implications when the aetiology of intra-operative strokes is taken into consideration.

II

Haemostasis and Platelet Physiology

2.1 Normal haemostasis

Improved understanding of the pathophysiology of various clinical clotting disorders has increasingly led to recognition of the limitations of the classical "cascade" theory of haemostasis. Traditionally coagulation was presented as two pathways: the *intrinsic* involving factor XII (FXII), FXI, FIX and FVIII; and the *extrinsic* involving FVII. These relatively independent pathways converge into a common pathway, which includes FX, FV, FIIa (thrombin) and fibrinogen. Although this model is supported by laboratory observations, it fails to adequately explain the situation observed *in vivo*.[128] Current concepts have adopted a cell-based approach to haemostasis, with the cascade of protease reactions tending to occur on cell surfaces, in a parallel, rather than fixed sequence.

The formation of a platelet/fibrin mass at a site of injury, and the localisation at that site of the powerful procoagulants activated in the process, is a delicate balance, without which overwhelming, widespread thrombosis would readily ensue.[129] The equilibrium is accomplished, in part, by the confinement of the coagulation reactions on specific cell

surfaces, and a further key regulatory mechanism is the separation of the constituent cell types until such time as an injury makes activation of coagulation desirable.[130]

It has been suggested that haemostasis occurs in overlapping, yet distinct steps, namely: *initiation*, *amplification* and *propagation*,[131] and primarily requires the contribution of two cell types: tissue factor (TF)-bearing cells, and platelets. Uncontrolled thrombotic dissemination is prevented by *localisation*, while fibrinolytic dispersion of the thrombus results in *resolution*.

2.1.1 Initiation phase

Initiation is localized to cells that express tissue factor (TF). TF is a membrane-bound protein structurally unrelated to the rest of the coagulation proteins.[132,133] It is normally found on cells which are not ordinarily in contact with blood, although it can also be expressed by monocytes and endothelial cells in inflammatory states.[131] It is clear that normal circulating platelets neither contain nor express TF.[131] After vascular injury, the coagulation protease cascade is initiated when TF within the vessel wall is exposed to, and forms a complex with, FVII in plasma (*Figure 13*).[134,135] The activated FVII (FVIIa)/TF complex subsequently triggers the activation of small amounts of FIX and FX.[130] "Prothrombinase" (FXa/Va) complexes are formed on the TF-bearing cells when small amounts of FXa associate with FVa,[136] which itself can originate from several sources. FV can not only be activated by FXa directly,[137] but also by other non-coagulation proteases.[138] The prothrombinase complex of FXa and FVa cleaves prothrombin (FII) to generate thrombin (FIIa).[128]

Whilst the FXa localized to the cell surface is relatively protected from inactivation by plasma protease inhibitors, any FXa that dissociates from the TF-bearing cell is rapidly inhibited by TF pathway inhibitor (TFPI) or antithrombin (AT). Through this inhibitory mechanism, FXa activity is effectively localized to the surface on which it was formed. Conversely, FIXa can move from the TF-bearing cell to a neighbouring platelet or other cell surface, because it is not susceptible to TFPI and although it is inhibited by AT, it is affected much more slowly than FXa.[130]

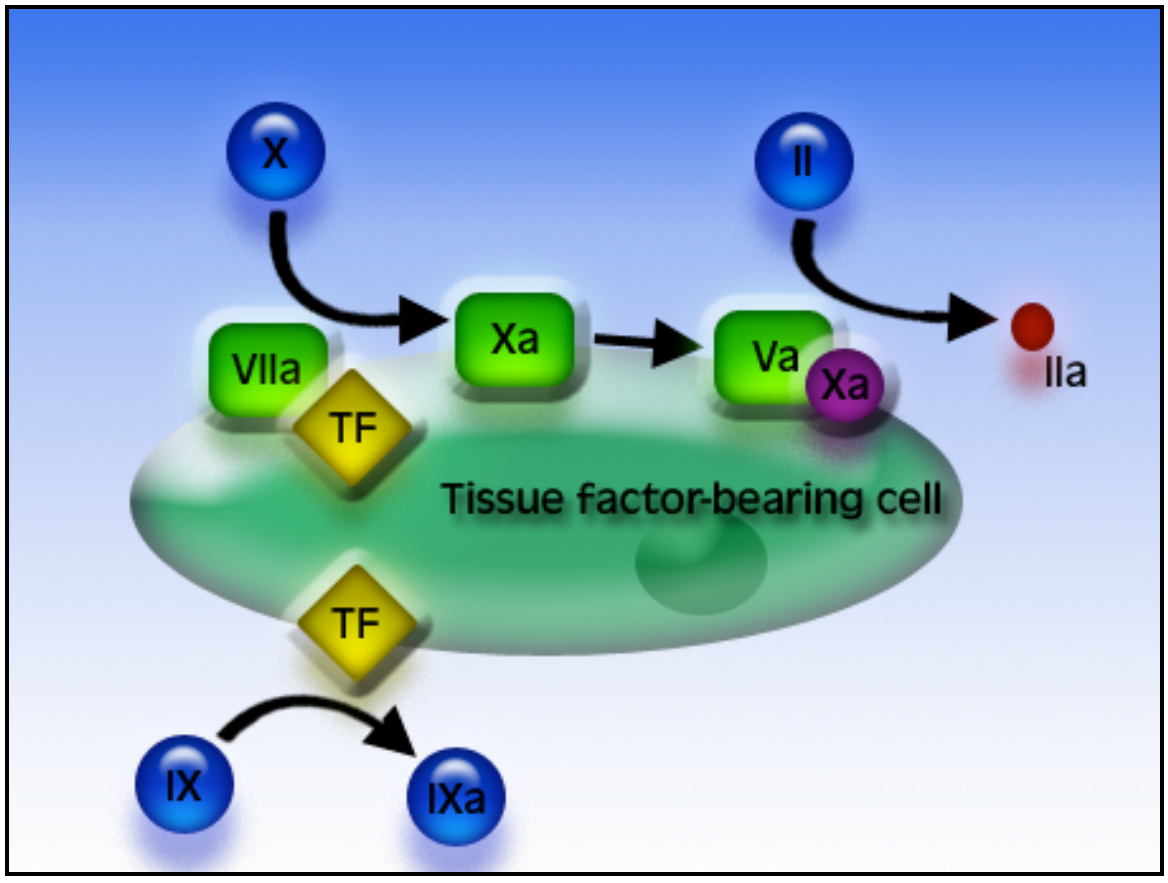

Figure 13 Initiation - the "extrinsic pathway" occurs on TF-bearing cells. Adapted from Monroe et al. Arterioscler Thromb Vasc Biol 2006;26:41-8

2.1.2 Amplification phase

Although the amount of thrombin generated on TF-bearing cells is relatively small, it is critical in amplifying the initial procoagulant signal, with one major function being the activation of the collagen-bound platelets,[139,140] mediating further activation via the protease activated receptor (PAR) mechanism.[128] The dual stimulation of platelets by thrombin and collagen has a synergistic effect on platelet activity.[141] Thrombin also moves from the TF-bearing cell to nearby platelets, where it binds to their high-affinity receptor, GPIb.[142] In addition, the thrombin formed during the initiation phase activates FV, FVIII (releasing it from vWF) and FXI on the platelet surface.[136,143-146] Thus, the platelet is now activated, and has FVa and FVIIIa bound to its surface such that assembly of the procoagulant complexes and the generation of much larger amounts of thrombin can ensue.[131]

2.1.3 Propagation phase

This phase takes place on activated platelets, and results in large-scale thrombin generation (*Figure 14*). Fully activated platelets change shape and degranulate, secreting partially activated FV from their α granules, which as before, is activated by FXa and thrombin.[147] The FIXa which was activated during the initiation phase on TF-bearing cells, and diffused to the platelet surface, now binds to FVIIIa on that membrane, forming a "tenase" (FVIIIa/IXa) complex.[130,131] In addition, plasma FXI can bind to activated platelets, facilitating its activation by thrombin[148] - the accompanying FXIa can then provide further FIXa directly on the platelet surface. Since FXa has no means of effectively moving from the TF-bearing cell to the platelet, it is instead provided directly on the platelet surface by

the FVIII/IXa complex. FXa rapidly associates with platelet surface FVa and produces a burst of thrombin generation of sufficient magnitude to clot fibrinogen and form a haemostatic fibrin clot.

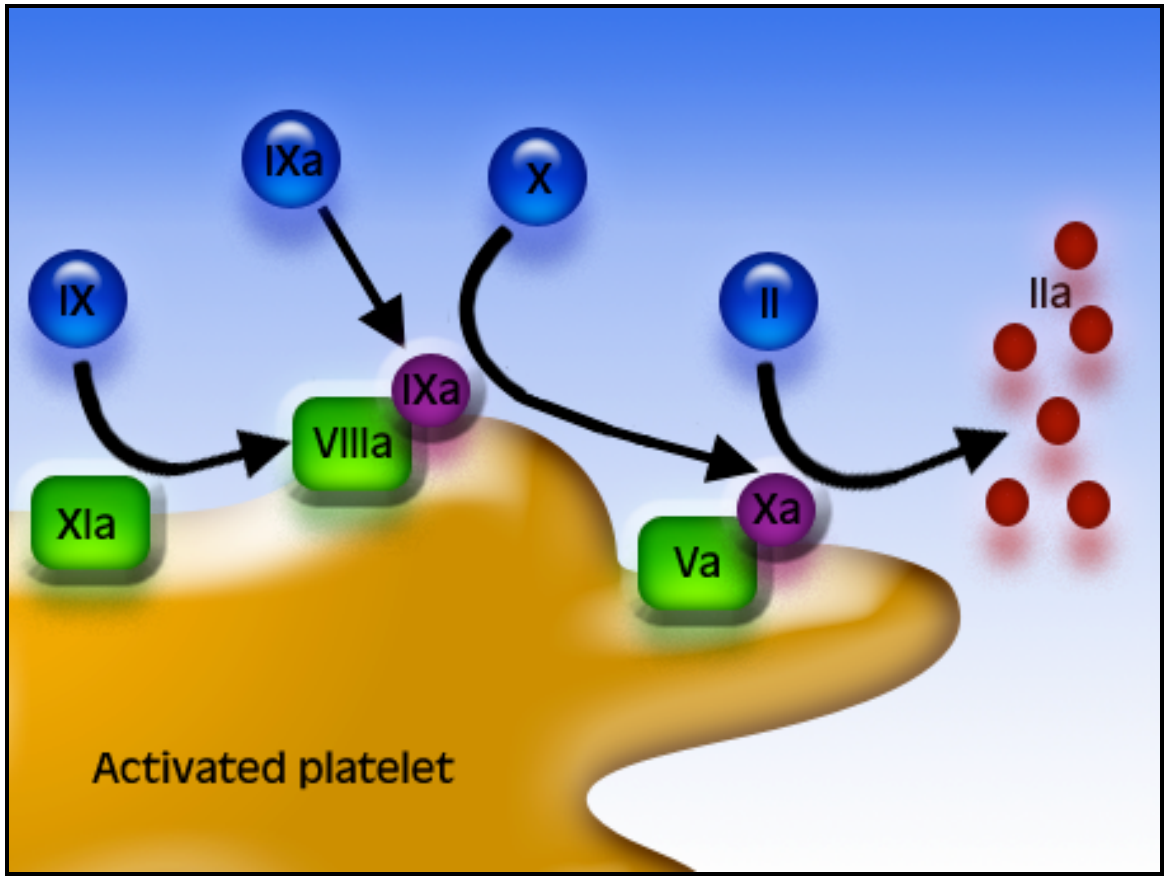

Figure 14 Amplification and propagation - the "intrinsic pathway" occurs on activated platelets. Adapted from Monroe et al. Arterioscler Thromb Vasc Biol 2006;26:41-8

2.1.4 Localization

The balance between preserving blood in the fluid phase until injury occurs, then producing a haemostatic thrombus, which is prevented from proliferation to the extent of vascular compromise, is maintained primarily by localization. The rapid and effective adhesion of

platelets at the site of injury allows the production of concentrated activated factors exactly where a clot is desirable. During this process, the platelet surface and the extravascular initiating cells are brought into close proximity, thus permitting the direct transfer of procoagulant proteases between the initiating cell and platelet surfaces.[130]

Membrane-bound procoagulant proteases are much less susceptible to inactivation by the plasma protease inhibitors than when they are in solution. As a result, activated factors that diffuse away from the appropriate cellular location are rapidly inhibited. Furthermore, healthy, intact endothelial cells express a range of unique anticoagulant mechanisms which tend to preclude thrombotic proliferation. These include the endothelial thrombomodulin (TM)/protein C/protein S system that inactivates FVa and FVIIIa.[149] TM is a receptor for thrombin, and is expressed at high levels on endothelial cells. When it binds to TM, the specificity of thrombin is altered, such that it is no longer able to clot fibrinogen or activate platelets.[150] Endothelial cells also express cell-surface ADP-ase activity that suppresses amplification of platelet activation by ADP release.[151,152] Furthermore, endothelial cell surfaces express heparanoid glycosaminoglycans that can bind and enhance the activity of plasma AT and TFPI.[153,154]

2.1.5 Fibrin clot formation

Activation of the clotting system results in thrombin generation. Thrombin catalyses cleavage of the fibrinogen molecule at precise points, thus revealing specific polymerisation sites. This results in a dramatic change in solubility that causes the molecules to aggregate and form fibrin fibres.[155] Double-stranded, twisting fibrils form,

which are arranged in a half-staggered, overlapping arrangement.[156] Fibrils branch out and create structures that result in a complex network of fibres, and there are likely two different types of branching that define the structure of the clot,[157] with FXIIIa providing a degree of covalent cross-linking.[158]

2.2 Platelets

Platelets are derived from the progenitor megakaryocyte in the bone marrow. The megakaryocyte is a highly specialised precursor cell that functions solely to produce platelets and release them into the circulation. The platelet has an equatorial diameter of 2-3 μm, and survives in the circulation for between 7 and 10 days. They have a relatively small, discoid character, which results in a haemodynamic marginalisation to the vessel edge, thus ensuring the rapid detection and response to vascular wall damage.

Circulating platelets detect damage to the walls of blood vessels through the expression of surface receptors that recognize exposed connective tissue components, normally covered by endothelial cells, and by the release of soluble factors by endothelial and other connective tissue cells that attract platelets. Once activated, platelets rapidly attach, change shape and spread over the damaged area.

2.2.1 Platelet structure

The platelet membrane is an asymmetrical phospholipid bilayer in which a range of proteins are embedded, notably adhesive, stimulatory and inhibitory receptors.[159] The

platelet does not possess a nucleus, but does nonetheless contain a number of intracellular organelles, including a circumferential microtubular band, a pair of membrane systems (the dense tubular system and the plasma membrane associated surface connected canalicular system) and three types of secretory granules (α granules, dense granules and lysosomes).[159] The discoid shape of the circulating platelet is maintained by an internal cytoskeleton composed of actin and tubulin polymers and their associated proteins.

2.2.1.1 Platelet receptors

Although there is some evidence for residual protein synthetic capacity from mRNA disseminated from the megakaryocyte, platelets cannot generally adapt by *de novo* protein synthesis because they are anucleate.[160,161] A vast array of physiological functions and potential pathological events necessitate the platelet to be equipped with a multitude of pre-synthesized molecules and receptors (*Table 4*). A variety of different types of receptor are present in the platelet storage granule membranes, to be expressed on the platelet surface itself only after specific activation.

2.2.1.1.1 Integrins

Integrins are a class of adhesive and signalling molecules present on most cell types, generally involved in linking adhesive molecules to the cytoskeleton.[162] They usually exist in either high or low affinity states, dependent on cytoplasmic signalling and phosphorylation of their cytoplasmic domains. Platelets possess members of three families of integrins (β1, β2 and β3) and in total six different integrins: α2β1,α5β1, α6β1, αLβ2,

αIIbβ3 and αvβ3. αIIbβ3 (the GPIIb-IIIa complex) is the only integrin unique to platelets, and is the major platelet integrin (and receptor) with 50,000-80,000 copies per platelet.

2.2.1.1.2 Seven trans-membrane receptors

The seven trans-membrane receptor group is the major agonist receptor family in cells generally, and is also very well represented on platelets.

2.2.1.1.2.1 Thrombin receptors

Thrombin is a critical platelet agonist. Thrombin receptors are major representatives of the seven trans-membrane receptor group on platelets. The first receptor to be identified and characterized was protease activation receptor-1 (PAR-1), which is the predominant receptor for thrombin-mediated platelet activation.[163] Other PAR class receptors have been identified on platelets as well.[164] Human platelets possess PAR-4,[164] which is sensitive to 10 times higher concentrations of thrombin than PAR-1 – possibly in order to handle situations where platelets are exposed to high doses and PAR-1 is down-regulated.

2.2.1.1.2.2 ADP receptors

ADP is an important primary platelet agonist. In addition, platelet dense granules contain a high concentration of ADP, and the release of ADP by platelets stimulated by other agonists such as thrombin and collagen contributes substantially to platelet aggregation. The accepted model for ADP receptors on platelets involves three separate components: $P2Y_1$ and $P2Y_{12}$ belonging to the seven trans-membrane family, and $P2X_1$, which is a

calcium channel (and probably an ATP receptor) belonging to a different structural family.[165]

2.2.1.1.2.2.1 $P2Y_1$

Research has shown that $P2Y_1$ couples with G_q to mobilize calcium, resulting in shape change and aggregation.[166] A second receptor, $P2Y_{12}$, is necessary for aggregation by lowering cAMP levels through down-regulation of adenylate cyclase.[166]

2.2.1.1.2.2.2 $P2Y_{12}$

This second ADP receptor couples to G_i to inhibit adenylate cyclase.[167] ADP-induced platelet aggregation is initiated by the $P2Y_1$ receptor and amplified in a synergistic fashion by the $P2Y_{12}$ receptor.[166]

2.2.1.1.2.3 Prostaglandin family receptors

2.2.1.1.2.3.1 Thromboxane receptors (TXA_2/PGH_2)

Like ADP, thromboxane A_2 (TXA_2) functions as a positive-feedback mediator during platelet activation. It is known to induce platelet aggregation and to constrict vascular and respiratory smooth muscle.

TXA_2 is produced from arachidonic acid (AA) through conversion by cyclooxygenase-1 (COX-1) and thromboxane synthase. The action of TXA_2 is locally restricted by its short

half-life of 32 seconds – it is transformed into the stable metabolite, thromboxane B_2 (TXB_2).

The TXA_2/PGH_2 receptor is coupled to signal transduction via several G proteins including G_q, G_{12} and G_{13}, leading to a rise in intra-cellular Ca^{2+}, activation of phospholipase A_2 and phospholipase C, and exposure of I/IIIa binding sites.[168] TXA_2 receptor agonists induce tyrosine phosphorylation of several signalling proteins including protein-tyrosine kinase $p72^{SYK}$.[169]

2.2.1.1.2.4 Lipid receptors

2.2.1.1.2.4.1 Platelet-Activating Factor Receptor

Platelet-activating factor (PAF) is a potent pro-inflammatory phospholipid, the action of which is mediated through a G protein coupled membrane receptor. It has wide-ranging effects on a number of tissues, and acts as a strong agonist in the platelet.

2.2.1.1.2.5 Other seven trans-membrane receptors

2.2.1.1.2.5.1 β_2-adrenergic receptor

Adrenaline only induces platelet aggregation in the presence of other agonists and its cloned β_2-adrenergic receptor is thought to couple to only the G_S class of G proteins.[170]

2.2.1.1.2.5.2 Serotonin (5-HT_{2A}) Receptor

The major platelet serotonin receptor is 5-HT_{2A}. In platelets, 5-HT_{2A} is coupled to G-proteins and its occupation by serotonin leads to calcium signaling.[171] Serotonin is a major component of dense granules and is released on platelet activation, therefore acting as a further autocrine agonist.

2.2.1.1.3 Immunoglobulin superfamily

2.2.1.1.3.1 GPVI

GPVI exists in platelets in a complex with the F_c receptor (F_cR) γ chain. The GPVI/F_cRγ chain complex serves, along with $\alpha_2\beta_1$, as the major activating receptor for collagen. GPVI belongs to the immunoglobulin gene (Ig) superfamily[172] and is predominantly a signalling molecule.

2.2.1.1.3.2 FcγRIIA

FcγRIIA (CD32) is a major member of the immunoglobulin family present on platelets, and has a role in immunological defence against bacteria, viruses and parasites. The FcγRIIA receptor is of interest because of its role in heparin-induced thrombocytopenia (HIT)[173] - antibodies develop against complexes of heparin and the platelet α-granule chemokine, platelet factor 4, and the complexes cause platelet activation via FcγRIIA, leading to thrombocytopenia.

Agonist	Receptor
Von Willebrand factor	GPIbα
Collagen	α2β1, GPVI
ADP	$P2Y_1$, $P2Y_{12}$, $P2X_1$
Thromboxane A_2	TP
Thrombin	PAR1, PAR4, GPIb
Platelet-activating factor	PAF receptor
Serotonin	$5\text{-}HT_2$
Vasopressin	V_1
IgG	FcγRIIA
Epinephrine	α_2
Fibrinogen	αIIbβ3

Table 4 Receptors currently identified on human platelets. Reproduced from McNicol A et al. J Pharmacol Sci 2003;93:381-96

2.2.1.2 Dense tubular system

The dense tubular system (DTS) is derived from the smooth endoplasmic reticulum found in the parent megakaryocyte.[174] In human platelets, it is organized in a core-coil structure maintaining the discoid shape of the resting platelet.[175] The DTS is the major calcium sequestrating organelle in platelets and acts to maintain the Ca^{2+} concentration, holding the major releasable Ca^{2+} store.[175,176] The DTS also has a major role in AA metabolism. AA-liberating enzymes and the enzymes involved in the stepwise conversion of AA to TXA_2, prostaglandin PGH-synthase-1 (PGHS-1) and thromboxane synthase, are all found in the DTS.[177,178]

2.2.1.3 Granules

Platelet granule exocytosis plays a major role in thrombosis and wound healing. Platelets contain three types of secretory granule, the contents of each of which may be released following stimulation. α-Granules are the largest and most numerous and contain fibrinogen, growth factors, and cytokines.[179,180] Furthermore, α-granules have glycoprotein receptors embedded in their limiting membranes which promote adhesion between platelets and the matrix. In particular, P-selectin, not expressed on the surface of the resting platelet, is stored in the membranes of the α-granules as well as a portion of the major platelet adherence receptors, GPIbIXV (a receptor for vWF) and the integrin αIIbβ3 (the receptor for fibrinogen).[181] Dense granules contain high concentrations of small molecules, such as ADP/ATP, calcium, magnesium and serotonin.[180,182] Lysosomal granules contain acid proteases, acid glycosidases, acid phosphatases and aryl sulphatases.[180,183]

2.2.2 Platelet activation

The formation of a haemostatic plug requires platelets to undergo rapid morphological changes as they convert from their latent discoid shape into their active, flattened form. Platelets initially adhere to both subendothelial collagen and von Willebrand factor at the site of injury to form a monolayer. This is mediated by the $\alpha_2\beta_1$ integrin and GPVI collagen receptors and the vWF receptor on the platelet surface. Spreading occurs to allow platelets to flatten over the damaged surface, while the elaboration of filopods and the engagement of the specific cell surface receptors facilitate the recruitment of additional platelets. Recruitment is accomplished by both the delivery of P-selectin receptors to the platelet surface and by the release of attractive molecules, such as ADP and serotonin

during secretion, the production and release of TXA_2 and a conformational change in the structure of the major surface platelet integrin, αIIbβ3.[181,184,185]

In the process of activation, the platelet changes shape. This requires the remodelling of the platelet cytoskeleton via the reorganisation of cytoplasmic actin filaments. The discoid shape of the platelet is lost after it makes contact with the surface, and a spherical transformation is induced by the transient rise in cystolic Ca^{2+} that follows receptor ligation. Next, as the platelet flattens over the surface, lamellae are produced, followed by a final, dynamic phase of membrane motility at various points along the lamellae - filopods cored by actin filaments originating in the cell centre extend and are rotated around the periphery of the flattened platelet.[181]

2.2.2.1 Exocytosis of granular products

Dense granules release ADP, serotonin and Ca^{2+}, whilst fibrinogen is secreted from α-granules, all of which are important in the recruitment of platelets to the site of injury. α-granules release growth factors which initiate vascular repair and cytokines, which in conjunction with lysosomal enzymes from lysosomal granules, provide a link to the immune system.[184]

2.2.2.2 Expression of granular membrane proteins

In addition to secreting the contents, the membranes of the platelet granules comprise a number of adhesive proteins (e.g. GPIb, P-selectin, CD36 and several integrins – αIIbβ3,

αVβ3, α5β1, α6β1, α2β1) which are expressed on the surface of the activated platelet, thereby increasing the surface receptor density.[182,186,187]

2.2.2.3 Eicosanoid formation

The availability of non-esterified AA represents the rate-limiting step in the biosynthesis of eicosanoids.[188] The AA cascade is initiated, leading to TXA_2 synthesis from the COX pathway. TXA_2 is a platelet agonist that plays a pro-aggregatory role. It is formed by the action of thromboxane synthase on the prostaglandin endoperoxide H_2 (PGH_2), mainly in activated platelets and macrophages (*Figure* 15).[189,190]

Fatty acid hydroperoxides are the primary intermediates of the non-enzymatic lipid peroxidation process. They are also intermediate metabolites in the enzymatic peroxidation of AA. In platelets they are formed via the bifunctional prostaglandin endoperoxide H synthase (PGHS), which leads to prostaglandin G_2 formation, and via the 12-lipoxygenase (12-LOX) enzyme, which catalyses the formation of 12-(S)-hydroperoxy-eicosatetraenoic acid (12-(S)-HPETE).[191] This is further reduced to 12-hydroxyeicosatetraenoic acid (12-HETE) by glutathione peroxidase.[192] It has been shown that the addition of hydroperoxides, especially 12(S)-HPETE to platelets that have been primed with a non-aggregating concentration of AA results in an increased production of TXB_2 and a potentiation of platelet aggregation.[193]

2.3 Pharmacological manipulation of haemostasis

2.3.1 Anti-platelet therapy

2.3.1.1 Inhibitors of prostaglandin-mediated platelet activation

2.3.1.1.1 Aspirin

The most widely used inhibitor of platelet function, aspirin irreversibly inhibits COX-1 by acetylating the hydroxyl group of serine at position 530, thereby preventing AA binding to the active site of the COX-1 component of PGHS-1 and prohibiting the conversion of arachidonate to the unstable prostaglandin intermediate PGH_2, which is converted to TXA_2 (*Figure* 15). A single dose of 160mg completely abolishes the platelet TXA_2 production (measured as its stable analogue TXB_2), and the evidence suggests that there is generally no greater benefit in the reduction of clinical thrombotic events by using chronic aspirin doses higher than 75mg (*Table 5*).[194] Whilst the major side effects of aspirin (gastrointestional irritation and haemorrhage) are dose-related, its anti-thrombotic effects are not.[195] The effective onset is rapid because aspirin can acetylate platelet COX-1 in the portal circulation.[184]

Because platelets are anucleate and cannot synthesize new proteins in large volumes, the aspirin effect is permanent, lasting for the 7-10 day lifetime of the platelet. COX-1 activity returns only as new platelets are produced and released into the circulation.[196]

Pathology	Lowest effective dose/mg
Transient ischaemic attacks	50
Hypertension	75
Angina	75
Significant carotid stenosis	75
Acute myocardial infarction	160
Acute ischaemic stroke	160

Table 5 Vascular disorders for which aspirin has been shown to be effective and lowest effective dose. Reproduced from Patrono C et al. Arterioscler Thromb Vasc Biol 2008;28:25s-32s

Approximately 100 randomized clinical trials have established the efficacy and safety of aspirin in the prevention of myocardial infarction, ischaemic stroke and vascular death among both high- and low-risk patients.[197] It has been evaluated in both primary and secondary prevention of vascular events. The meta-analysis of the Antiplatelet Trialists' Collaboration (ATC) demonstrated a 25% reduction of vascular death, myocardial infarction or stroke for anti-platelet therapy (primarily aspirin) versus placebo in patients with acute or previous cardiovascular or cerebrovascular events.[194] The Second International Study of Infarct Survival (ISIS-2) found a similar (25%) reduction in mortality at 5 weeks, and a 50% reduction in non-fatal stroke for patients who received aspirin starting within 24 hours of acute myocardial infarction, when compared to placebo.[198] Analysis of the published literature demonstrated a reduced incidence of serious vascular events in specific high risk groups, and suggested that the indications for aspirin use should be expanded to secondary prevention in these populations, such as those with diabetes, peripheral vascular disease and carotid stenosis.[199,200] Anti-platelet therapy

decreased the risk of non-fatal MI by one third, non-fatal stroke by one quarter, and vascular mortality by one sixth.

The results of the Physician's Health Study are similar, showing a 44% reduction in the incidence of first myocardial infarction over a 5 year span in middle-aged men taking aspirin compared to those receiving placebo.[201]

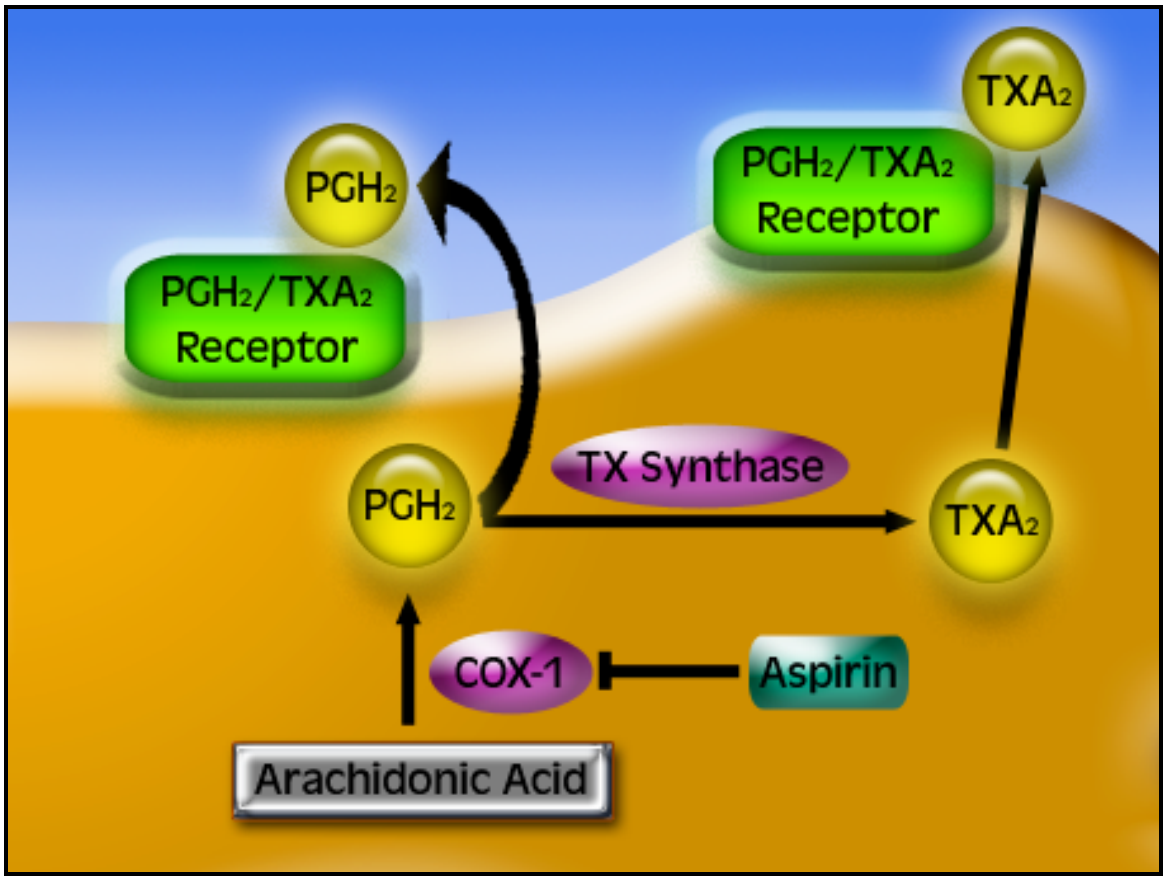

Figure 15 Pharmacological site of action of aspirin

2.3.1.1.1.1 Aspirin resistance

The term "aspirin resistance" has variously been used to describe the ability of aspirin to produce a measurable response on *ex vivo* tests of platelet function, to inhibit TXA_2 biosynthesis *in vivo*, or to protect individual patients from recurrent thrombotic complications.[202]

2.3.1.1.1.1.1 Failure of aspirin to prevent clinical events

This phenomenon has been called "clinical aspirin resistance",[203] but the term "treatment failure" is probably more accurate.[202] However, given the multi-factorial nature of pathological thrombus formation, and given the fact that thrombus formation is by far the most common, but not the only mechanism leading to vascular occlusion, it would be unrealistic to expect aspirin, or any other anti-thrombotic drug, to prevent clinical events in all patients at risk.[204]

2.3.1.1.1.1.2 Failure of aspirin to inhibit platelet function in vivo or in vitro

None of the methods currently available for the assessment of *ex vivo* platelet function is ideal, and debate exists as to the suitability of the various tests to define aspirin resistance. The prevalence of aspirin resistance is largely dependent on the assay utilized and the population studied, ranging anywhere from 8-45%.[205]

2.3.1.1.1.1.3 Failure of aspirin to inhibit TXA_2 production

Studies correlating biochemical aspirin resistance to clinical outcomes have shown that where it is present, patients are more likely to sustain future ischaemic events. Eikelboom et al. demonstrated that patients with atherosclerosis who had elevated levels of urinary 11-dehydrothromboxane B_2 (the excreted stable TXA_2 metabolite) while on aspirin were more likely to sustain future ischaemic events.[206]

2.3.1.2 Thienopyridine derivatives

These anti-platelet drugs have no *in vitro* effect on agonist-induced platelet aggregation, requiring hepatic metabolism by cytochrome P450.[207] They selectively and irreversibly bind to $P2Y_{12}$, thereby preventing ADP-induced activation of the αIIbβ3 integrin, fibrinogen binding and platelet aggregation.[208]

2.3.1.2.1 Ticlopidine

Thebault showed in 1975 that the thienopyridine derivative ticlopidine inhibits ADP-induced primary platelet aggregation with maximum effect after 15 days oral administration and with evidence of irreversibility of action.[209] The Stent Anticoagulation Restenosis Study (STARS),[210] the Multi-centre Aspirin and Ticlopidine Trial after Intracoronary Stenting Study (MATTIS),[211] and the Intracoronary Stenting and Antithrombotic Regimen Study (ISAR)[212] each demonstrated that there were significantly fewer restenoses in patients undergoing stent insertion given a ticlopidine/aspirin combination compared to aspirin alone.

2.3.1.2.2 Clopidogrel

The Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) trial demonstrated an 8.7% relative risk reduction in patients receiving clopidogrel for myocardial infarction, ischaemic stroke or vascular death.[213] If myocardial infarction was taken as the outcome, there was a 19.2% relative risk reduction in the patients receiving clopidogrel. The Clopidogrel in Unstable Angina to Prevent Recurrent Ischaemic Events (CURE) study compared the effects of aspirin with that of a clopidogrel/aspirin combination in patients with acute coronary syndromes.[214] There was a 20% relative risk reduction for patients given the clopidogrel/aspirin combination, and although this was associated with an increased risk of bleeding, the incidence of severe haemorrhage was similar in the two arms.

The Clopidogrel Aspirin Stent International Cooperative (CLASSICS) Study compared the effects of clopidogrel/aspirin combinations versus ticlopidine/aspirin combinations in patients undergoing coronary stenting, and showed that the clopidogrel/aspirin combination was at least as effective as the ticlopidine/aspirin combination in preventing post-stenting cardiovascular events; clopidogrel was superior with respect to adverse effects.[215]

In contrast, the results of the Management of Atherothrombosis with Clopidogrel in High-Risk Patients with Recent Ischaemic Attacks or Ischaemic Stroke (MATCH) study looked at the benefit of combination aspirin and clopidogrel treatment for the prevention of recurrent stroke in high-risk patients. The relative risk reduction in the primary end-point was not significant, but there was an increased incidence of life-threatening bleeding in the

group of patients receiving dual therapy.[216] The Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial proposed that there might be a clinical benefit from adding clopidogrel to low-dose aspirin treatment in patients with documented cardiovascular, cerebrovascular or peripheral vascular disease or those with recognised combinations of atherosclerotic risk factors. Although sub-group analyses showed some potential benefit of dual anti-platelet therapy in patients with established cardiovascular disease, increased bleeding tendency and higher mortality rates were revealed when patients with multiple risk factors alone were analysed. Overall, addition of clopidogrel to the standard low-dose aspirin was not significantly more effective than aspirin mono-therapy in reducing the rate of myocardial infarction, stroke or death from cardiovascular causes in patients with stable cardiovascular disease or multiple cardiovascular risk factors.[217]

2.3.1.2.3 Dipyridamole

Dipyridamole has anitplatelet and vasodilatory properties, and although its efficacy has been debated, in the ESPS-2 trial of secondary prevention in over 6000 patients with prior stroke or TIA, the combination of aspirin and dipyridamole was more effective in preventing recurrent stroke than either drug alone.[218] It works by inhibiting the nucleoside transporter of cell membranes, thus increasing the plasma concentration of adenosine because it is no longer taken up and metabolized.[219] Since adenosine counteracts platelet aggregation, this is probably the primary mode of action of the drug.[220]

2.3.2 Heparin

A strong acid; heparin is a mixture of highly sulphated glycosaminoglycans of different chain lengths, the molecular weights of which vary from 1800 to 30000 daltons.[221] Chemical and enzymatic fragmentation of heparin gives rise to low molecular weight heparin (LMWH) fractions, the mean molecular weights of which range from 3000 to 9000 daltons.[222]

Antithrombin (AT) III is the main physiological inhibitor of thrombin and other serine proteases of the coagulation system.[223] Both unfractionated (UFH) and LMWH induce a conformational change in the ATIII molecule, converting it into a high affinity inhibitor of clotting factors IIa, IXa, Xa, XIa and XIIa.[224] Of these factors, thrombin (IIa) and FXa are most susceptible to binding with the ATIII-heparanoid dimer. The binding of the ATIII-UFH occurs non-specifically and inactivates FIIa and FXa in equal proportions. In contrast, LMWH via the ATIII-LMWH dimer binds preferentially to FXa (Xa:IIa ratio 4:1). Although both forms provide an anticoagulant effect, LMWH generates a more targeted inhibition of FXa with a more predictable anticoagulant response.[225]

2.3.2.1 Heparin and platelets

Beyond its anticoagulant influence on ATIII, heparin has additional biological effects which depend on its capacity to interact with a number of soluble or surface ligands. Heparin has long been recognized to interact with platelets although there is still controversy as to the exact effects, and how they occur.[226-228] In the 1940's studies demonstrated that a single injection of heparin resulted in an immediate, but transient drop

in the platelet count.[229,230] Heparin has subsequently been reported to both reduce[231] and enhance platelet aggregation.[232] Studies have shown that heparin causes platelet aggregation and activation, as measured in vitro with platelet aggregometry,[233] or in vivo with surface markers of activation.[234]

2.3.2.1.1 Heparin induced thrombocytopenia

Perhaps the best understood interaction between heparin and platelets occurs with heparin induced thrombocytopenia (HIT). This phenomenon was first described in 1958, with a report of a series of 10 patients who developed occlusion of lower limb arteries between 7 and 15 days after commencement of heparin therapy. Rather than this being a direct result of heparin administration, it was hypothesized that heparin anticoagulation had disrupted pre-existing aortic thrombi leading to distal embolisation.[235] Fifteen years later the link between thrombocytopenia and thrombosis induced by heparin was recognized, when a study of two patients who developed thrombocytopenia during heparin therapy showed resolution on cessation of heparin and recurrence with heparin re-challenge.[236] Although uncommon (between 0.03% and 0.09% of patients exposed to heparin experience the marked platelet activation and severe thrombo-embolic crises[224]), this is a potentially life-threatening complication of heparin therapy.[237]

In an unpredictable fashion, around 17% patients treated with UFH and 8% of patients treated with LMWH will form antibodies against complexes of platelet factor 4 (PF4) and heparin.[225,238] Most patients who form anti-heparin-PF4 antibodies suffer no clinical consequences, yet in as many as 20% of the patients who develop anti-heparin-PF4

antibodies, the antibody complexes are functionally active, which triggers platelet activation, additional PF4 release and destabilization of endothelial cells.

PF4 is released during platelet activation, and is available to bind to heparin. When heparin and PF4 combine they undergo a conformational change that creates a highly antigenic neo-epitope.[224] Binding of anti-PF4/heparanoid IgG to the platelet surface facilitates subsequent platelet activation and degranulation with release of procoagulant microparticles via the IgG F_c and platelet-bound FcγRIIA. This leads to further generation of platelet and monocyte activation, endothelial perturbation and TF release. Furthermore, the heparin-PF4 dimer sustains positive feedback binding to the platelet PF4 receptor, which promotes additional platelet cross-linking, aggregation and activation. Ultimately this pro-inflammatory cycle provokes procoagulation with some patients developing thromboembolic consequences.[224]

Approximately 20% of patients presenting for vascular surgical procedures either already have pre-existing or develop heparin-induced antibodies.[239]

2.3.2.1.2 Other heparin-platelet interactions

The work of Sobel et al. revealed that heparin binds to the platelet integrin, αIIbβ3. Experiments showed that heparin binds directly to the αIIbβ3 integrin on intact, live platelets. They also found that low pharmacologic concentrations of heparin enhance fibrinogen binding. Heparin-mediated aggregation is accompanied by a hallmark of outside-in platelet signalling, the translocation of Rap2B to the cytoskeleton. Importantly,

Sobel et al. showed that there was a non-immune-mediated stimulation of platelets by heparin; a more subtle problem than heparin-induced thrombocytopenia and thrombosis.[226]

Heparin also enhances the pro-platelet aggregating effect of ADP and collagen.[240] During coronary angiography, after administration of 100 $IUkg^{-1}$ body weight of UFH, there is a significant fall in platelet count, a significant enhancement of platelet aggregation in platelet rich plasma and significant release of platelet TXA_2.[241] In a cohort of intensive care patients, the pro-aggregating effects of UFH and two types of LMWH were compared. UFH caused significant enhancement of platelet aggregation, whilst the LMWH caused intermediate aggregation. Substantial inter-patient variability in the platelets reactions to the drugs, in particular, the UFH, was also noted, with a suggestion that certain individuals have platelets that are more highly reactive to heparin.[242]

Intravenous heparin is considered the drug of choice for the prevention of arterial thromboembolic events.[243] Yet whilst it inhibits thrombin-induced platelet aggregation, heparin paradoxically potentiates platelet aggregation induced by a range of platelet agonists, including ADP, TRAP, PAF and epinephrine.[244-247]

2.3.2.1.3 Heparin and lipid metabolism

Heparin activates lipoprotein lipase (LPL),[248] and both UFH and LMWH have been shown to increase LPL release with a corresponding increase in free fatty acids. The plasma lipolytic effect of LMWH is significantly weaker than that of UFH.[248]

III

Carotid Endarterectomy – a Local Perspective to Systematic Risk Reduction

In 1992 the 30-day risks of intra-operative and post-operative stroke/death related to CEA in the Department of Vascular Surgery in Leicester, UK were 4% and 2% respectively. A prospective audit process was started to determine if introducing a policy of monitoring and quality control assessment would lead to a sustained reduction in the operative risks. A phased research programme commenced, characterized at each stage by a change in protocol followed by further auditing.[249]

3.1 Addressing the intra-operative stroke with a policy of intra-operative monitoring and quality control

In the early 1990's, it became recognised that intra-operative assessment might detect those "technical errors" that could proceed to serious peri-operative morbidity and mortality. There was, however, little consensus as to the optimal method of intra-operative assessment.[100,126] In light of this, Gaunt et al. conducted a comparison of several of the then-available monitoring techniques to investigate which would provide the most effective

and feasible quality control.[126] The techniques studied were angioscopy, B-mode ultrasound and continuous transcranial Doppler ultrasound monitoring (TCD), with the recognition that whilst the technique of on-table angiography could be well-applied to detect technical defects, there were significant problems with adequate image capture and morbidity associated with the method itself.

3.1.1 Clinical relevance of intra-operative embolization detected by TCD[250]

A prospective study was undertaken of 100 consecutive patients undergoing CEA between April 1992 and January 1994. Patients underwent a combination of post-operative investigations, including cerebral CT scanning (with correlation of new ischaemic lesions with TCD-detected embolic events), fundoscopy and visual field testing, neurological examination and cognitive assessment.

Continuous TCD monitoring of the ipsilateral middle cerebral artery was performed throughout each operation. There were 2 patients (2%) with permanent intra-operative neurological deficits and a further 2 patients (2%) with temporary deficits. TCD monitoring was successfully carried out in 91 operations, and intra-operative embolization was detected in 92% of monitored procedures. This embolization tended to occur most often after shunt opening (n=71 patients) and final restoration of flow (n=78). However, these emboli were predominantly characteristic of air and were shown not to be associated with adverse clinical outcome. Conversely, emboli detected during the carotid dissection

phase in 23 patients were shown to be associated with important clinical outcomes (*Table 6*).

Stage of operation	Patients	Mean emboli (range)	Predominant emboli character
Total operation	91	61 (0-672)	
Dissection	23	3 (0-32)	Particulate
Shunt-opening	71	6 (0-101)	Air
During shunting	48	8 (0-254)	Air
Restoration of external carotid flow	48	7 (0-100)	Air
Restoration of internal carotid flow	78	9 (0-72)	Air
Manipulation of artery	45	6 (0-228)	Air

Table 6 Results of ultrasonographically detected emboli related to operative stage. Reproduced from Gaunt et al. Br J Surg 1994:81:1435-9

Whilst there was no overall correlation between the total number of intra-operative emboli and psychometric deterioration, when the number of emboli from each specific operative stage was correlated with psychometric assessment, there was found to be an association with particulate embolization during the dissection phase. Of the 91 patients, 23 experienced embolization during dissection and 11 of these 23 had a decrease in one or more psychometric scores post-operatively. Furthermore, 8 of the 23 patients registered more than 10 emboli, and 7 of these 8 had a significant decrease in psychometric score.

3.1.2 Role of completion angioscopy[251]

There are several potential methods for evaluating the technical results of CEA, including duplex scanning, ultrasonography and intra-operative angiography, but each has its limitations, such as difficulty in interpreting the images. Perhaps more importantly, however, these methods are all employed *after* clamp release and restoration of flow.[252] By contrast, angioscopy enables visualisation of the inside of the artery using a flexible fibreoptic angioscope, facilitating the detection of technical defects after endarterectomy, but before flow restoration.

In order to evaluate the potential benefits of completion angioscopy, the technique was employed in 30 consecutive patients undergoing CEA between January and October 1992. The 2.8mm angioscope was inserted into the clamped internal carotid artery before final stitching of the patch and after shunt removal; ie immediately before restoration of flow (*Figure 16*). Back-bleeding and flushing of all carotid vessels and branches was performed in the standard manner with heparinised saline to remove any thrombus.

Irrigation was achieved through the angioscope thus providing a clearer view by eliminating the small amount of blood from the vasa vasora. This also slightly distended the vessel and, simulating blood flow, elevated and highlighted any intimal flap or frond. It also demonstrated adherence of any thrombus. The endarterectomy site was visualised first, followed by the midpoint and finally the proximal endarterectomy site and common carotid artery. When a defect was encountered, an attempt was made to correct it, with re-

insertion of the angioscope used to verify the correction. The time taken to perform a routine angioscopy was less than 2 minutes.

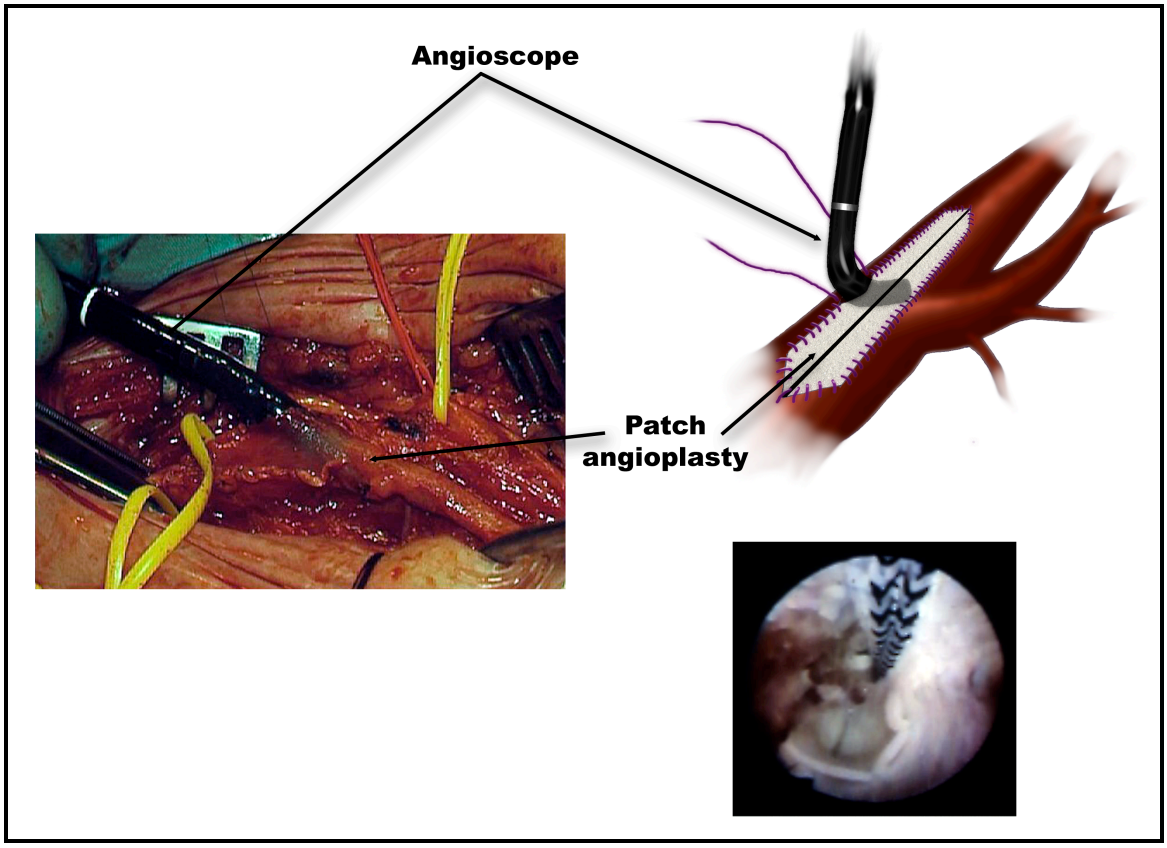

Figure 16 Completion angioscopy. With the proximal internal carotid artery and distal branches clamped, the angioscope is inserted prior to completion of the patch angioplasty. From this vantage point, both proximal and distal aspects of the endarterectomy zone can be assessed (inset)

Angioscopy revealed no technical abnormality in 21 of the 30 patients, with unsuspected abnormalities demonstrated in 9 patients. These included 2 major intimal flaps, 2 major thrombi and 5 minor thrombi. The intimal flaps were formally sutured after taking down part of the anastomosis. Major thrombi were removed with forceps, while minor thrombi could be flushed out with heparinised saline.

Angioscopy proved to be a simple technique to perform, enabling the surgeon to operate independently of technicians; the visual output on the monitor was easy to interpret. The major advantage of the technique was that defects were identified prior to restoration of flow, avoiding the need to re-open the artery, and preventing small fragments of thrombus embolising. This technique proved to be superior to B-mode ultrasound, which was often compromised by the synthetic arteriotomy patch closure. Furthermore, B-mode ultrasound and continuous wave Doppler ultrasound necessitated the presence of a vascular technologist to obtain and interpret the images.

In Gaunt's study, the combination of TCD and completion angioscopy offered the maximum yield in terms of identifying technical error and detecting significant causes of peri-operative morbidity. The results also suggested that TCD could have an important role as a continuous quality control measure detecting errors of operative technique as they occurred.

Completion angioscopy and continuous intra-operative TCD monitoring were then introduced into routine practice in Leicester in 1995, and in a total of 252 patients undergoing CEA, the combination resulted in 6 patients (2.5%) requiring repair of an intimal flap and 6 patients (2.5%) having residual luminal thrombus expelled prior to restoration of flow. The result of implementation was the effective abolition of intra-operative stroke; the rate declined to 0%.[253] However, 7 patients died or suffered a stroke in the 30-days post CEA, to give an overall death and disabling stroke rate of 1.6% and a death/any stroke rate of 2.8%.

3.2 The post-operative stroke[250]

With the effective elimination of intra-operative stroke by the introduction of these monitoring and quality control procedures, attention turned to preventing post-operative stroke. Despite technical error being excluded as a cause for CEA-related stroke, certain patients nonetheless seemed destined to suffer a post-operative stroke.

Intra-operative TCD monitoring was continued into the early post-operative recovery period. In Gaunt's study, carotid thrombosis was encountered in 3 patients in the early post-operative period. The first patient experienced persistent particulate embolization (672 particulate emboli) while in Theatre Recovery, which was associated with a gradual decline in the magnitude of middle cerebral artery blood velocity suggesting that thrombus in the internal carotid artery was progressively occluding the lumen. For most of this time period, the patient was neurologically asymptomatic but after 672 emboli were detected he developed a right arm monoplegia. At re-exploration, fresh thrombus was found to have occluded the endarterectomy zone. In the second case, middle cerebral artery blood velocity did not diminish because whilst the thrombus forming in the internal carotid artery did not completely occlude the vessel, it did act as a source of embolization into the middle cerebral artery. A total of 157 particulate emboli were detected in Theatre Recovery and at 110 minutes post-flow restoration, this patient developed a right arm monoparesis. On re-exploration of the endarterectomised internal carotid artery, fresh thrombus was removed but the neurological deficit did not resolve.

Based on the adverse experience in these two patients, it was decided to immediately re-explore a third patient who had increasing rates of particulate embolization and a falling middle cerebral artery blood velocity even though he was neurologically asymptomatic. At re-exploration, fresh thrombus was removed and upon recovery from anaesthesia the patient was noted to have a mild right hand monoparesis that resolved completely within 24 hours.[254]

Pre- and post-operative CT head scans were obtained in 91 patients in Gaunt's study. There was evidence of pre-operative infarction in the hemisphere ipsilateral to the operated carotid in 39% of scans. New post-operative infarcts occurred in the 3 patients with post-operative particulate embolization, and this was confirmation that TCD monitoring had not missed clinically relevant episodes of intra-operative embolization or haemodynamic insufficiency. Subsequent MRI studies suggested that post-operative microembolic event rates of >5 per 15 minutes were associated with ischaemic neurological changes.[255]

3.2.1 High microembolic signal loads predict post-operative cerebral ischaemia[256]

Levi et al. hypothesized that micro-emboli signal counts of >50/hour were likely to be associated with the development of cerebral ischaemia. Patients were studied prospectively with post-operative TCD monitoring which was correlated with clinical neurological assessments during the first 24 hours, repeated at 30 days post-operatively.

A total of 75 consecutive patients were studied between November 1993 and October 1995, with 65 cases (87%) successfully studied with post-operative TCD monitoring and 59 cases (79%) successfully monitored both intra- and post-operatively.

Thirty-minute periods of monitoring were performed during the 0-1 hour phase, 2-3 hour phase, 4-6 hour phase and at 24 hours post-operatively, with time zero being defined as skin closure. The overall 30 day stroke rate was 6.1% (4 patients) and the major stroke rate was 1.5% (1 patient), the major stroke having been associated with prolonged MCA signal loss after shunt insertion

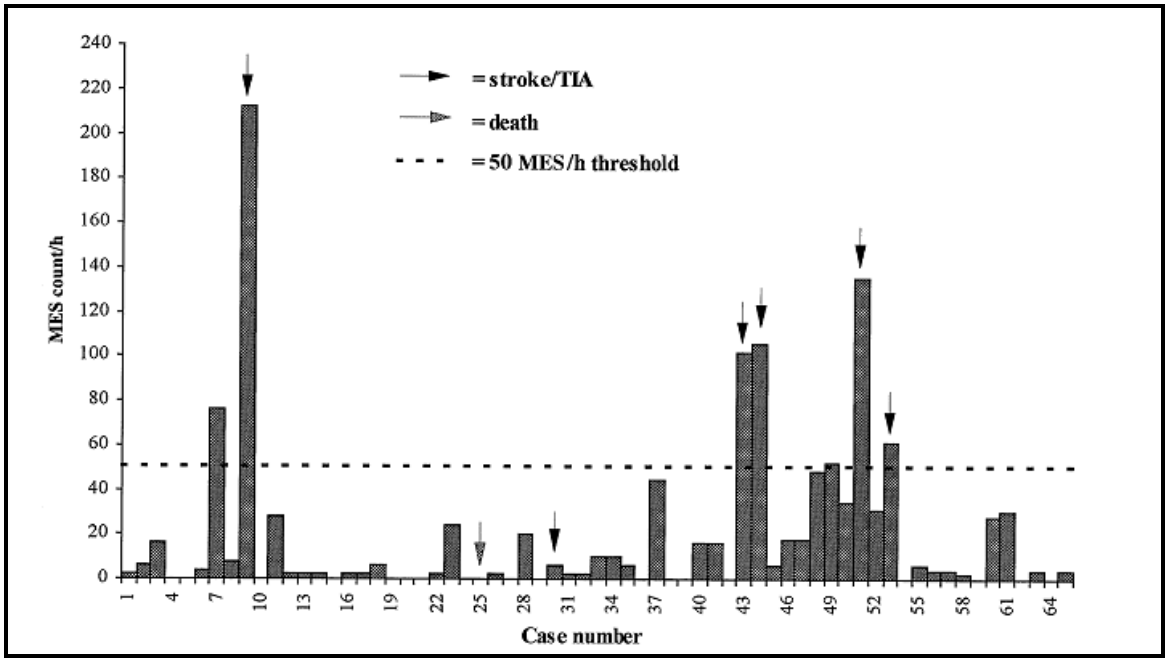

Figure 17 Embolic signal counts and clinical outcomes (patients arrowed) during the 0-1 hour post-operative phase for each of the 65 patients. The hypothetical threshold of 50 emboli/hour is shown. Reproduced from Levi et al. Brain 1997;120:621-9

Embolic signal counts for each patient at the 0-1 hour post-operative phase are shown in Figure 17. A strong association was evident between counts of >50 emboli/hour during the 0-1 hour phase (7 patients) and the development of ischaemic stroke (n=3) and TIA (n=2). The positive predictive value for cerebral ischaemia of detecting counts of >50 emboli/hour was 0.71, and the negative predictive value for counts of <50 emboli/hour was 0.98.

3.2.2 Prevention of post-operative thrombotic stroke after CEA: the role of TCD[95]

In January 1994, it was decided to administer intravenous Dextran (thought to be an effective and fast acting anti-platelet agent) to all patients following restoration of flow. The hypothesis was that this would reduce thrombus formation within the endarterectomy zone. However, while this policy was associated with the abolition of stroke due to post-operative carotid thrombosis, there was an increase in the risk of neck haematoma formation, cardiac failure and one diabetic patient, unfortunately, died of multi-organ failure thought to be attributable to the use of Dextran.

Accordingly, in September 1995, a further protocol change was made to the monitoring strategy. Wherever possible, all patients underwent intra-operative TCD monitoring and completion angioscopy. However, TCD monitoring was now continued for 6 hours following recovery from anaesthesia. In order to minimise prolonged exposure to ultrasound, the ipsilateral middle cerebral artery was monitored for 10 minutes in every 40 minute period. Anyone with >25 emboli detected in any 10 minute period (this threshold

was derived from Gaunt's earlier work[125]) was considered to be at high risk for progressing on to a thrombotic stroke.[125,126,255-257] All such patients received a bolus intravenous dose of Dextran (20ml) followed by an incremental infusion (starting at 20mlhr^{-1}). The dose increased if there was no reduction in the magnitude of embolization. Once embolization was controlled, Dextran was continued for a further 12 hours.

Table 7 summarizes the number of patients in whom emboli were detected during the 6-hour period of monitoring.

Number of emboli	Number of patients	%
0	52	52
1 to 10	23	23
11 to 25	10	10
26 to 50	7	7
51 to 75	2	2
76 to 100	1	1
>100	5	5

Table 7 Incidence of postoperative embolization in 100 patients. Reproduced from Lennard et al. J Vasc Surg 1997:26:579-84

There was an almost equal split between patients experiencing no emboli at any time (52 patients, 52%) and those experiencing 1 or more embolus (48 patients, 48%). Overall, 23% had fewer than 10 emboli, 17% had between 11 and 50, 3% had between 51 and 100, and

only 5% of patients had more than 100 emboli detected during the 6-hour period of monitoring.

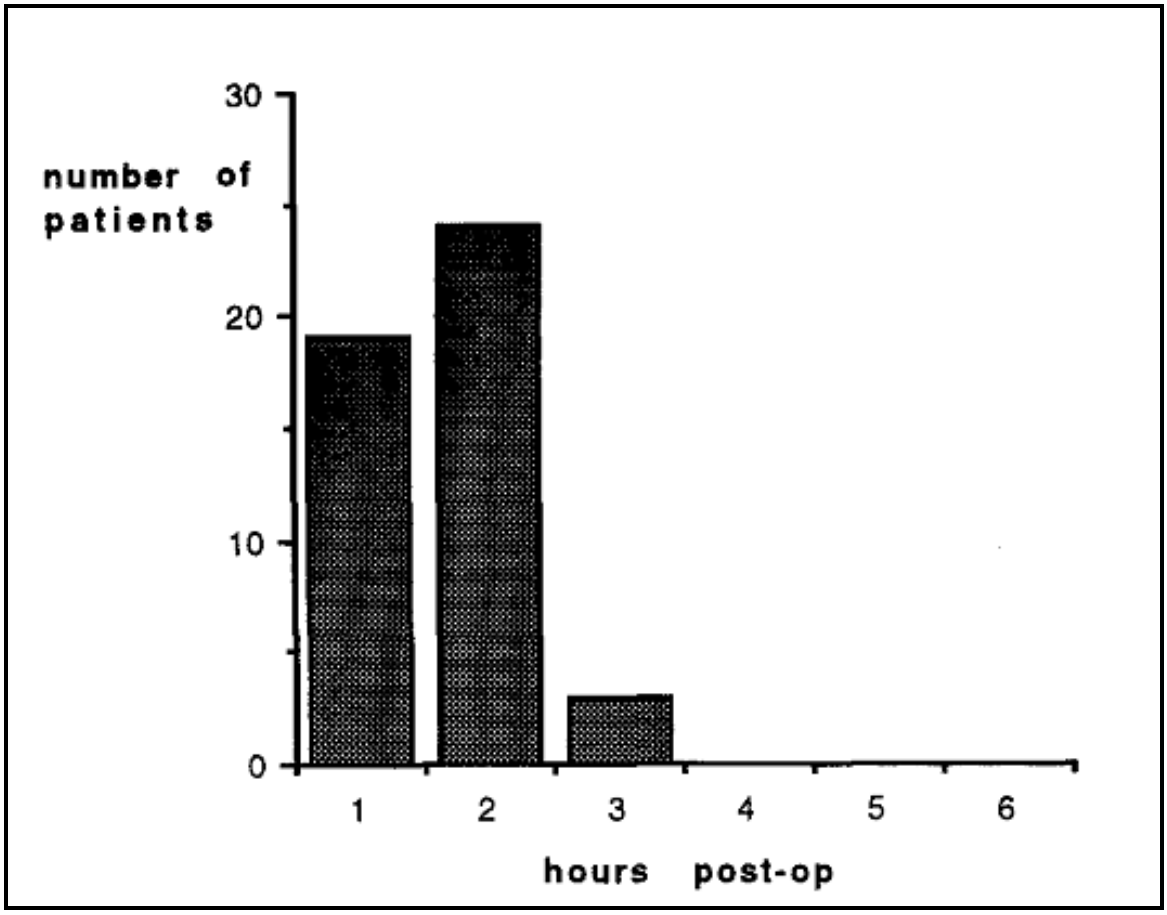

Figure 18 Onset of embolization after CEA. Reproduced from Lennard et al. J Vasc Surg 1997:26:579-84

The timing of embolization is shown in Figure 18, demonstrating that the onset of embolization occurred primarily within the first two post-operative hours. Within the first post-operative hour, 19 patients began embolizing, whereas a further 24 began to experience embolization during the second hour. Thereafter, the chances of any patient beginning to embolize were very small. Critically, it could be demonstrated that if

embolization had not started by the end of the third post-operative hour, it would not do so thereafter. Conversely, 10% of patients in whom embolization had begun in the first 3 post-operative hours had more than 10 emboli detected during the fourth to sixth post-operative hours.

3.2.3 Patient specificity

Intra-operative TCD monitoring, coupled with completion angioscopy, all but eliminated intra-operative stroke. Continuation of TCD monitoring into the recovery phase enabled the detection of clinically significant post-operative embolization, thereby enabling Dextran to be administered to these high risk patients. However, it was still unclear why a small proportion of patients developed high rate embolization prior to suffering their stroke. In the past, it was believed that post-operative thrombosis was the direct consequence of uncorrected technical error. However, Gaunt's preceding work had shown this not to be the case. Could the patient, somehow, be more susceptible to peri-operative thrombosis?

3.2.3.1 Patients' thromboembolic potential between bilateral CEAs remains stable over time[258]

It was hypothesized that the individual patient possessed an inherent tendency to form thrombus, and that therefore, their potential to generate microemboli after CEA would be consistent over time. To investigate this, aspirinated patients undergoing staged, bi-lateral CEAs were identified, and the magnitude of embolization after each procedure compared.

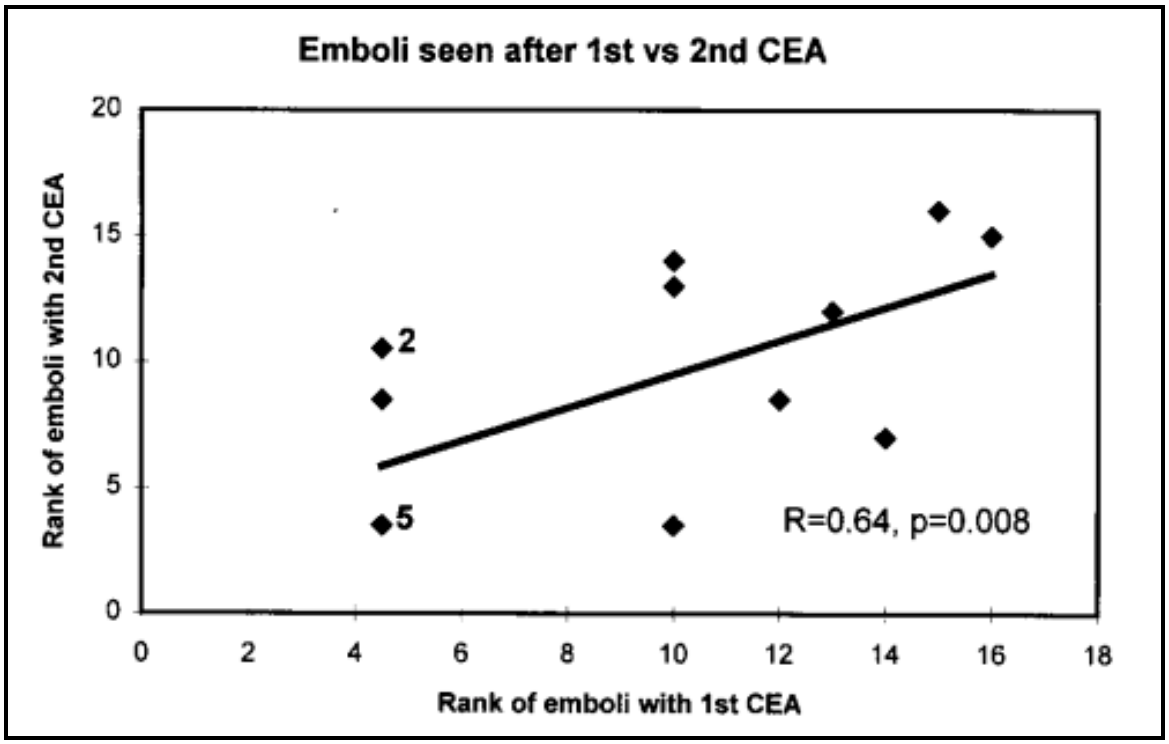

Figure 19 Correlation between number of emboli seen with the first as opposed to the second CEA. Numbers 2 and 5 indicate number of identical observations. Reproduced from Hayes et al. Eur J Vasc Endovasc Surg 2001:22:496-8

Sixteen patients underwent staged bilateral CEAs (on average) 13 months apart. Dextran therapy was required in a single patient to control high post-operative embolization after the first operation. Interestingly, this patient also experienced high rates of embolization after the second operation. The principle finding was that if the patient was a high rate embolizer after the first operation, they were likely to be a higher rate embolizer after the second procedure. Conversely, patients with zero or low rate embolization after the first procedure had similarly low rates of embolization after the second. This correlation could be illustrated by ranking and comparing the number of emboli seen after each CEA (r=0.64; p=0.008, *Figure 19*).

Given that staged, bi-lateral CEAs were often separated by a significant period of time, it appeared that the attribute of embolization had an element of specificity. Certainly the correlation between emboli counts after the first and second CEA suggested technical error was not necessarily a significant cause of post-operative embolization: it would be highly unlikely that the same technical error would be repeated twice in the same individual. Hayes et al. proposed that there was a patient-specific component to post-operative embolization, a so-called "thromboembolic potential".

3.2.3.2 Role of the platelet

Given that the material complicating CEA is invariably platelet-rich, and there was consistency over time in terms of post-operative embolization, it was hypothesized that there might indeed be an inherent, patient-specific enhancement of platelet activity.

3.2.3.2.1 Patients' thromboembolic potential after CEA is related to the platelets' sensitivity to adenosine diphosphate (ADP)[259]

Hayes et al. studied the pre-operative platelet response to incremental doses of various physiological platelet agonists (ADP, collagen and thrombin) and correlated the results with the number of post-operative emboli.

Pre-operative blood samples were collected on the morning of surgery from 110 aspirinated patients undergoing CEA. Patients were monitored with TCD for 3 hours post-operatively,

and Dextran was administered to any patient who had (1) ≥ 25 emboli in any 10-minute period or (2) emboli that distorted the MCA waveform, which suggested they were large.

The blood samples were prepared for Born-type platelet aggregometry, which was performed in platelet-rich plasma (PRP), with aggregation measured in response to final concentrations of ADP (0.5, 1, 2 and 4$\mu moll^{-1}$), collagen (10, 20 and 50$mgml^{-1}$), and AA (3 or 6$\mu moll^{-1}$). Flow cytometry was also performed, with fibrinogen-binding measured in unstimulated blood samples and samples stimulated with ADP (0.1, 1, 10$\mu moll^{-1}$) or thrombin (0.02, 0.04, 0.08, 0.16$\mu molml^{-1}$) the latter in the presence of GPRP peptide to prevent clot formation. The results from the pre-operative aggregometry and flow cytometry studies were then correlated with the number of post-operative emboli. To facilitate analysis, the patients were split into two groups: a "high embolizing" group, with >25 postoperative emboli (n=22, 20%); and a "low-embolizing" group with <25 post-operative emboli (n=88, 80%).

There was no significant difference in the response to AA between the high- and low-embolizing groups, in terms of magnitude of aggregation. However, when ADP was used as the platelet agonist, there were significant differences observed between the two groups (*Figure 20*).

The platelets of the "high-embolizing" patients were significantly more sensitive to the effects of ADP than the platelets of the "low-embolizing" patients, a pattern echoed in the aggregation response to collagen (*Figure 21*).

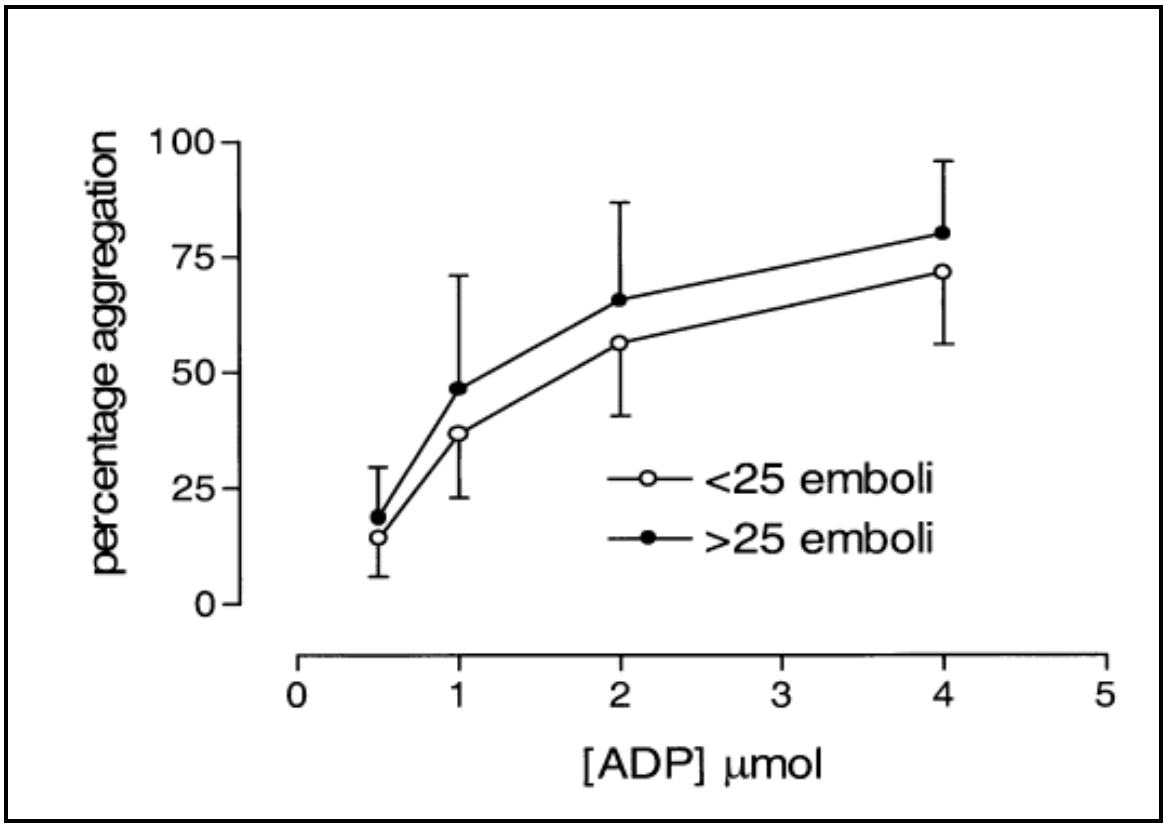

Figure 20 The percentage of platelets aggregating in response to ADP. Reproduced from Hayes et al. J Vasc Surg 2003:38:226-31

The flow cytometry data were analysed using the same definitions of "high embolizer"/"low embolizer", and there was (again) no difference between the two groups with regard to the level of expression of the platelet surface receptors GPIb or GPIIb/IIIa.

The percentage of unstimulated platelets with bound fibrinogen on their surface was not significantly different between the high- and low-embolizing groups. Stimulation of the platelets with ADP before incubation with the fibrinogen demonstrated that the patients who had most post-operative emboli bound significantly increased amounts of fibrinogen, in line with the aggregometry data.

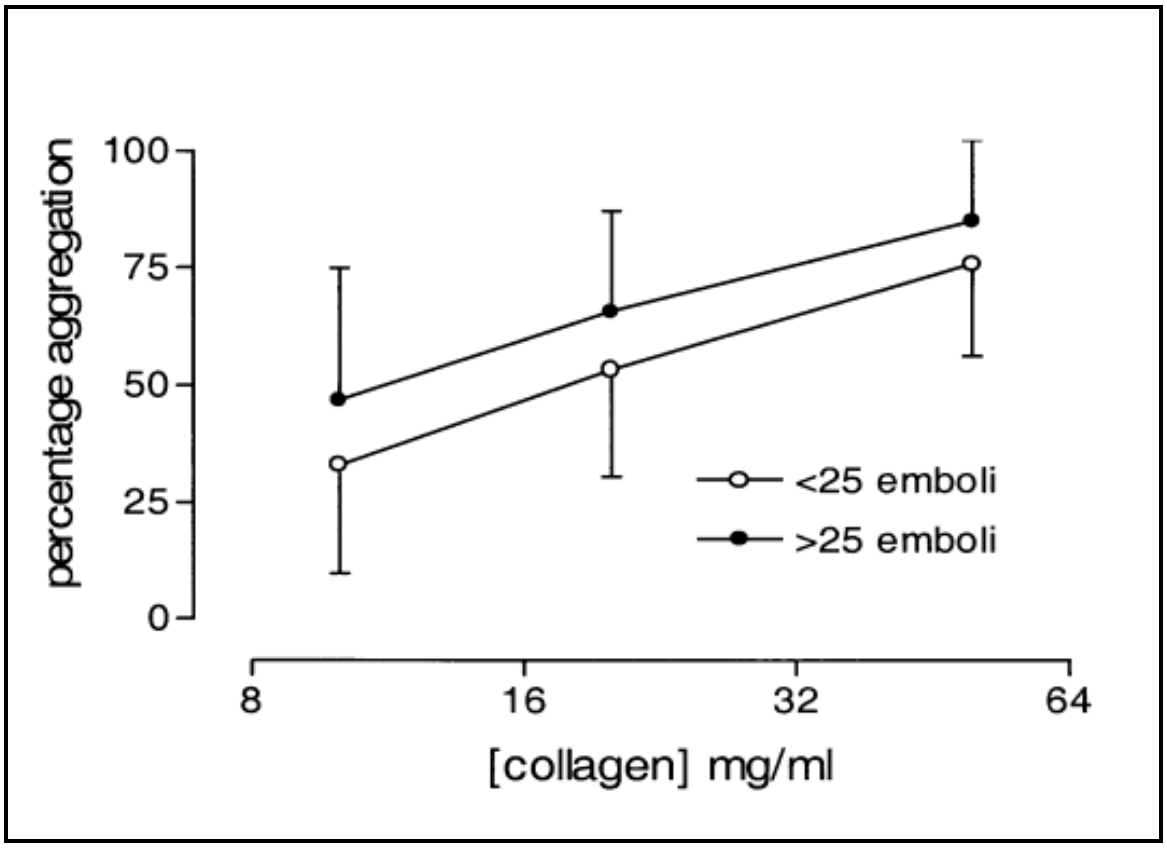

Figure 21 The percentage of platelets aggregating in response to the agonist collagen. Reproduced from Hayes et al. J Vasc Surg 2003:38:226-31

3.2.3.3 Towards targeted anti-platelet therapy – beneficial effects of clopidogrel combined with aspirin in reducing cerebral emboli in patients undergoing CEA[260]

Given the apparent link between pre-operative platelet reactivity to ADP and post-operative embolization, it seemed reasonable to pose the question: could pre-operative ADP-inhibition reduce post-operative embolization? This hypothesis was tested in a double blind randomised controlled trial, in which aspirinated patients undergoing CEA received

either a single 75mg dose of the ADP-inhibitor clopidogrel or placebo 12 hours prior to surgery.

Blood was sampled prior to administration of the trial drug and then immediately before surgery. Both platelet aggregometry and flow cytometry were performed, with Born-type aggregometry used to assess platelet aggregation in response to AA ($2.5 \times 10^{-3} moll^{-1}$). For flow cytometry, fibrinogen binding to platelets was measured in unstimulated blood samples and in samples stimulated with ADP ($1 \times 10^{-6} moll^{-1}$) by the binding of an FITC-conjugated rabbit antibody to fibrinogen to the platelets.

All patients underwent a standardized CEA with systemic heparinisation 5000IU UFH administered intravenously prior to carotid clamping. As a way of estimating whether the addition of clopidogrel increased bleeding, the time taken from flow restoration to closure of the neck wound and removal of the drapes was recorded as an indirect marker of haemostasis. Other indirect markers of blood loss included the incidence of neck haematomata, wound drainage volumes and the number of patients requiring re-exploration for post-operative bleeding.

One hundred patients were randomized to either clopidogrel (n=46) or placebo (n=54). In order to perform a meaningful statistical analysis, an arbitrary cut-off point of 20 post-operative emboli was used to separate "high-embolizing" from "low-embolizing" patients. The risk of a patient being in the "high-embolizing" group was reduced ten-fold in the

clopidogrel group (1 of 46; 2.2%) compared with patients receiving placebo (10 of 54; 18.5%; odds ratio 10.23, p=0.01, *Figure 22*).

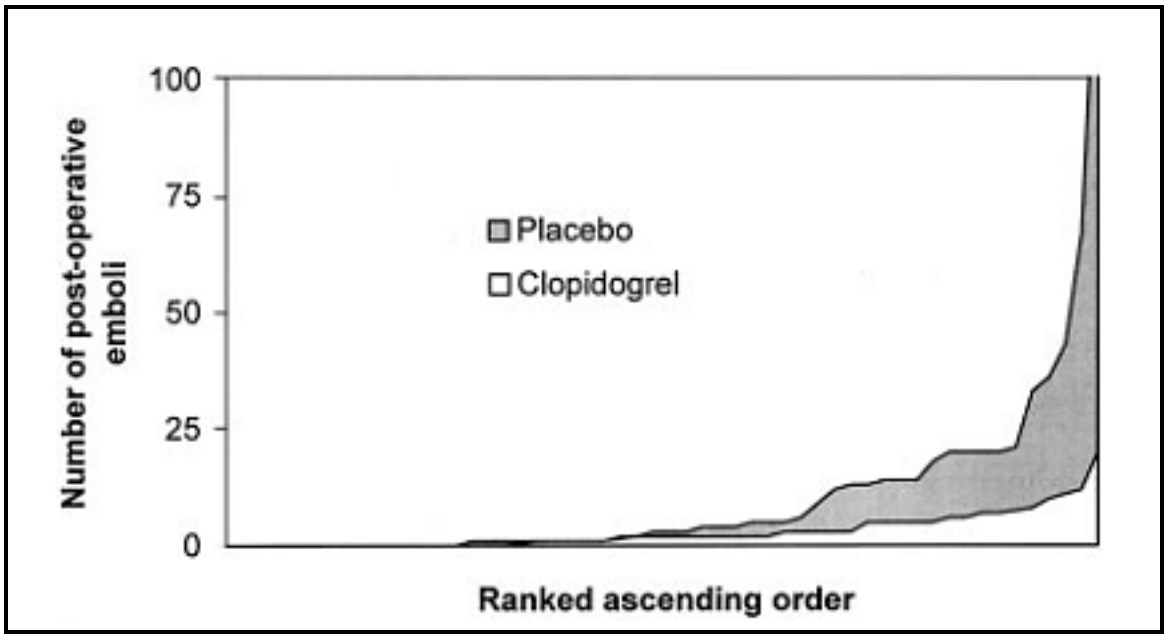

Figure 22 Post-operative emboli during 3 hours of monitoring after surgery, ranked in ascending order for patients randomised to either aspirin (150mg) plus placebo or aspirin (150mg) plus clopidogrel (75mg). Reproduced from Payne et al. Circulation 2004:109:1476-81

No patient who had taken clopidogrel had >25 emboli detected. Two patients in the placebo group required Dextran to control high-grade embolization; none in the clopidogrel arm required it.

Before drug administration there was no significant difference in platelet fibrinogen binding in response to ADP between the two groups (73.2±2.9% vs 73.6±3.1%; p=0.73, *Figure 23*). However, after clopidogrel administration, there was an 8.8% reduction in

platelet fibrinogen binding in response to ADP, which was significant in comparison to the placebo group (66.76%±2.9% vs 75.52±2.7%; p=0.03, *Figure 23*).

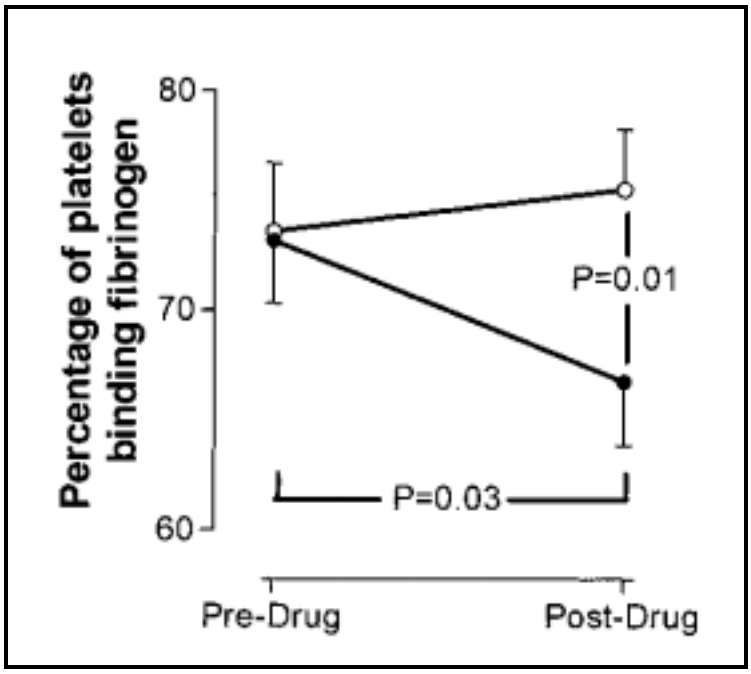

Figure 23 Fibrinogen binding to platelets in response to ADP before and after clopidogrel but before surgery. Binding was analysed in whole blood from patients treated with aspirin alone (open circles) or aspirin plus clopidogrel (closed circles) 12 hours before testing. Reproduced from Payne et al. Circulation 2004:109:1476-81

Whilst ADP inhibition with clopidogrel led to a significant reduction in post-operative embolization there were no significant differences in the markers of blood loss compared with the placebo group.

3.2.3.4 Platelet inhibition by aspirin is diminished in patients during CEA: an unexpected form of "transient aspirin resistance"[261]

During the randomised study, the ability of aspirin to prevent AA-mediated platelet aggregation in patients undergoing CEA was also examined. All patients were on daily aspirin therapy and were standardised to 150mg for at least 2 weeks prior to surgery, including the morning of the operation.

Blood was sampled immediately before surgery and at the end of the operation following restoration of blood flow. Born-type platelet aggregometry was carried out, with aggregation measured in PRP as the percentage of maximum aggregation at 10 minutes compared to autologous platelet poor plasma (PPP). PRP was prepared and stimulated with AA 2.5 and 5mmoll^{-1}. As well as the ADP experiments, PRP was stimulated with collagen 0.5 and 1.0μgml^{-1} and thrombin receptor agonist peptide (TRAP) 6μmoll^{-1}. Spontaneous platelet aggregation was also measured in a sub-set of 28 patients.

Predictably, pre-operative aggregation in response to AA (5mM) was low (7.6±5.5%) in all subjects, with none having an aggregation response >25%. Despite the effective blockade of the COX-1 pathway in all patients prior to CEA, the response of platelets to AA (5mM) was significantly increased by the end of surgery to 50.8±29.5%; an increase of 570% (*Figure 24*). A similar pattern was seen with 2.5mM AA, rising from 3.0±2.2% prior to surgery to 10.9±8.6% at the end of the procedure.

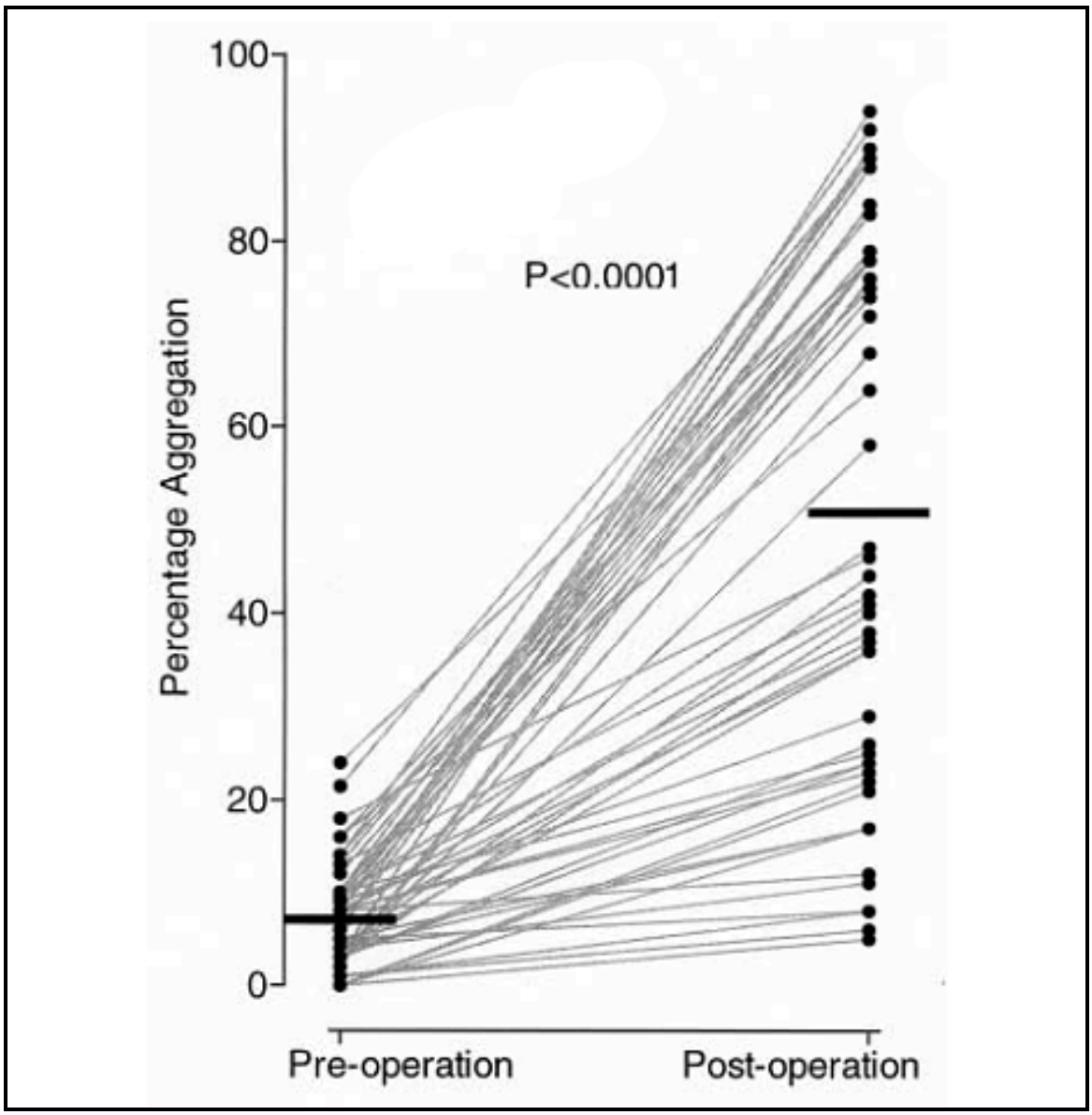

Figure 24 Platelet aggregation in response to 5mM AA prior to and at the end of surgery. Reproduced from Payne et al. Thromb Haemost 2004:92:89-96

Supplemental studies were undertaken in sub-sets of the CEA patients to determine whether the increased platelet activation to AA could be the result of increased levels of ADP, thrombin or epinephrine *in vivo*. The ADP-degrading enzyme apyrase ($4\mu gml^{-1}$), the thrombin inhibitor hirudin (4μM) and the α2-adrenergic receptor antagonist yohimbine

(10μM) were added to PRP samples taken at the end of surgery, which were then stimulated with AA. The effects of epinephrine 200ngml^{-1} and heparin 1Uml^{-1}, alone or in combination, were also assessed by their addition to PRP from pre-operative blood samples then stimulated with AA.

Platelet aggregation in response to ADP, collagen and TRAP also increased after surgery but not to the same extent as the increase observed with AA (*Figure 25*), suggesting that it was unlikely that the release, or generation of these physiological agonists *in vivo* could account for the large increase in platelet aggregation seen in response to AA.

Inhibitor	Without inhibitor	With inhibitor	p value
Apyrase (n=18)	56.2±32.4	61.2±24.6	0.49
Hirudin (n=22)	55.2±32.2	54.1±26.3	0.87
Apyrase + Hirudin (n=12)	61.8±29.8	63.6±21.3	0.82
Yohimbine (n=15)	41.5±28.6	43.6±30.0	0.84

Table 8 Effects of inhibitors on AA-induced platelet aggregation in samples taken at the end of the operation. Platelet aggregation (percentage of maximum) in response to AA (5mM). Mean ± SD. Reproduced from Payne et al. Thromb Haemost 2004:92:89-96

This was substantiated by the fact that the *in vitro* addition of agents known to prevent the platelet response to ADP (apyrase), thrombin (hirudin) and epinephrine (yohimbine), either alone or in combination, to samples taken at the end of surgery, did not significantly reduce the response to AA (*Table 8*).

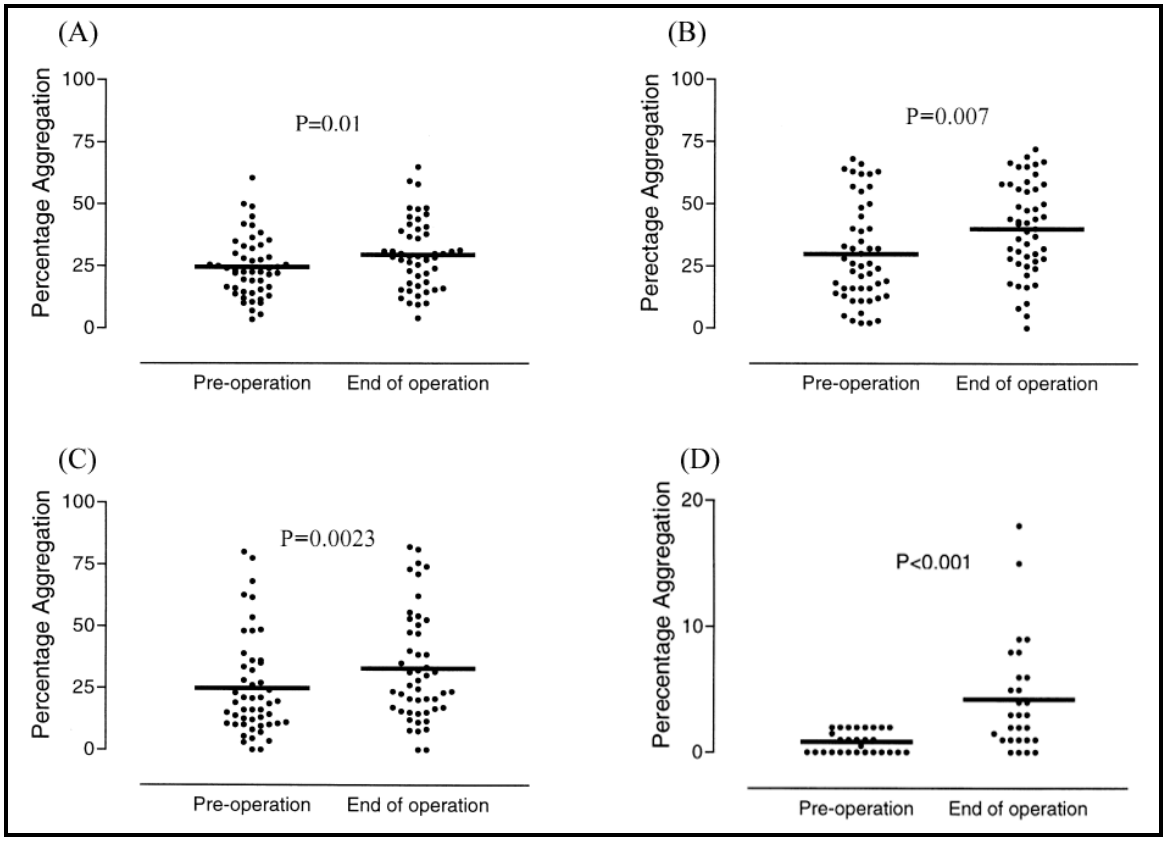

Figure 25 Platelet aggregation in response to (A) ADP 1μM, (B) collagen 0.5μgml^{-1}, (C) TRAP 6μM and (D) spontaneous platelet aggregation prior to and at the end of surgery. Reproduced from Payne et al. Thromb Haemost 2004:92:89-96

Spontaneous platelet aggregation, studied in a subset of 28 CEA patients, increased from a pre-operative level of 0.8±0.9% to 4.2±4.5% at the end of surgery (*Figure 25, D*). A correlation was observed between this and AA-induced aggregation (r=0.37, p=0.04), suggesting that those patients whose platelets were most activated during surgery were the same patients in whom the loss of aspirin effectiveness during surgery was greatest.

Only a small (12%), non-significant increase in AA-induced aggregation was observed with the *in vitro* addition of epinephrine (200ngml^{-1}) to pre-operative PRP. Similarly,

addition of UFH *in vitro* gave only a small (14.9%) and non-significant increase in AA response. Even with a combination of epinephrine and heparin, the increase was only 23.5% ($p>0.05$ for all).

3.2.3.4.1 The implication of intra-operative heparin in transient aspirin failure- anti-platelet effect of aspirin is substantially reduced after administration of heparin during CEA[262]

It appeared that at some stage during CEA, patients' platelets overcame the aspirin inhibition of COX-1, and started aggregating in response to AA stimulation. Subsequent work aimed to establish a more precise chronology and reason for this phenomenon.

In 41 patients undergoing CEA, blood was sampled at 8 time-points: pre-operatively; after induction of anaesthesia; after skin incision; 3 minutes after intravenous UFH administration; 3 minutes after shunt opening; immediately after flow restoration; 4 hours post-operatively and; 24 hours post-operatively, but prior to next aspirin administration. PRP was prepared for Born-type aggregometry which was used to assess platelet aggregation in response to increasing concentrations of AA (2.5 and 5mmoll^{-1}), ADP (2.5 and 5μmoll^{-1}) and TRAP (3 and 6μmoll^{-1}), with spontaneous aggregation also assessed. In all patients aggregation was measured over 10 minutes so that aggregation reached a maximum in all subjects, and compared to autologous PPP to set the value for 100%.

Before surgery the level of aggregation to 5mmoll^{-1} AA was low (3.9±2.2%, *Figure 26*). No patient exhibited greater than 10% aggregation, indicating that there was adequate

inhibition of patient platelets by aspirin before surgery. There was a small, but significant increase in aggregation after induction of anaesthesia (10.1±7.1%), but no significant increase in aggregation associated with incision and soft tissue dissection.

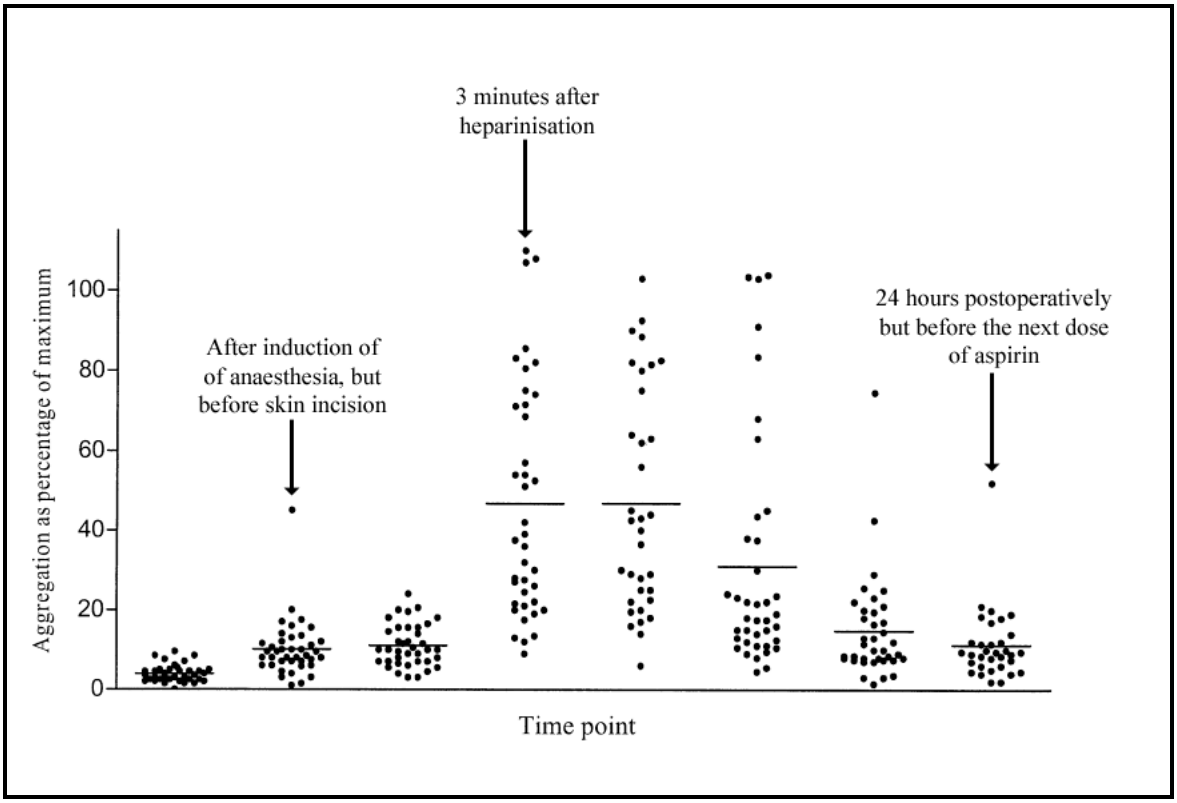

Figure 26 Platelet aggregation in response to AA (5mmoll^{-1}). Adapted from Webster et al. J Vasc Surg 2004:40:463-8

However, after heparinisation (but before insertion of the shunt), there was a ten-fold increase in aggregation to AA to 45.1±29.3%. This level of response did not increase further after restoring flow through the shunt. By 4 hours the response had decreased significantly, but to a level that was still higher than in the pre-operative or pre-heparinisation phases (14.9±13.2%). By 24 hours the response to AA had decreased to 11.2±9.0% without further administration of aspirin.

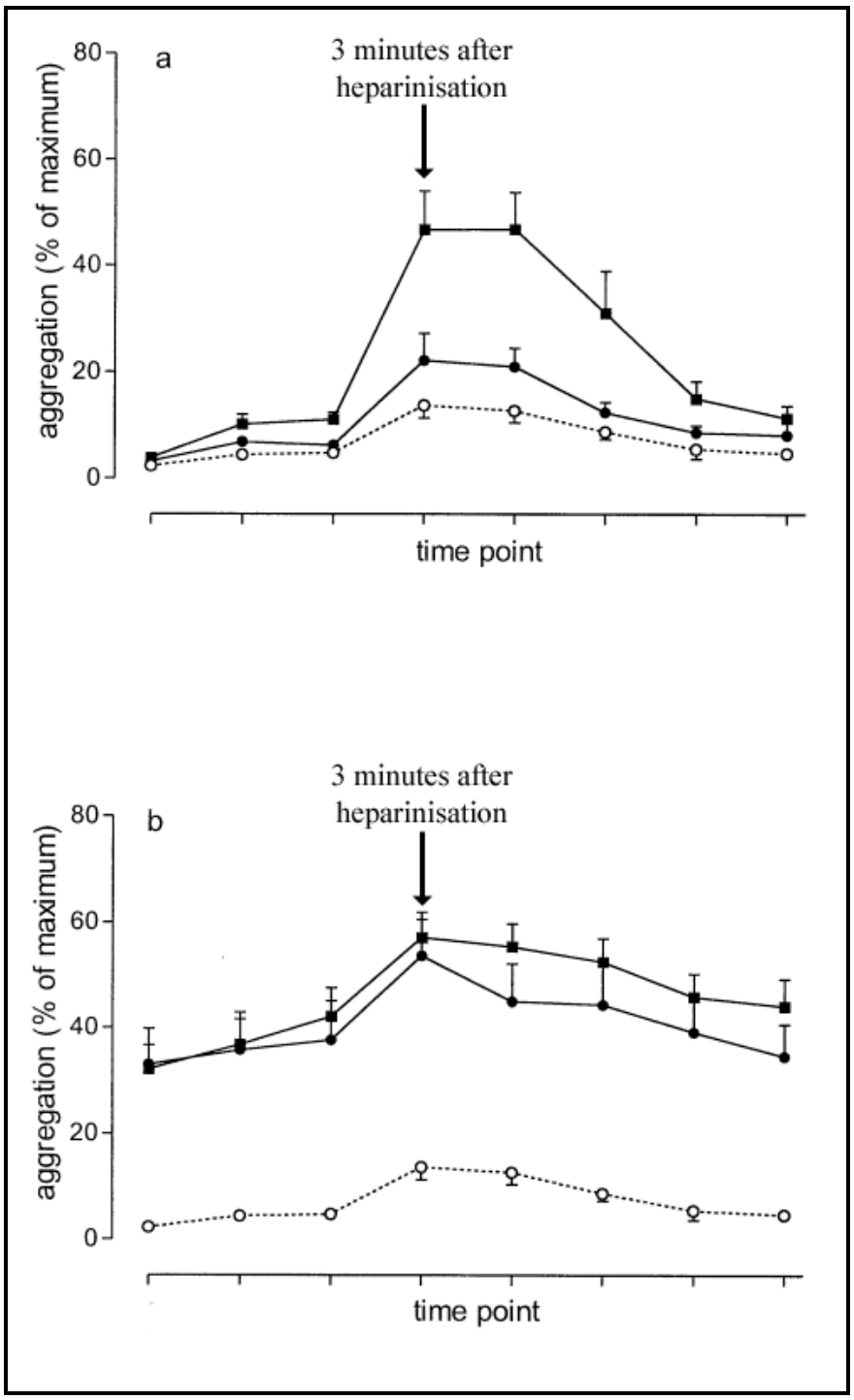

Figure 27 Platelet aggregation in 41 CEA patients. (a) response to AA (5mmoll^{-1}, solid squares, 2.5mmoll^{-1}, solid circles), and spontaneous platelet aggregation (open circles). (b) response to ADP (0.1µmoll^{-1}, solid squares), TRAP (0.3µmoll^{-1}, solid circles), and spontaneous platelet aggregation (open circles). Reproduced from Webster et al. J Vasc Surg 2004:40:463-8

Spontaneous aggregation showed a similar pattern of change throughout the procedure, but the levels of aggregation reached were significantly lower than those produced by either concentrations of AA and could therefore not account for the increased response (*Figure 27a*). Aggregation in response to an intermediate concentration of ADP or TRAP also rose by a small amount after administration of heparin (*Figure 27b*). However, these increases did not account for the large increase in response to AA, and could be accounted for by the increase in spontaneous aggregation, because subtraction of these values removed the increases in the responses to ADP and TRAP.

It was clear that there was a significant increase in the platelet aggregation response to AA immediately following the intravenous administration of UFH during CEA. The phenomenon was transient – the platelet activity significantly normalised at 24 hours, without further dosing of aspirin.

Experimental Work

IV

Heparin Increases Platelet Aggregation

4.1 Introduction

The material leading to thromboembolic complications after CEA is invariably platelet-rich,[100] and patients' thromboembolic potential after CEA is related to their platelet sensitivity to ADP.[259] The combined anti-platelet effect of aspirin and clopidogrel, inhibiting AA and ADP induced aggregation respectively, is associated with a reduction in the risk of high-level embolization following CEA – a proven surrogate marker for stroke risk.[260]

Platelets are integral to the development of the post-operative carotid thromboembolism that gives rise to CEA-related stroke. Webster et al demonstrated that the administration of intravenous UFH prior to carotid clamping resulted in a tenfold increase in the platelet aggregatory response to AA, from 3.9% pre-operatively to 45.1%.[262] Heparin exerts several pleiotropic effects on platelets, although debate persists as to the exact mechanisms and consequences. LMWH, however, is known to be less platelet-reactive, generating a more targeted inhibition of FXa and a more predictable anticoagulant response.

It was unclear what, if any, clinical relevance there might be to the sudden and transient increase in platelet aggregation response to AA during CEA. Nonetheless, loss of the primary anti-platelet mechanism at such a crucial point during CEA was an important finding. That this was seemingly related to intra-operative heparinisation, led to the hypothesis that the substitution of UFH with intravenous LMWH might reduce the platelet aggregatory response.

4.2 Aims

This study aimed to: demonstrate the reproducibility of the finding of a transient increase in the platelet aggregatory response to AA following intra-operative heparinisation during CEA; to determine the peri-operative behaviour of platelets in response to ADP; to show that intra-operative heparinisation with LMWH rather than standard UFH heparin would reduce the observed augmented platelet excitability, and; to investigate possible mechanisms by selectively inhibiting platelet aggregation with specific inhibitors.

4.3 Materials and Methods

Previous studies aimed at reducing post-operative embolization, in combination with a pilot study, were used to inform a power calculation prior to the establishment of a double-blinded, randomised controlled trial. It was hypothesized that the proportion of patients who would receive standard UFH, that would be "high-embolizers" would be in the region of 25%, whilst in the group who would receive LMWH, it was predicted that the proportion

of high-embolizers would be reduced to 10%. Thus, for the study to have 80% power for detecting a significant difference ($p<0.05$) between the two heparin types, it was calculated that two groups of 100 subjects would be required. To allow for patients who would not be eligible for inclusion in the trial, it was proposed to recruit a total of 230 patients. Pilot studies suggested that fewer subjects would be required for the laboratory arms of the study.

Approval was granted from the Leicestershire Local Research Ethics Committee and the University Hospitals of Leicester NHS Trust Directorate of Research and Development. Patients gave their written informed consent, and all received 75mg aspirin daily for at least 2 weeks prior to CEA. At commencement of the trial, computer-generated randomisation of treatment method (LMWH vs. UFH) were consecutively numbered and sealed in opaque envelopes. Starting with envelope number 1, these were allocated on a consecutive basis immediately following induction of anaesthesia. Prior to carotid clamping patients were anticoagulated by the anaesthetist either in our standard fashion, with 5000IU UFH (Multiparin, CP Pharmaceuticals, Wrexham, UK) or with 2500IU dalteparin (Fragmin, Pfizer, New York, USA) - both intravenously. Surgeon and anaesthetist were blinded to the type of heparin used. Our usual practice during CEA was to anticoagulate with a standard, patient-independent dose of UFH (5000IU). We therefore aimed to use a similarly standardised and effective dose of LMWH, and 2500IU was recommended by Pfizer, the manufacturer of dalteparin.

Patients presenting for CEA were not eligible for inclusion in the trial if they refused to consent. Other exclusion criteria were; aspirin intolerance; absence of a transcranial

window for TCD monitoring, and; the use of other anticoagulants or anti-platelet agents within 14 days pre-operatively (heparin, warfarin, dipyridamole or clopidogrel).

Platelet aggregometry studies were conducted in 65 patients (32 in the LMWH group, 33 in the UFH group).

4.3.1 Operation and monitoring

CEA was performed in a standardised manner, with normotensive, normocarbic general anaesthesia by, or supervised by, one of six consultant vascular surgeons. Pruitt-Inahara intra-luminal shunts were universally used to maintain cerebral flow during the procedure. Continuous TCD monitoring of the blood flow velocity in the ipsilateral middle cerebral artery was performed for the duration of the operation and for 3 hours post-operatively. The carotid arteriotomy was closed with a Dacron patch (W. L. Gore, Flagstaff, Arizona, USA) in all cases, and completion angioscopy was performed in patients prior to restoration of flow.[95]

4.3.2 Blood sampling

Blood samples were taken at 4 time-points: induction of general anaesthesia; then at 3, 120 and 330 minutes following systemic heparinisation. All blood samples were taken from an indwelling arterial line into vacutainer tubes (Becton Dickinson, Oxford, UK), with the first 3ml of blood taken into EDTA (0.184M) and used to obtain a full blood count (A^{c}T Diff^{TM}

Analyser, Coulter Electronics Ltd, Luton, UK), and subsequent samples taken into 0.105M buffered sodium citrate solution for immediate processing for platelet aggregometry.

4.3.3 Platelet aggregometry

"Resting" platelets suspended in plasma (platelet rich plasma, PRP) create a turbid solution that absorbs light. In a platelet aggregometer, light is passed through a vial containing PRP onto a receiver, with the proportion of light transmitted through the turbid solution quantified. The addition of a physiological agonist to the PRP induces platelet aggregation, causing the aggregates to coalesce and fall to the bottom of the tube, resulting in an increase in light transmittance through the sample as it becomes less opaque. This change in light transmission is detected, measured and compared to the light transmission through autologous platelet poor plasma (PPP) by the aggregometer, with the resulting data output to an attached computer. Aggregation is performed over a 10-minute period, and a graph constructed demonstrating the changing percentage of platelet aggregation in comparison to PPP, with time in minutes along the *x* axis and percentage platelet aggregation along the *y* axis (*Figure 28*).

The platelets show a different aggregation response based upon the strength of the activating stimulus. In the first wave of the aggregation, the platelets change shape without degranulating. If the activation is sufficiently strong, the platelets go on to release their granules and experience an irreversible platelet aggregation response.[263]

This Born-type aggregometry[261] was used to assess platelet aggregation in response to the physiological agonists AA and ADP at each of the four time points, on a PAP4 platelet aggregometer (BioData Corporation, Horsham, USA). To produce PRP, 9ml of blood, dispersed across 2 citrate tubes was centrifuged at 150×g for 20 minutes, and the resulting platelet rich supernatant was aspirated and decanted to specialised aggregometer vials. Simultaneously, 4.5ml of blood in a third citrate tube was centrifuged at 1500×g for 20 minutes to initiate production of autologous PPP. After 20 minutes, the supernatant from this tube was aspirated and transferred to vials to be spun on a microfuge at 9300×g for a further 10 minutes to produce PPP. Supernatants were aspirated with a Pasteur pipette from the centre, and to within 1-2mm of the buffy coat to avoid aspirating platelets and white cells.

The aggregometer was calibrated to autologous PPP by placing 450µl PPP in an aggregometer test tube, without a magnetic stir bar, and placing it in the first, then second, third, and fourth test wells. To perform aggregometry, 450µl aliquots of PRP were added to clean aggregometer test tubes, and a magnetic stir bar was added to each. The samples were incubated in the aggregometer at 37°C for 3 minutes, with the stir bars revolving at 900rpm. Aggregation was commenced on the aggregometer, and recording begun on the attached computer. After 1 minute, the agonists were added to each of the wells, and aggregation was recorded over the following 9 minute period. These procedures were repeated at each of the four peri-operative time points. All aggregometry tests were performed in duplicate, with the final result taken as a mean of the maximum percentage aggregation reached at 10 minutes.

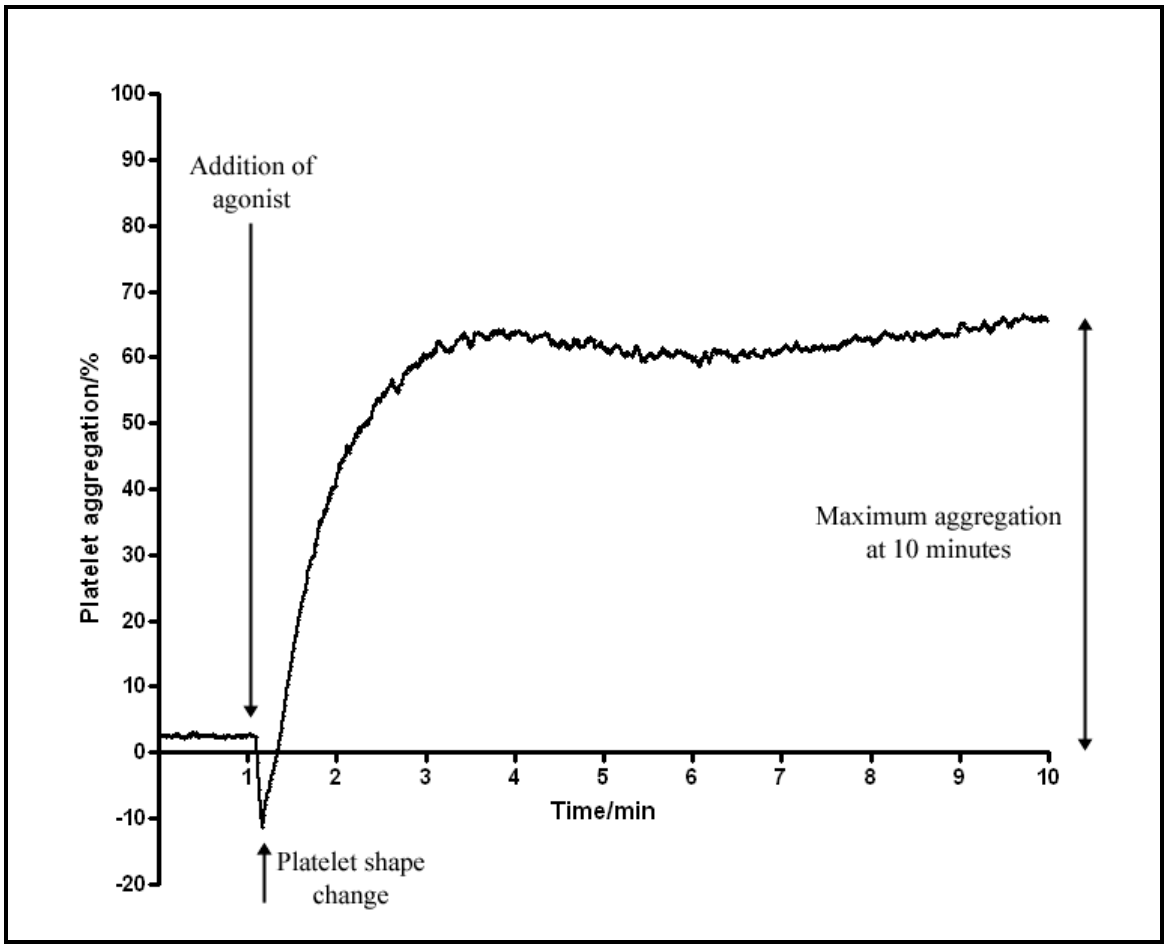

Figure 28 Example of graphical output from the platelet aggregometer

4.3.4 Platelet agonists for aggregometry

Platelets were stimulated with the physiological agonists AA and ADP, at the four time points. The volume of agonist added to each 450µl aliquot of PRP was 40µl.

HEPES buffered saline (HBS) was used as the aqueous diluent. It was made up of 150mM NaCl, 5mM KCl, 1mM $MgSO_4.7H_2O$ and 10mM Hepes (Sigma, Poole, Dorset, UK) and stored at 4°C. It provided for a more physiological buffered environment for *ex vivo* platelet studies.

4.3.4.1 Adenosine Diphosphate (ADP)

A final concentration of 3.3×10^{-6}moll^{-1} ADP (Sigma, Poole, Dorset, UK) was used to stimulate the platelets. This was based on previous titrations[260] and was designed to test the efficacy of the platelet response to an intermediate concentration of ADP. The final concentration was achieved by serially diluting the supplied ADP, from its concentration of 10^{-2}M to a final concentration of 4.0×10^{-5}moll^{-1}. A stock of 4.0×10^{-5}M ADP solution was aliquoted on ice into 600 marked vials using a step pipette delivering 50µl aliquots. These were subsequently stored at -80°C until required for platelet aggregometry studies. 40µl of these 4.0×10^{-5}moll^{-1} aliquots was used in each PRP vial.

4.3.4.2 Arachidonic Acid (AA)

A final concentration of 4.0×10^{-3}moll^{-1} AA was used to stimulate the platelets. This concentration was based on previous titrations[260-262] and was designed to fully test the efficacy of the patients' aspirination with maximal AA stimulation.

100mg of AA (Sigma, Poole, Dorset, UK) was homogenised in HBS rather than being dissolved in its usual solvent, ethanol, so as not adversely affect the platelets in the aggregometry studies. To achieve the final concentration of 4.0×10^{-3}moll^{-1} AA, 40µl of AA at a concentration of 5×10^{-2}moll^{-1} would need to be added to 450µl of PRP in the aggregometer. Serial dilutions were used to achieve a final stock AA concentration of 5×10^{-2}moll^{-1}, and this was divided into 100µl aliquots and stored at -80°C in labelled vials until required for platelet aggregometry.

4.3.5 Platelet inhibitors for aggregometry

In order to assess which pathways were active during platelet aggregometry, a range of platelet inhibitors were incubated with the PRP prior to aggregometry. The inhibitors tested were aspirin (COX-1 inhibition), baicalein (12-LOX inhibition) and SQ 29548 (thromboxane receptor antagonist, TRA).

4.3.5.1 Aspirin

A final concentration of aspirin of 4×10^{-4}moll^{-1} was used *in vitro* incubated in the PRP vials prior to platelet stimulation with AA. The ID_{50} of aspirin is in the order of 1μM for platelet COX-1.[264]

The molecular weight of aspirin is 180.16 and it was obtained from Sigma, Poole, Dorset, UK. An initial stock solution of 1×10^{-2}moll^{-1} was produced by dissolving 18.016mg in 10ml distilled water. Serial dilutions were then produced in HBS, and the solutions were kept at 4°C.

4.3.5.2 Baicalein

Baicalein is a potent and selective inhibitor of platelet 12-LOX. The concentration for 50% inhibition (ID_{50}) has been found to be 0.12μM for platelet 12-LOX and 0.83mM for platelet COX.[265]

The molecular weight of baicalein (Biomol, Exeter, UK) is 270.2, and because it has a tendency to oxidize, fresh aliquots were made up as required and stored at 4°C. 2.702mg of baicalein was fully dissolved in 10µl dimethyl sulphoxide 4% (DMSO) and then made up to 1ml with HBS to give a stock solution of 1×10^{-2}moll^{-1} from which aliquots could be added to the PRP vials during aggregometry.

4.3.5.3 SQ 29548

The thromboxane receptor antagonist SQ 29548 (Alexis Biochemicals Corp., San Diego, USA) was used in the aggregometry studies to specifically block the platelet thromboxane receptor. It has a molecular weight of 387.5, and an ID_{50} of 6×10^{-8}moll^{-1} [266] and in order to produce aliquots of 1×10^{-3}moll^{-1}, 3.875mg was dissolved in 1.2ml of ethanol before being further diluted in 8.8ml HBS. 100µl of this was then diluted in 4.3ml of HBS to give a final concentration of 2.3×10^{-5}moll^{-1}. Aliquots were then stored at -20°C.

4.4 Results

Data were analysed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and Graphpad Prism version 4.03 for Windows (Graphpad Software Inc., La Jolla, California, USA). Paired continuous data were analysed with the use of a 2-tailed paired *t*-test, with non-paired observations analysed with a 2-tailed Mann-Whitney U test. Multiple continuous observations were analysed with a Kruskal-Wallis test. Categorical variables were analysed with chi-square tests. Data are presented as mean ± SD. Probability values <0.05 were considered statistically significant.

4.4.1 Demographics

Variable	LMWH (n=32)	UFH (n=33)	p value
Age/years	68±9.4	64±8.8	0.08
Sex			
Male	21 (66%)	23 (70%)	0.73
Female	11 (34%)	10 (30%)	
Weight/kg	79±15.2	74±13.3	0.20
Hypertension	26 (81%)	23 (69%)	0.28
Diabetes	6 (19%)	6 (18%)	0.95
Current smoker	9 (28%)	11 (33%)	0.65
Ex-smoker	18 (56%)	20 (61%)	0.72
Non-smoker	5 (16%)	2 (6%)	0.21
Presentation			
Asymptomatic	6 (19%)	13 (39%)	0.07
Stroke	5 (16%)	9 (27%)	0.25
Transient ischaemic attack	16 (50%)	5 (15%)	*0.003*
Amaurosis fugax	5 (16%)	6 (18%)	0.78
Mean carotid stenosis	79±8.4%	77±7.9%	0.34

Table 9 Characteristics of 65 patients undergoing CEA in whom platelet aggregometry was performed

Aggregation in response to the physiological agonists AA and ADP was performed on a total of 65 patients undergoing CEA (LMWH: 32; UFH: 33). Additional aggregation studies were performed in sub-groups.

For a range of demographic variables, the LMWH and UFH groups were reasonably well-matched (*Table 9*). There were differences observed in the mode of presentation of the patients between the two groups; 39% of the UFH patients were asymptomatic, versus 19% of the LMWH group (p=0.07); 27% of the UFH had suffered a stroke, versus 16% of the LMWH group (p=0.25), and; 16% of the LMWH patients presented with amaurosis fugax, versus 18% of the UFH group (p=0.78) but these were not statistically different. However, there was a statistically significant difference between the two groups with regards to TIAs; only 15% of the UFH group presented with TIA's, whereas 50% of the LMWH group presented in this manner (p=0.003).

4.4.2 Full blood counts

Blood was sampled into EDTA (0.184M) from the patients undergoing platelet aggregometry at each of the four time-points (induction of anaesthesia, 3, 120 and 330 minutes after heparinisation), to obtain a full blood count (A^{c}T Diff^{TM} Analyser, Coulter Electronics Ltd, Luton, UK).

4.4.2.1 Leucocyte counts

The mean leucocyte counts at induction of anaesthesia were not significantly different in the LMWH and UFH groups, at 7.8±2.6×10^3l^{-1} and 6.8±2.3×10^3l^{-1} respectively (p=0.13). After heparinisation, the mean leucocyte counts were not significantly increased at 8.0±2.6×10^3l^{-1} and 7.7±2.4×10^3l^{-1} (p=0.52). At 120 minutes post-heparinisation, the mean leucocyte counts remained stable at 8.3±2.4×10^3l^{-1} and 7.6±2.2×10^3l^{-1} respectively (p=0.19).

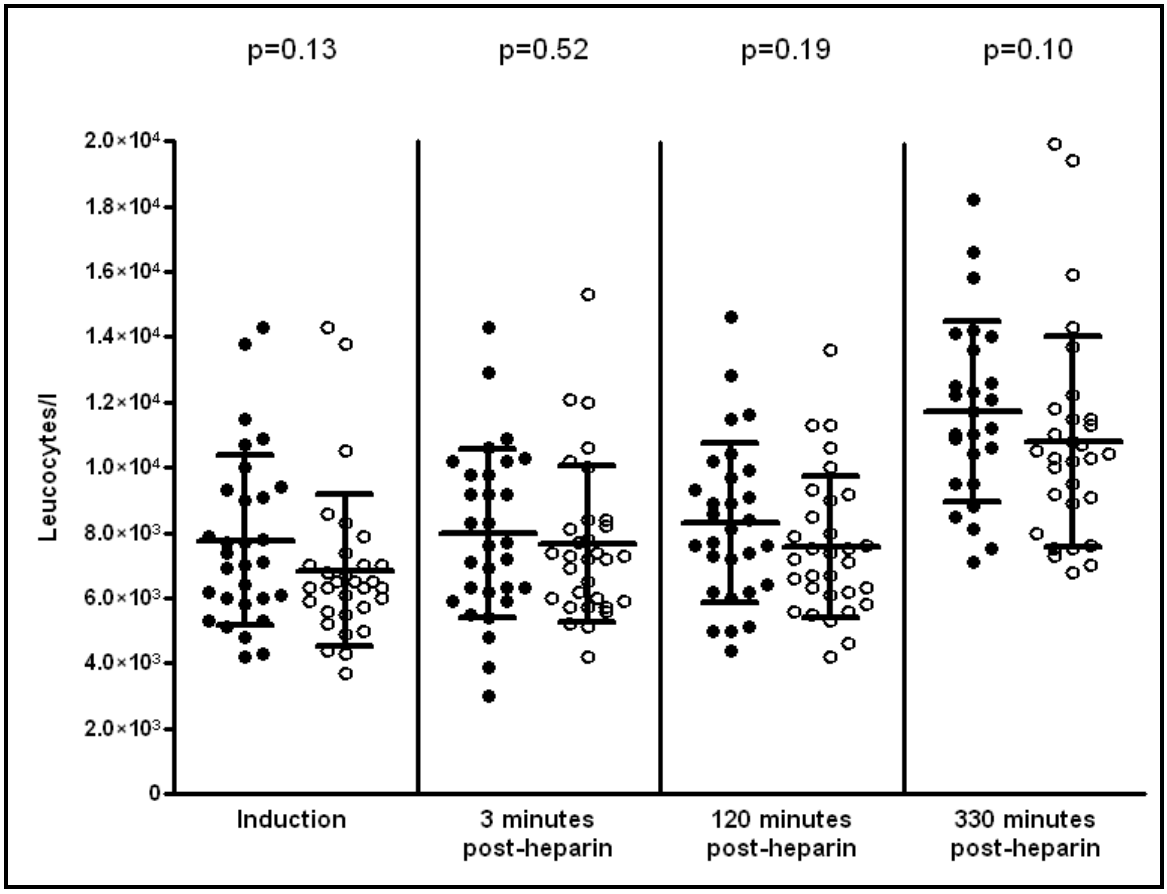

Figure 29 Leucocyte counts in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD).

By 330 minutes post-heparinisation, the leucocyte counts had increased significantly to $11.7\pm2.8\times10^{3}l^{-1}$ in the LMWH group ($p<0.0001$) and $10.8\pm3.2\times10^{3}l^{-1}$ in the UFH group ($p<0.0001$), although there was no significant difference in the magnitude of this increase between the two groups ($p=0.10$, *Figure 29*).

4.4.2.2 Erythrocyte counts

Figure 30 shows the erythrocyte counts for the LMWH and UFH groups at induction of anaesthesia, 3, 120 and 330 minutes post-heparinisation. At induction the mean erythrocyte counts were identical at $3.9\pm0.5\times10^{6}l^{-1}$ ($p=0.89$). After heparinisation, the erythrocyte counts dropped significantly to $3.5\pm0.5\times10^{6}l^{-1}$ in the LMWH group ($p=0.0013$) and $3.5\pm0.4\times10^{6}l^{-1}$ in the UFH group ($p=0.0015$), but there was no difference in this drop between the groups ($p=0.96$). At 120 minutes the counts remained significantly lower than at induction, at $3.5\pm0.6\times10^{6}l^{-1}$ ($p=0.005$) and $3.5\pm0.4\times10^{6}l^{-1}$ ($p=0.002$) in the LMWH and UFH groups respectively. Again, there was no statistical difference between the two groups ($p=0.70$). At 330 minutes the erythrocyte count in the LMWH group remained significantly lower than at induction at $3.6\pm0.5\times10^{6}l^{-1}$ ($p=0.03$) and at $3.6\pm0.4\times10^{6}l^{-1}$ in the UFH group ($p=0.005$), but there was no significant difference between the two groups ($p=0.51$).

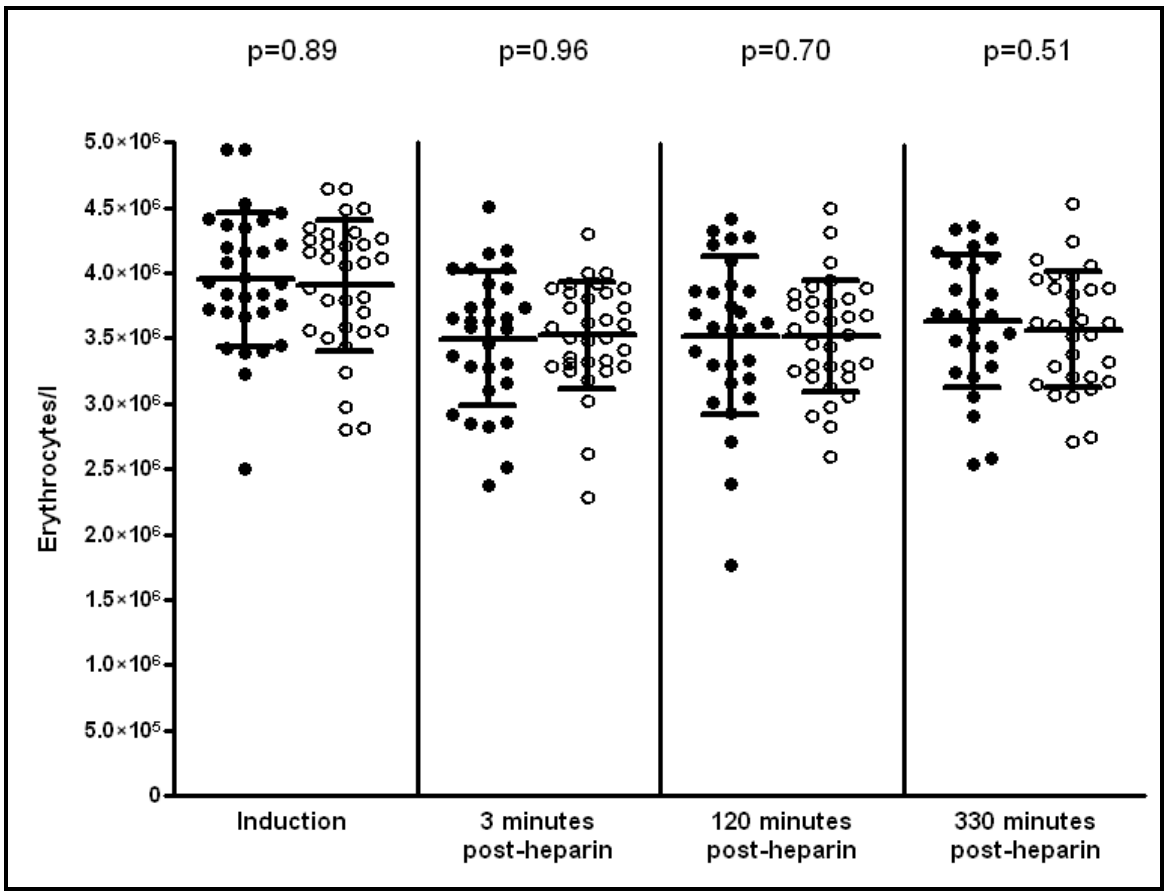

Figure 30 Erythrocyte counts in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD)

4.4.2.3 Haemoglobin

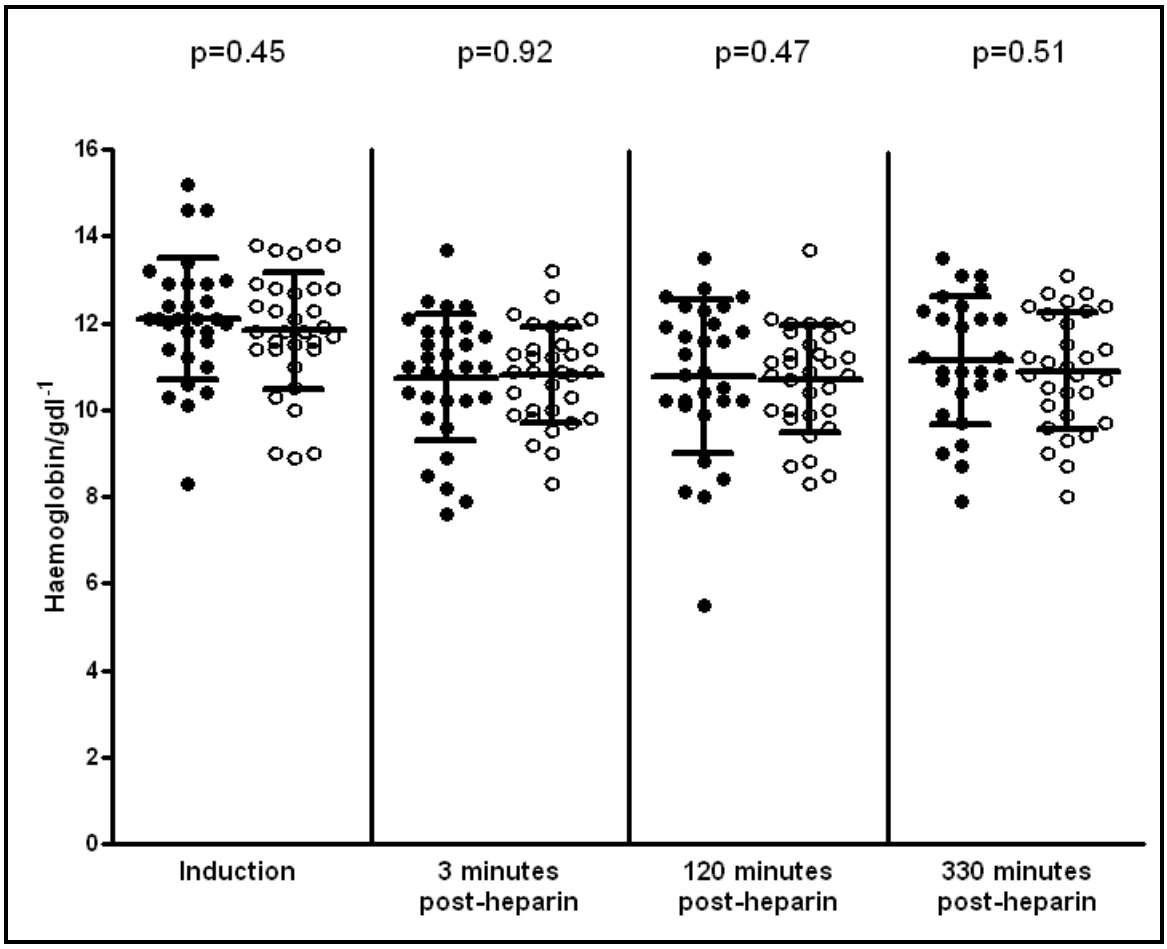

Figure 31 Haemoglobin concentrations in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD)

The mean haemoglobin concentrations at induction of anaesthesia were 12.1±1.4gdl^{-1} in the LMWH group and 11.8±1.4gdl^{-1} in the UFH group (p=0.45). These concentrations both fell significantly to 10.7±1.5gdl^{-1} (p=0.0005) and 10.8±1.1gdl^{-1} (p=0.001) in the LMWH and UFH groups respectively 3 minutes after heparinisation, although there was no difference between the two groups (p=0.92). At 120 minutes, the haemoglobin concentrations remained significantly lower than at induction, at 10.8±1.8gdl^{-1} (p=0.004) in the LMWH group and 10.7±1.2gdl^{-1} in the UFH group (p=0.0006), but the difference

between the groups was not significant (p=0.47). At 330 minutes, the mean haemoglobin concentration in the LMWH group was 11.2±1.5gdl^{-1} (p=0.04) and 10.9±1.3gdl^{-1} (p=0.009) in the UFH group, with the two groups statistically equivalent (p=0.51, *Figure 31*).

4.4.2.4 Platelets

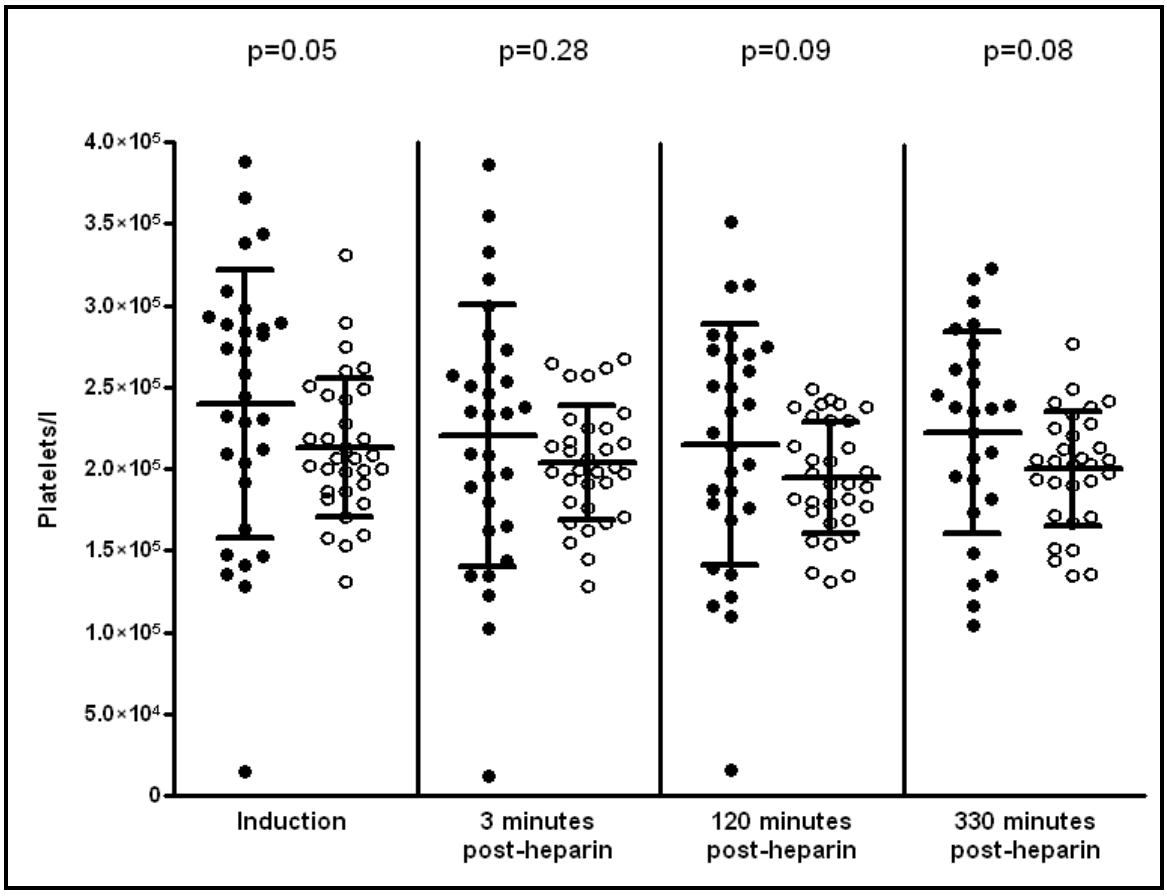

Figure 32 Platelet counts in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD)

The mean platelet counts at induction of anaesthesia were not significantly different in the LMWH and UFH groups, at 240±82×10^{3}l^{-1} and 213±43×10^{3}l^{-1} respectively (p=0.05). The platelet counts did not significantly alter after heparinisation in either group, at

$220\pm80\times10^{3}l^{-1}$ (p=0.26) for the LMWH group and $204\pm35\times10^{3}l^{-1}$ (p=0.46) for the UFH group. There was no difference between the two groups at this stage (p=0.28). At 120 minutes post-heparinisation, the platelet counts were $215\pm74\times10^{3}l^{-1}$ (p=0.15) and $195\pm34\times10^{3}l^{-1}$ (p=0.07) respectively, and were not significantly different (p=0.09). 330 minutes after heparinisation, the platelet counts were $222\pm62\times10^{3}l^{-1}$ in the LMWH group (p=0.30) and $200\pm35\times10^{3}l^{-1}$ in the UFH group (p=0.31), with no statistical difference between the two groups (p=0.08, *Figure 32*).

4.4.2.5 Haematocrit

Figure 33 illustrates the haematocrit of the 65 patients undergoing CEA at each of the four time-points. At induction of anaesthesia, the mean haematocrit in the LMWH group was 38±4.5%, versus 37±4.1% in the UFH group (p=0.46). After heparinisation, the haematocrit dropped significantly to 33±4.5% (p=0.0004) in the LMWH group and to 33±3.1% (p=0.0004) in the UFH group, although there was no significant difference between these drops (p=0.62). At 120 minutes, the haematocrit remained significantly lower in both groups, at 33.5±5.3% (p=0.002) and 33.1±3.7% (p=0.0007) respectively. There was no difference between haematocrits at this time-point (p=0.41). By 330 minutes, the haematocrits remained significantly reduced compared to induction, at 34±4.3% (p=0.01) for the LMWH group, and 34±3.8% (p=0.006) for the UFH group, although there was no difference between the groups at this point either (p=0.44).

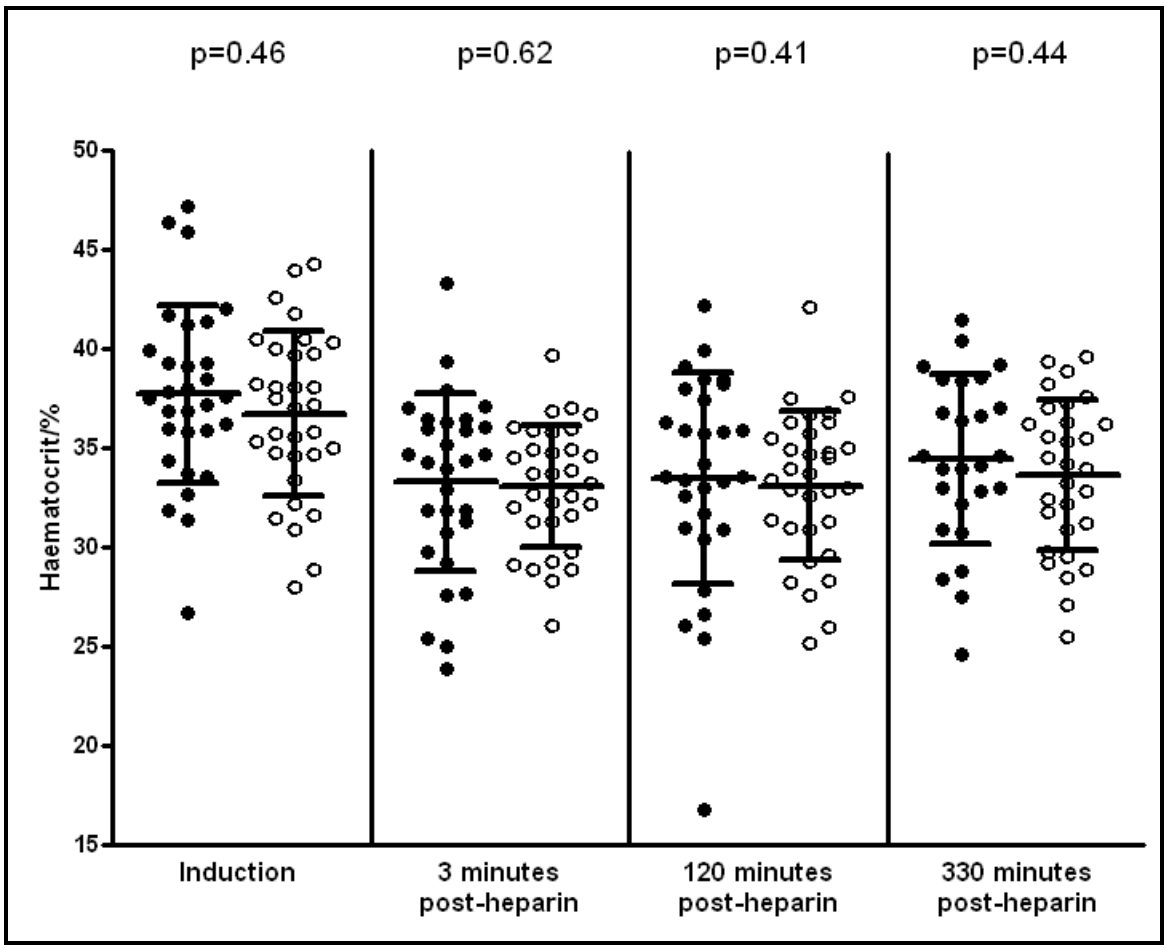

Figure 33 Haematocrit in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD)

4.4.3 Platelet aggregation to AA

Platelet aggregometry was performed in 65 patients undergoing CEA (32 patients in the LMWH group and 33 patients in the UFH group). At induction of anaesthesia, prior to heparinisation, platelet aggregation to AA (4×10^{-3}moll^{-1}) was less than 20% of maximum in 50 of the 65 (77%) patients, indicating them to be adequately aspirinated for the purposes of this study. Seven patients in the LMWH group and 8 patients in the UFH group were still responsive to AA (range 22.1%-90.3%), suggestive of "aspirin failure"[205]

and were subsequently excluded from the analysis of the platelet response to AA (*Figure 36*).

Variable	**Aspirin responders (n=50)**	**Aspirin resistants (n=15)**	**p value**
Age/years	66±9.6	66±8.3	0.93
Sex			
Male	33 (66%)	11 (73%)	0.59
Female	17 (34%)	4 (27%)	
Weight/kg	74±13.1	83±16.4	0.06
Hypertension	38 (76%)	11 (73%)	0.83
Diabetes	9 (18%)	3 (20%)	0.86
Current smoker	15 (30%)	5 (33%)	0.80
Ex-smoker	29 (58%)	9 (60%)	0.89
Non-smoker	6 (12%)	1 (7%)	0.56
Presentation			
Asymptomatic	15 (30%)	4 (27%)	0.80
Stroke	12 (24%)	2 (13%)	0.38
Transient ischaemic attack	15 (30%)	6 (40%)	0.47
Amaurosis fugax	8 (16%)	3 (20%)	0.71
Mean carotid stenosis	78±8.1%	76±8.0%	0.26

Table 10 Characteristics of 50 "aspirin-responding" and 15 "aspirin-resistant" patients undergoing CEA in whom platelet aggregometry was performed

The level of aggregation in response to AA prior to heparinisation in the remaining 50 patients was similar in both groups (8.3±4.2% for LMWH vs. 8.7±4.4% for UFH; p=0.66, *Figure 36*).

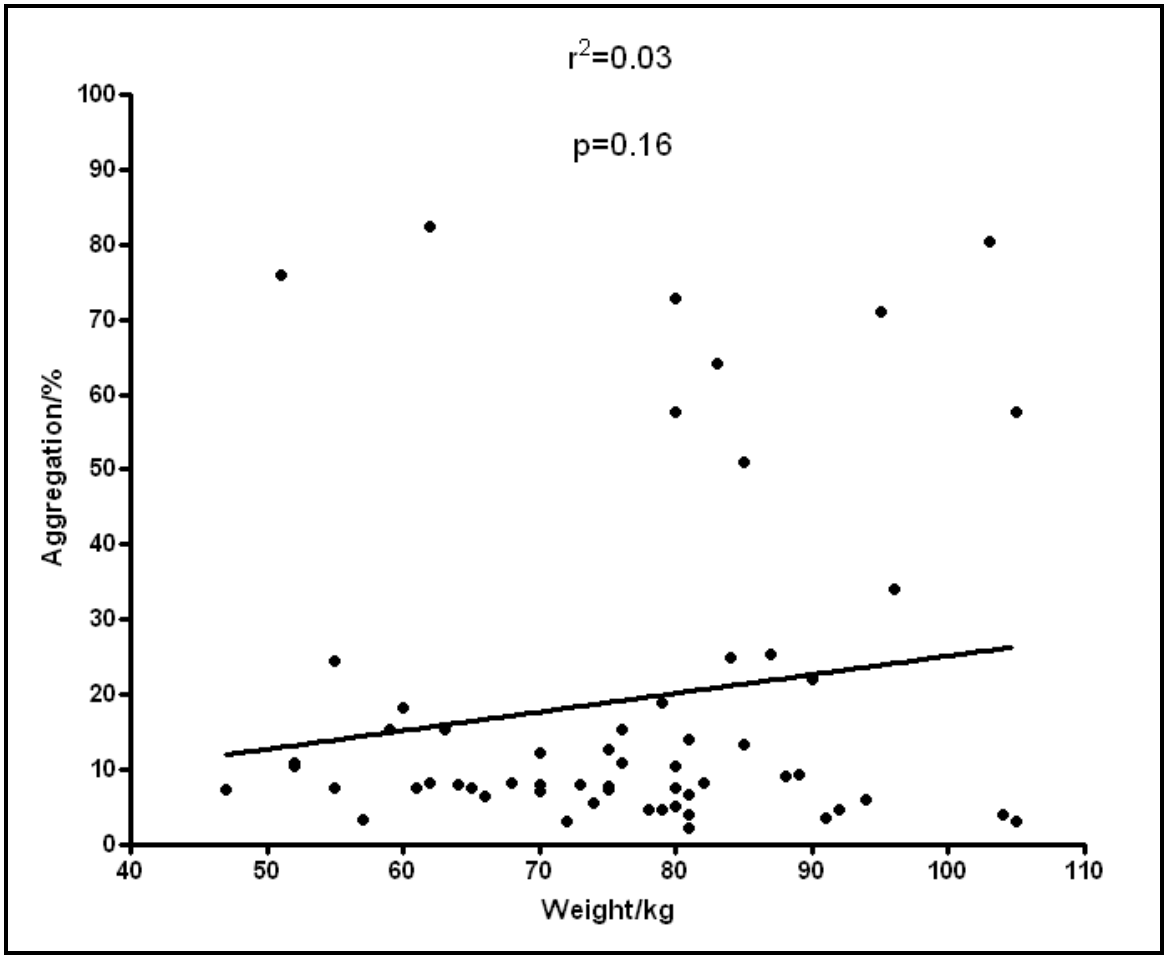

Figure 34 Relationship between platelet aggregation in response to 4×10^{-3}moll^{-1} AA in 65 patients undergoing CEA at induction of anaesthesia and patient weight

There were no statistically significant differences between the characteristics of the aspirin responders versus those patients who displayed aggregation to AA greater than 20% (*Table 10*). Interestingly, the mean weight of patients who were deemed "aspirin-resistant" at induction of anaesthesia was 83kg, compared to 74kg in the aspirin responding group,

although this was not a statistically significant difference (p=0.06), and there was no correlation between platelet response to AA at induction of anaesthesia and patient weight (*Figure 34*). Three minutes after the administration of heparin, the platelet response to AA increased dramatically (six to seven-fold) to 56.9±28.2% in the LMWH group (p<0.0001) and to 57.6±26.8% in the UFH group (p<0.0001). However, this increase was not significantly different between the two heparin groups (p=0.75).

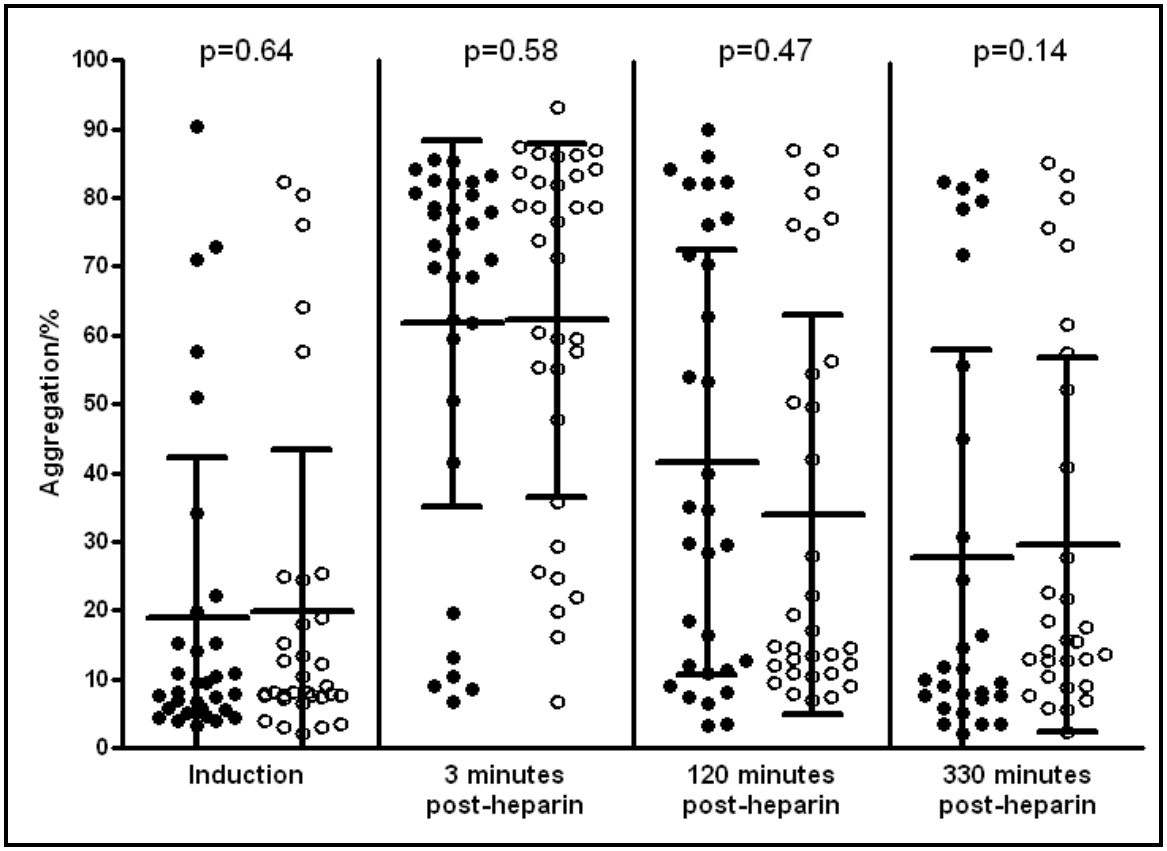

Figure 35 Platelet aggregation in response to 4×10^{-3}moll^{-1} AA in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD)

The platelet response to AA subsequently fell so that by 120 minutes it was 38.3±32.4% in the LMWH group and 27.3±26.1% in the UFH group (p=0.46), and at 330 minutes was

29.4±31.6% and 21.4±20.7% in the LMWH and UFH groups respectively (p=0.81). This fall in the AA response occurred without further aspirin therapy.[261,262]

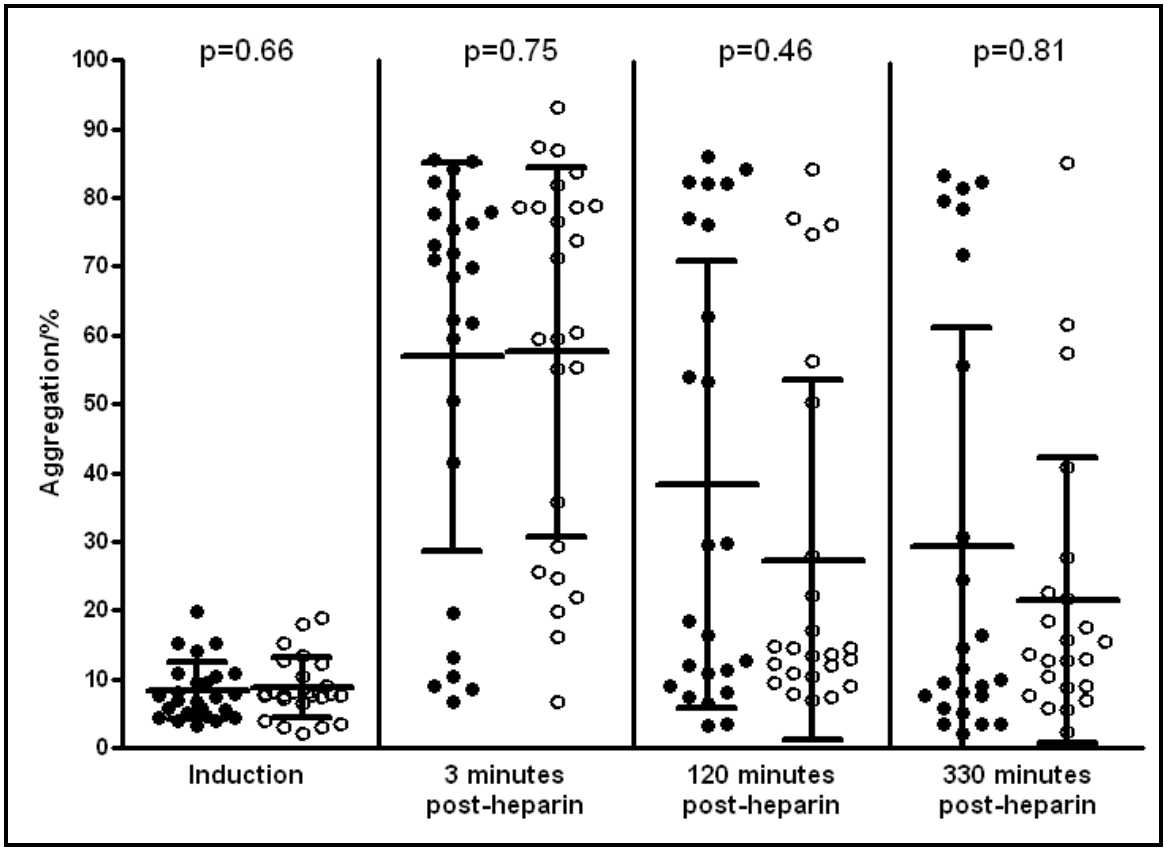

Figure 36 Platelet aggregation in response to 4×10^{-3}moll^{-1} AA in 50 patients undergoing CEA. Closed circles; LMWH group (n=25); open circles; UFH group (n=25), horizontal bar represents mean (±SD). 15 patients who exhibited greater than 20% platelet aggregation at induction have been excluded

Given the statistically significant difference observed between the LMWH and UFH groups in terms of presenting symptom (*Table 9*), the aggregation responses to AA were also analysed to look for potential confounding (*Figure 37*). At induction, the mean aggregation responses to 4×10^{-3}moll^{-1} AA were 14.4±16.5%, 18.4±25.6%, 23.6±27.6% and 21.1±22.4% for the asymptomatic, stroke, TIA and amaurosis fugax patients respectively

(p=0.63). After heparinisation, the responses to AA all rose significantly to 53.7±22.5% (asymptomatic, p<0.0001), 67.4±24.6% (stroke, p=0.002), 62.2±31.4% (TIA, p=0.0013) and 68.8±20.1% (amaurosis fugax, p=0.0008). These increases were not statistically significantly influenced by the presenting symptom however (p=0.13).

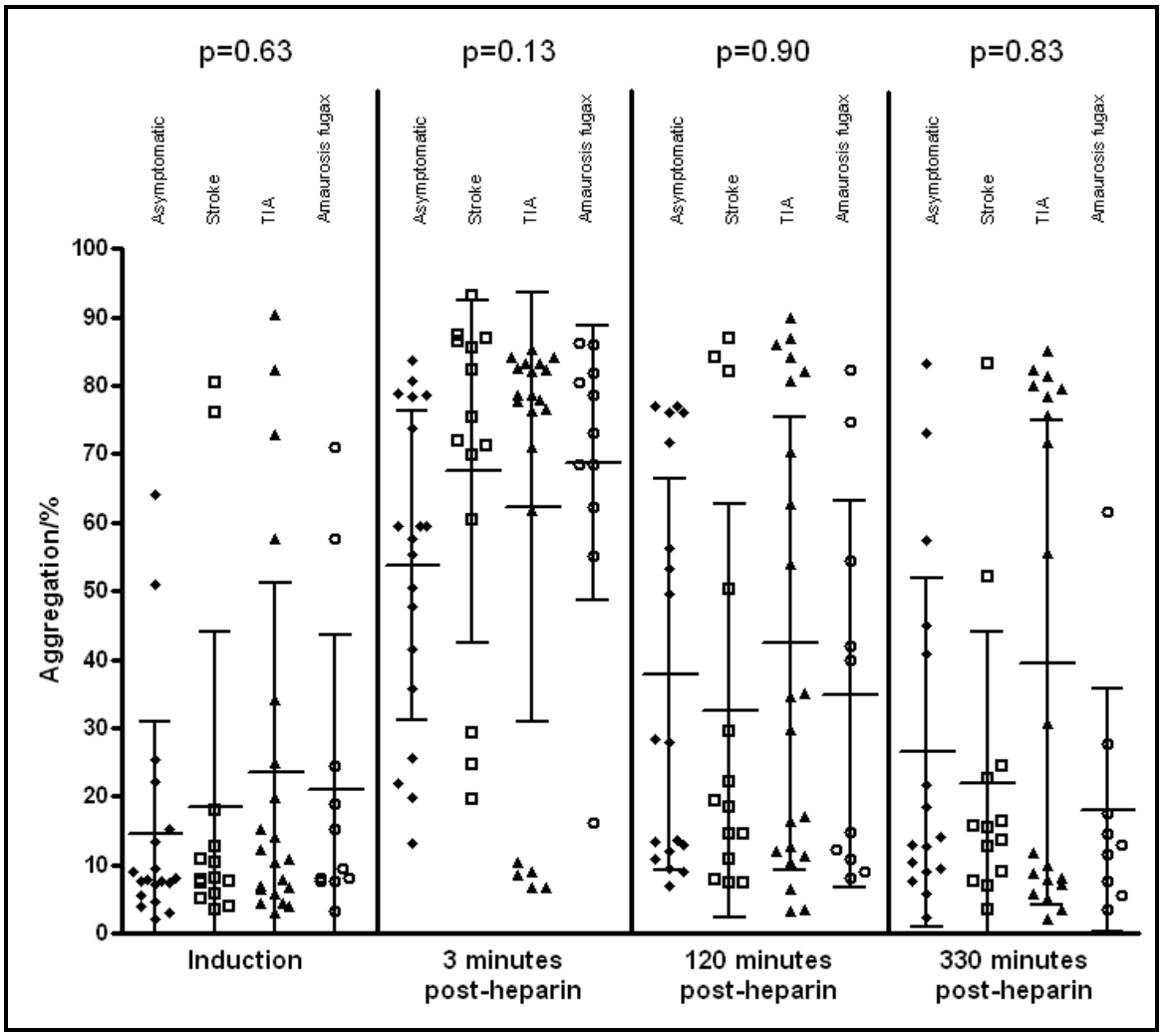

Figure 37 Platelet aggregation in response to 4×10^{-3}moll^{-1} AA in 65 patients undergoing CEA. Closed diamonds; asymptomatic patients (n=19); open squares; stroke patients (n=14); closed triangles; TIA patients (n=21); open circles; amaurosis fugax patients (n=11). Horizontal bar represents mean (±SD)

At 120 minutes post-heparinisation, platelet aggregation to AA was 37.8±28.5%, 32.5±30.2%, 42.3±33.1% and 34.8±28.2% for the asymptomatic, stroke, TIA and amaurosis fugax patients respectively (p=0.90).

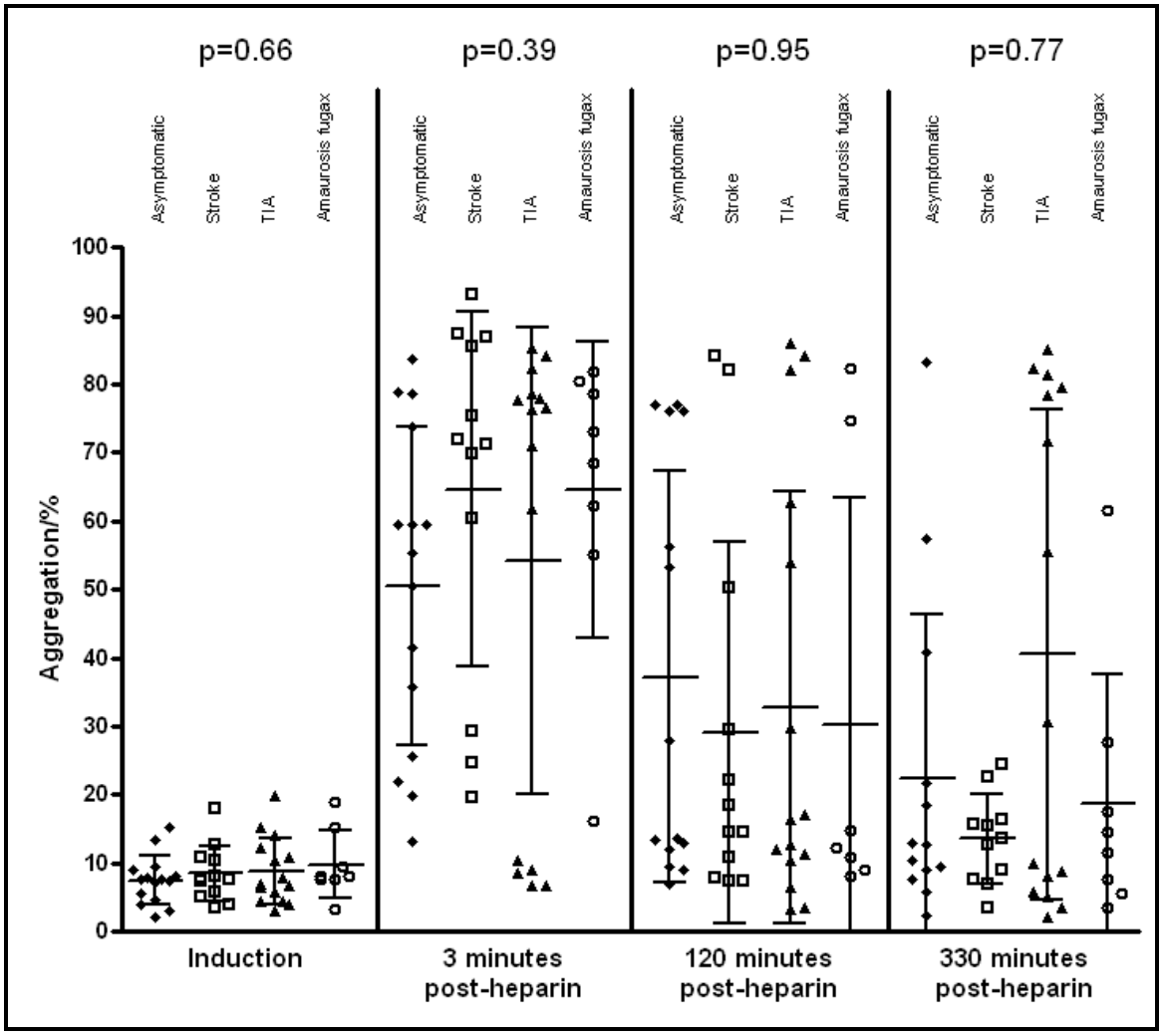

Figure 38 Platelet aggregation in response to 4×10^{-3}moll^{-1} AA in 50 patients undergoing CEA. Closed diamonds; asymptomatic patients (n=15); open squares; stroke patients (n=12); closed triangles; TIA patients (n=15); open circles; amaurosis fugax patients (n=8). Horizontal bar represents mean (±SD). 15 patients who exhibited greater than 20% platelet aggregation at induction have been excluded

There was also no statistical difference across presenting symptom at 330 minutes, with the respective aggregation responses at 26.5±25.5%, 21.9±22.2%, 39.5±35.3% and 18.1±17.8% (p=0.83). The potential confounding effect of presenting status was also analysed in only those patients who exhibited less than 20% platelet aggregation to AA 4×10^{-3}moll^{-1} at induction of anaesthesia (50 of the 65, 77% patients, *Figure 38*).

At induction of anaesthesia, there were no statistically significant differences in the mean platelet aggregation responses to 4×10^{-3}moll^{-1} AA, at 7.5±3.5% in the 15 asymptomatic patients, 8.5±4.1% in the 12 stroke patients, 8.8±4.8% in the 15 TIA patients, and 9.8±4.9% in the 8 amaurosis fugax patients (p=0.66). After heparinisation, these platelet responses increased significantly to 50.5±23.3% (p<0.0001), 64.4±25.9% (p<0.0001), 54.3±34.1% (p=0.0014) and 64.5±21.7% (p=0.0003) respectively. There was no statistical difference across the presentations, however (p=0.39). At 120 minutes post-heparinisation, the platelet aggregation responses to AA were 37.2±30.0%, 29.1±28.0%, 32.8±31.6% and 30.3±33.1% respectively (p=0.95). Similarly, although patients presenting with TIA exhibited a greater platelet aggregation response to AA at 330 minutes post-heparinisation, there was no statistical difference across the modes of presentation, with the respective aggregation levels 22.5±23.9%, 13.5±6.5%, 40.5±35.8% and 18.7±18.9% (p=0.77).

4.4.4 Platelet aggregation to ADP

Figure 39 shows platelet aggregation in response to ADP at a concentration of 3.3×10^{-6}moll^{-1} for all 65 patients. At induction of anaesthesia, the mean platelet response was similar in both groups (27.8±13.1% in the LMWH group and 31.7±12.2% in the UFH

group (p=0.10)). Three minutes after the administration of heparin, aggregation in response to ADP increased to 44.1±16.4% in the LMWH group (p<0.0001). The increase observed in the UFH group was even greater, rising to 63.3±11.6% (p<0.0001). By 120 minutes the platelet response to ADP had fallen slightly in both groups, but remained significantly greater in patients randomized to receive UFH (48.6±15.0%) compared to the LMWH group (40.0±14.4%; p=0.02).

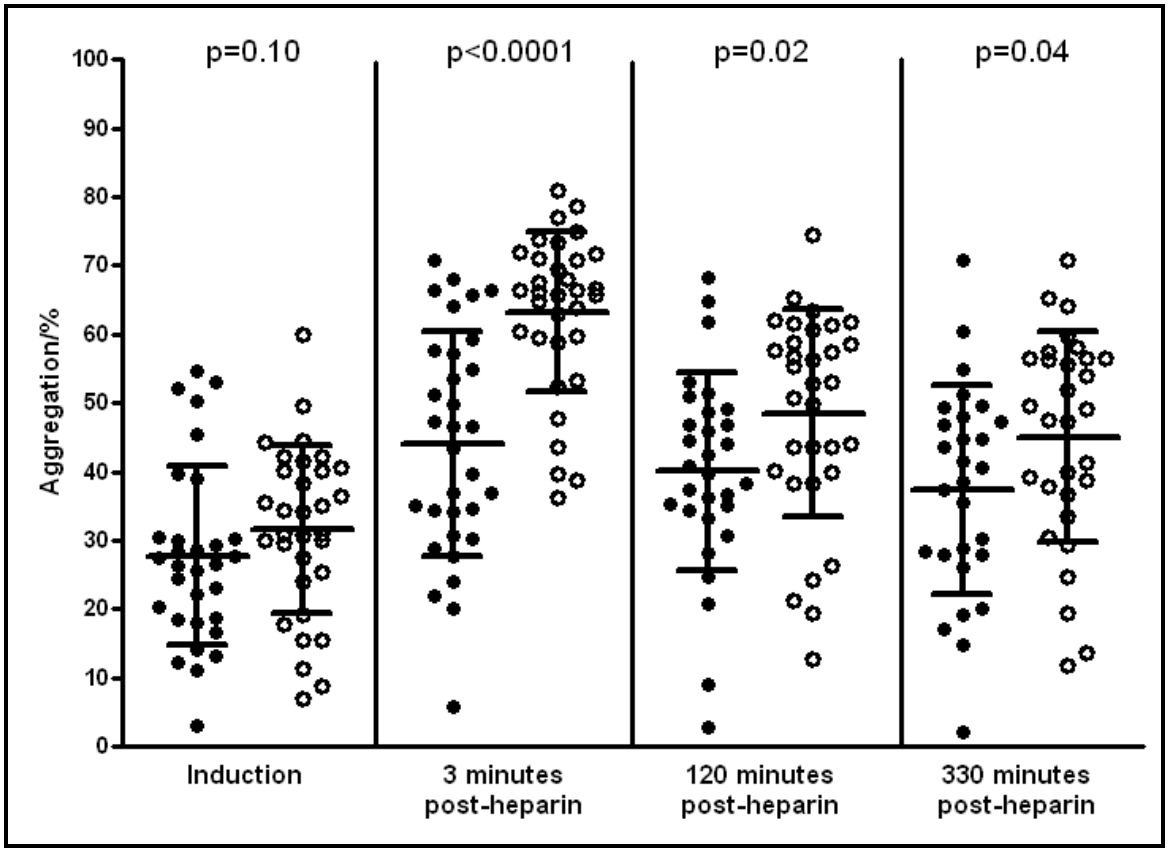

Figure 39 Platelet aggregation in response to 3.3×10^{-6}moll^{-1} ADP in 65 patients undergoing CEA. Closed circles; LMWH group (n=32); open circles; UFH group (n=33), horizontal bar represents mean (±SD)

At 330 minutes the UFH group still showed greater aggregation to ADP (45.1±15.3%) compared to the LMWH group (37.4±15.2%), which remained statistically significant (p=0.04).

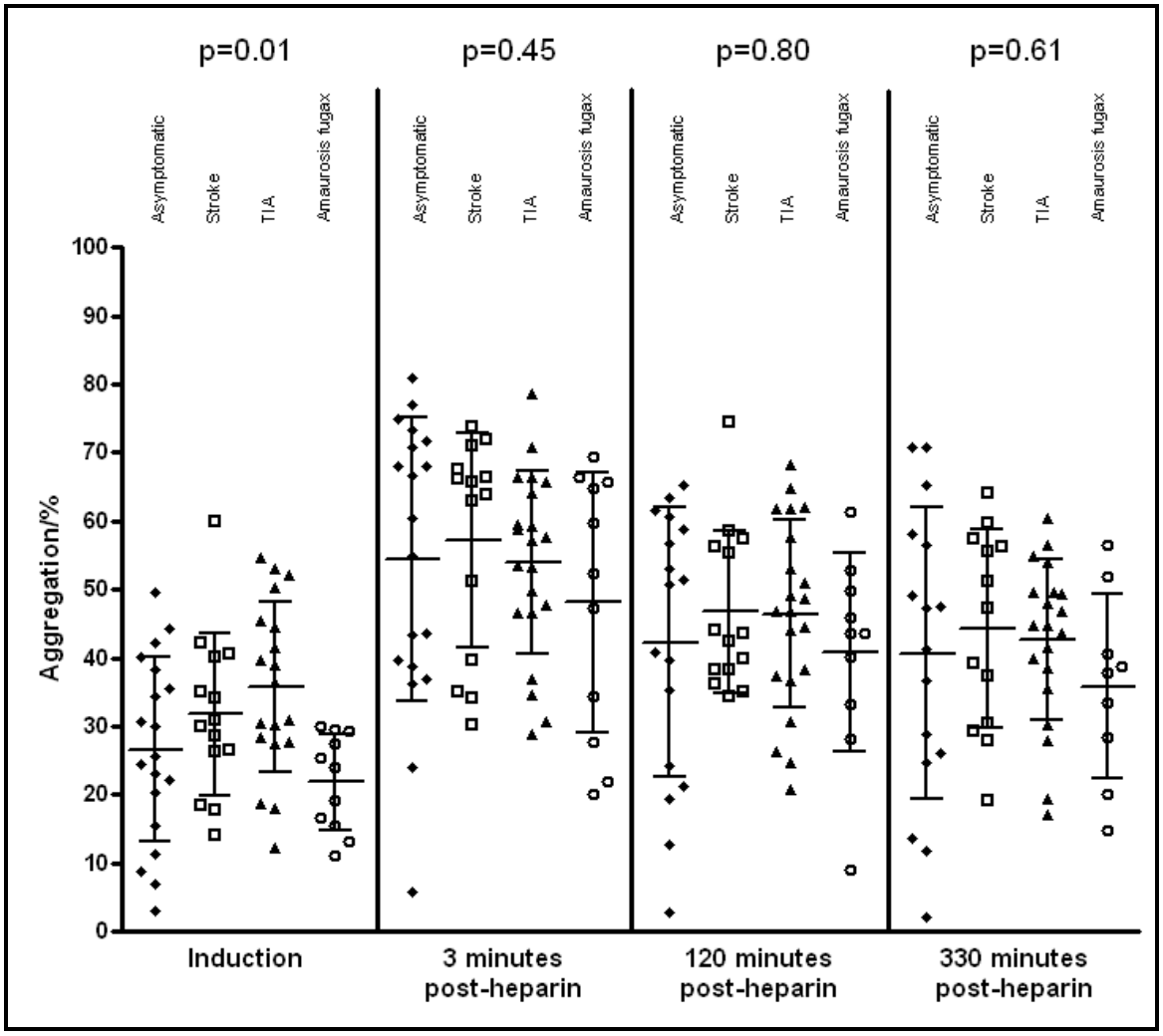

Figure 40 Platelet aggregation in response to 3.3×10^{-6}moll^{-1} ADP in 65 patients undergoing CEA. Closed diamonds; asymptomatic patients (n=19); open squares; stroke patients (n=14); closed triangles; TIA patients (n=21); open circles; amaurosis fugax patients (n=11). Horizontal bar represents mean (±SD)

In view of the statistically significant difference in presenting symptoms between the LMWH and UFH groups (*Table 9*), the platelet aggregatory response to ADP was also

analysed according to presenting symptom (*Figure 40*). At induction of anaesthesia, the mean platelet response to 3.3×10^{-6}moll^{-1} ADP was 26.6±13.5% in the asymptomatic, 31.8±11.9% in the stroke, 35.8±12.5% in the TIA and 22.0±7.0% in the amaurosis fugax patients. The level of aggregation in the amaurosis fugax patients was significantly lower than that in the TIA (p=0.002) and stroke (p=0.02) but not the asymptomatic patients (p=0.29). The ADP response at induction between asymptomatic and TIA patients was also statistically significantly different (p=0.04), but there was no difference between the response in asymptomatic and stroke patients (p=0.37). After heparinisation, the platelet aggregatory responses to ADP rose significantly to 54.5±20.7% (p=0.0002), 57.1±15.7% (p=0.0005), 53.9±13.3% (p=0.0002) and 48.2±19.0% (p=0.003) in the asymptomatic, stroke, TIA and amaurosis fugax patients respectively. There was no statistically significant difference between these increases (p=0.45). At 120 minutes post-heparinisation, the ADP responses were not statistically significantly different at 42.2±19.7%, 46.7±11.8%, 46.4±13.7% and 40.8±14.6% respectively (p=0.80). Similarly, at the 330 minute time-point, there was no significant difference in platelet response to ADP across the presentations, at 40.7±21.3%, 44.2±14.5%, 42.6±11.7% and 35.8±13.5% respectively (p=0.61).

4.4.5 Platelet aggregation to AA and inhibitors

4.4.5.1 Aspirin

In a sub-population of 20 patients undergoing CEA, the PRP was incubated with aspirin at a final concentration of 4×10^{-4}moll^{-1} prior to aggregometry in response to AA. The aim of

this experiment was to assess whether the *ex vivo* addition of the AA/COX-1-inhibitor aspirin to the platelets would overcome the increase in AA-stimulated aggregation in patients receiving either heparin type.

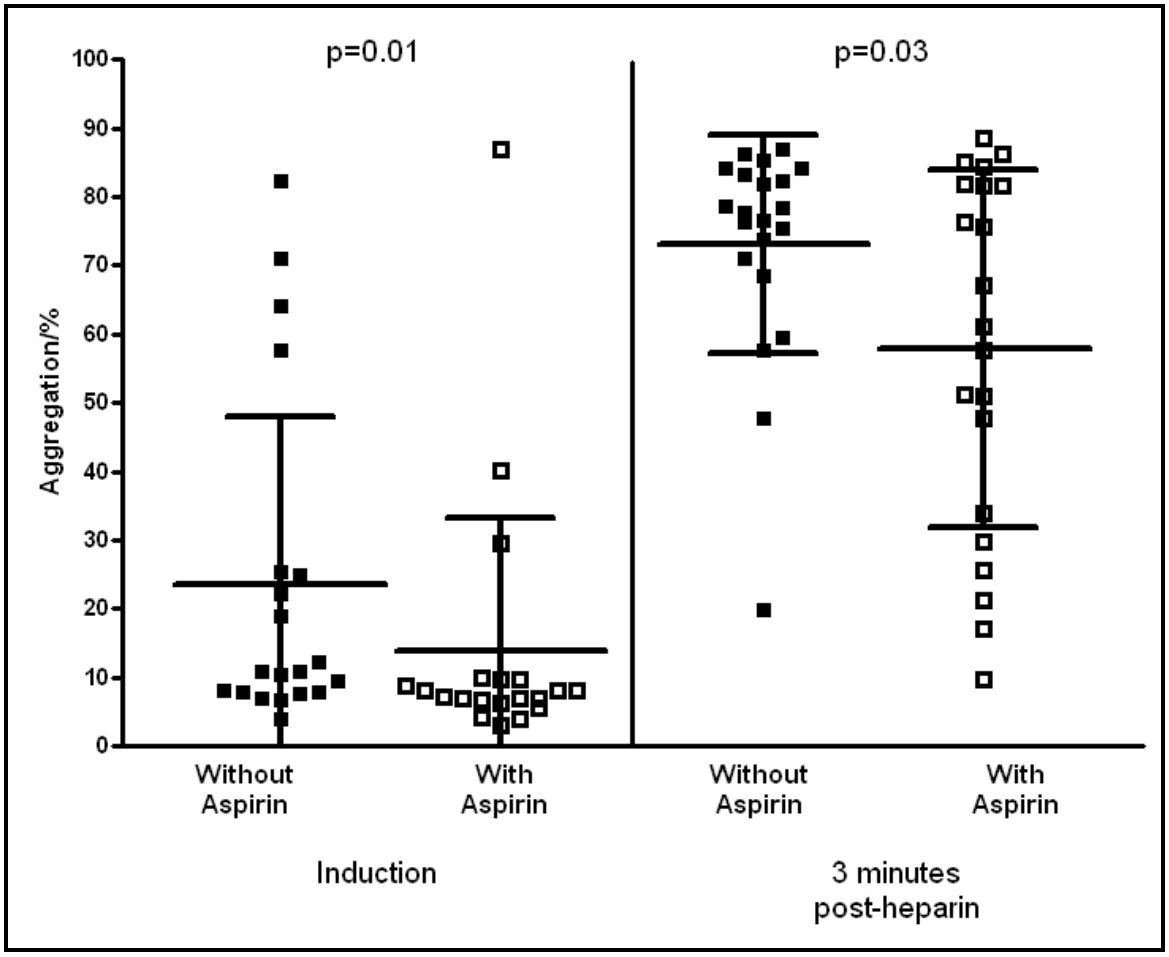

Figure 41 Platelet response to 4×10^{-3}moll^{-1} AA in 20 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with aspirin 4×10^{-4}moll^{-1}. Horizontal bar represents mean (±SD)

At induction of anaesthesia the mean aggregation to AA was 23.4±24.4%. Aggregation to AA at induction, after incubation with aspirin dropped significantly to 13.9±19.3% (p=0.01, *Figure 41*). After heparinisation with either LMWH or UFH the mean

aggregation response to AA rose significantly to 73.1±15.9% ($p<0.0001$). When the PRP was incubated with aspirin, the mean aggregation response to AA remained high at 57.8±26.0%, but this was nonetheless significantly lower than without *in vitro* addition of aspirin ($p=0.03$).

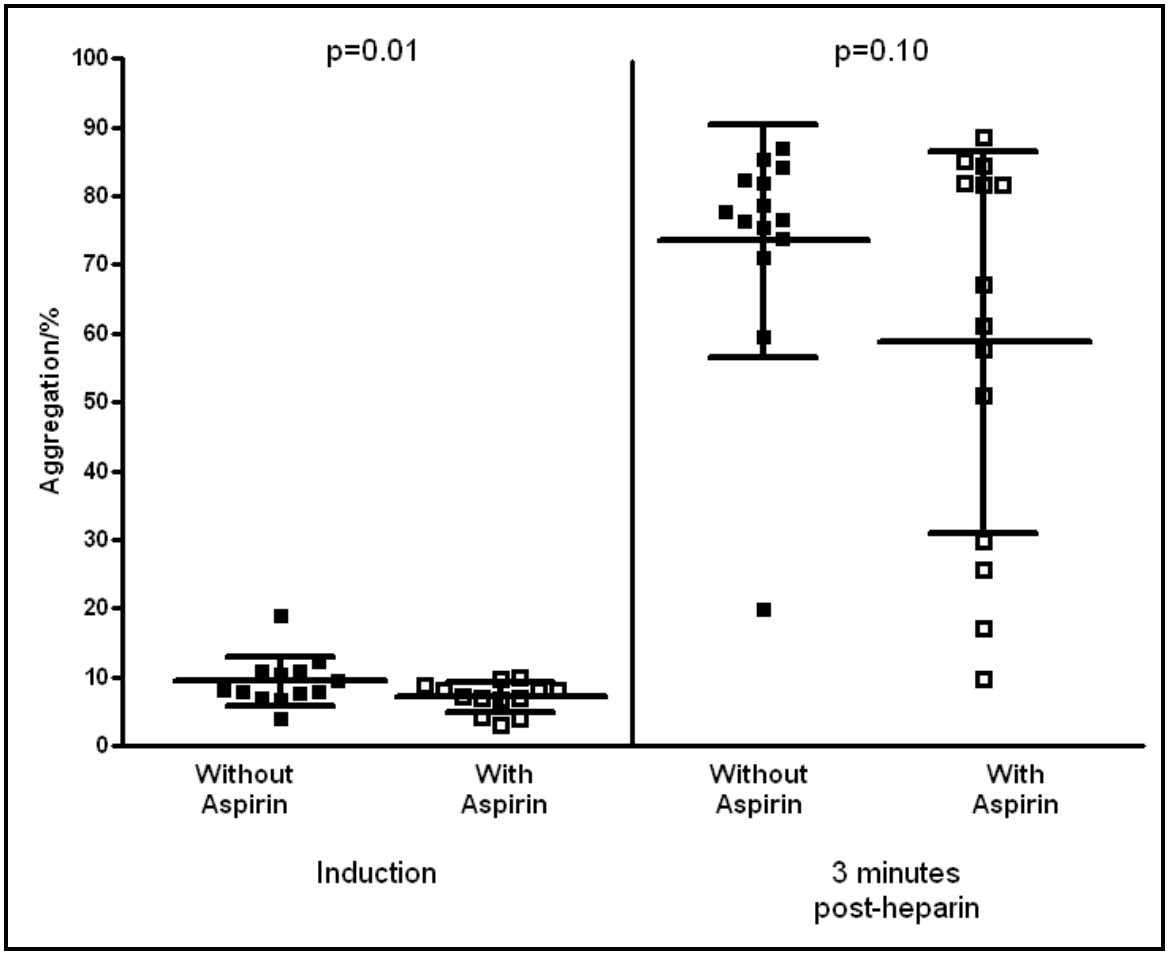

Figure 42 Platelet response to 4×10^{-3}moll^{-1} AA in 13 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with aspirin 4×10^{-4}moll^{-1}. Horizontal bar represents mean (±SD) Seven patients who exhibited greater than 20% platelet aggregation to AA at induction have been excluded

However, 7 of the 20 (35%) patients exhibited greater than 20% aggregation response to AA at induction of anaesthesia, suggestive of "aspirin failure".[205] When these subjects

were excluded, the mean aggregation response to AA at induction in the remaining 13 patients was low, at 9.4±3.6%. This was further reduced significantly by incubation with aspirin, to a mean aggregation response of 7.1±2.2% (p=0.01, *Figure 42*).

After heparinisation, the significant increase in aggregation response to AA was maintained, at 73.5±16.9%. The fall in mean aggregation response when the post-heparin PRP was incubated with aspirin, to 58.7±27.8% no longer reached statistical significance (p=0.10).

4.4.5.2 Baicalein

The IC_{50} of baicalein is $1.2\times10^{-7}moll^{-1}$. PRP from 26 patients undergoing CEA was incubated with two different concentrations of baicalein; $8.2\times10^{-7}moll^{-1}$ and $8.2\times10^{-6}moll^{-1}$ in order to ensure maximal 12-LOX inhibition. The aim of this study was to ascertain whether inhibition of platelet 12-LOX would reduce the increase seen in the platelet aggregatory response to AA after heparinisation with either UFH or LMWH.

At induction of anaesthetic, the mean platelet response to $4\times10^{-3}moll^{-1}$ AA was 22.3±26.3%. After incubation with $8.2\times10^{-7}moll^{-1}$ baicalein, this mean fell significantly to 13.1±16.3% (p=0.04, *Figure 43*).

After heparinisation, the mean aggregatory response to AA rose significantly to 68.0±24.1% (p<0.0001). With baicalein the mean aggregatory response to AA did not change significantly, at 63.7±25.4% (p=0.20).

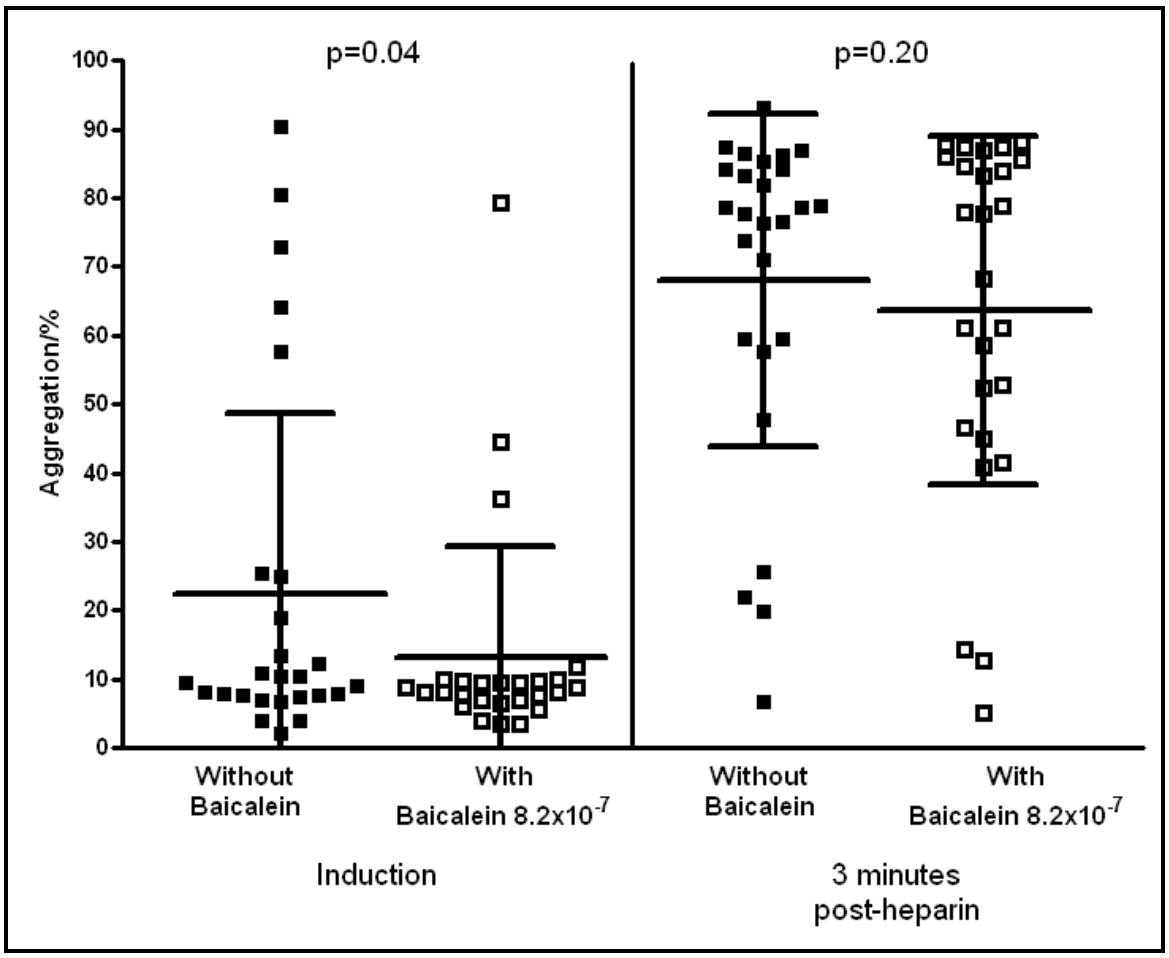

Figure 43 Platelet response to 4×10^{-3}moll^{-1} AA in 26 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with baicalein 8.2×10^{-7}moll^{-1}. Horizontal bar represents mean (±SD)

There were 7 of the 26 patients who exhibited greater than 20% aggregation to AA at induction, suggestive of "aspirin failure". When these patients were excluded from analysis, there was no significant difference in the mean aggregation response to AA at induction without and with baicalein, at 8.7±3.8% and 9.0±6.9% respectively (p=0.86). There was also no difference observed in the platelet response to AA without and with baicalein after heparinisation, at 65.5±26.5% and 63.6±25.5% respectively (p=0.57, *Figure 44*).

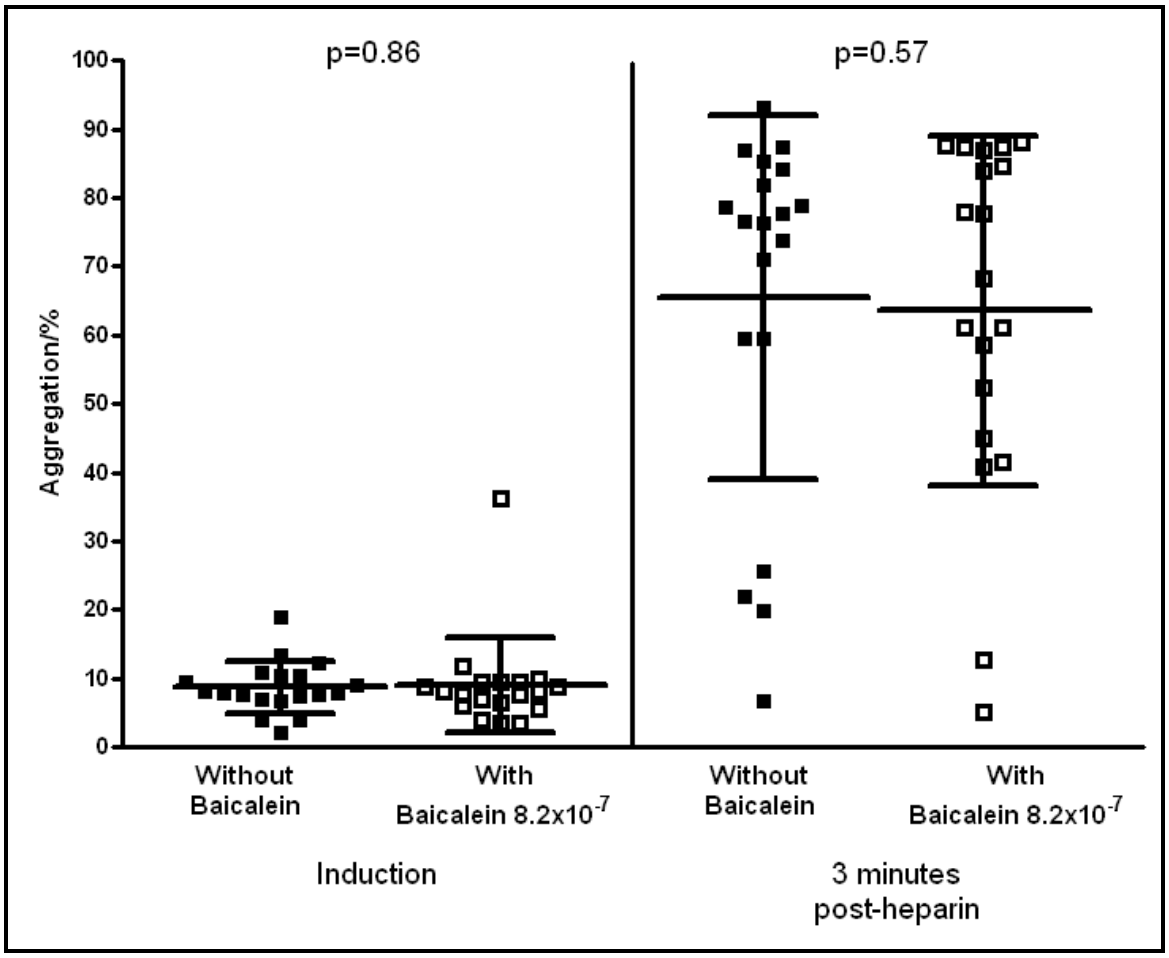

Figure 44 Platelet response to 4×10^{-3}moll^{-1} AA in 19 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with baicalein 8.2×10^{-7}moll^{-1}. Horizontal bar represents mean (±SD). Seven patients who exhibited greater than 20% platelet aggregation to AA at induction have been excluded

PRP was also incubated with baicalein at a final concentration of 8.2×10^{-6}moll^{-1} in 11 subjects undergoing CEA. At induction of anaesthesia, the platelet aggregatory response was 40.6±38.5%, which rose to 65.8±31.3% after heparinisation, although this did not reach statistical significance (p=0.06).

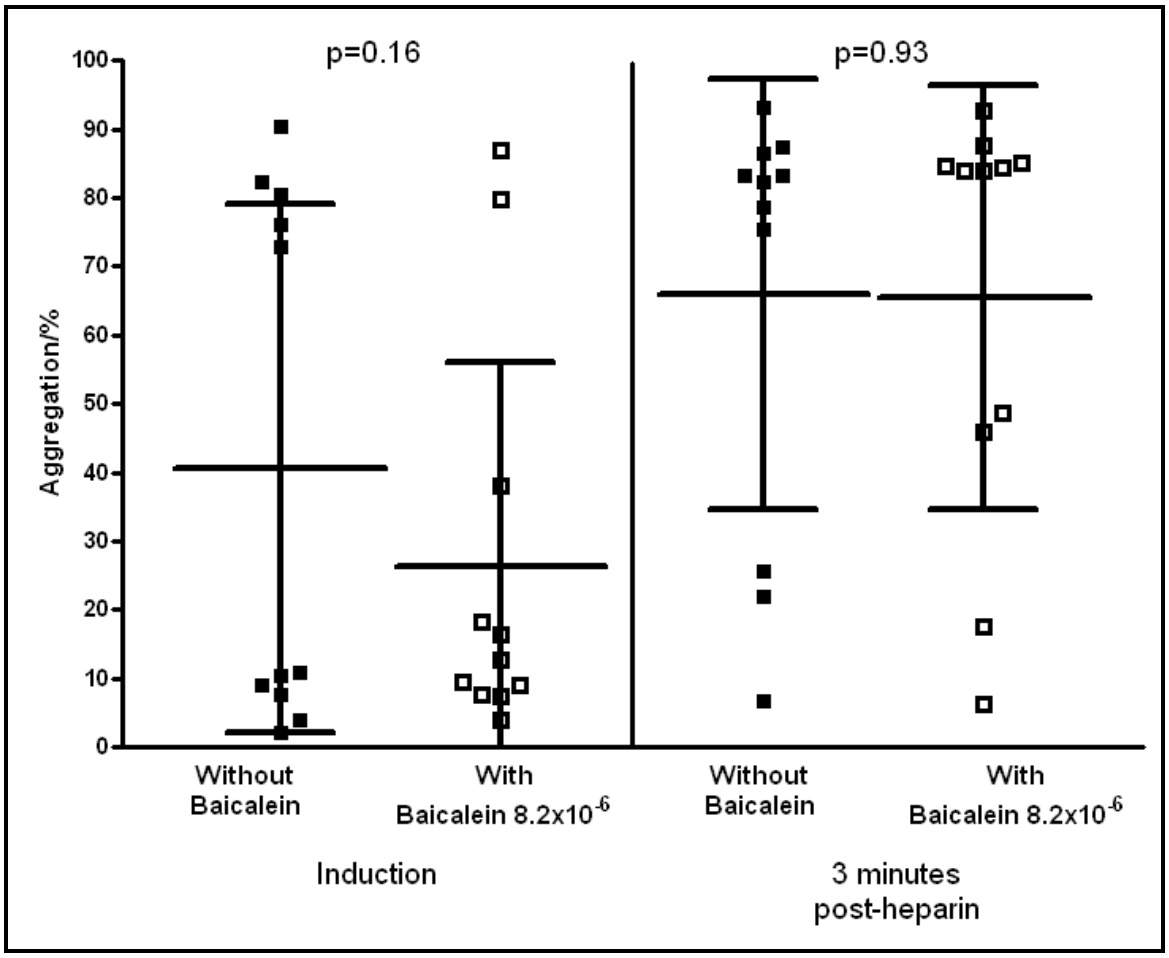

Figure 45 Platelet response to 4×10^{-3}moll^{-1} AA in 11 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with baicalein 8.2×10^{-6}moll^{-1}. Horizontal bar represents mean (±SD)

At induction, the incubation of PRP with 8.2×10^{-6}moll^{-1} baicalein reduced the platelet response non-significantly to 26.3±29.7% (p=0.16). Incubation of the PRP after heparinisation did not affect a change in the platelet response to AA, at 65.5±30.8% (p=0.93, *Figure 45*).

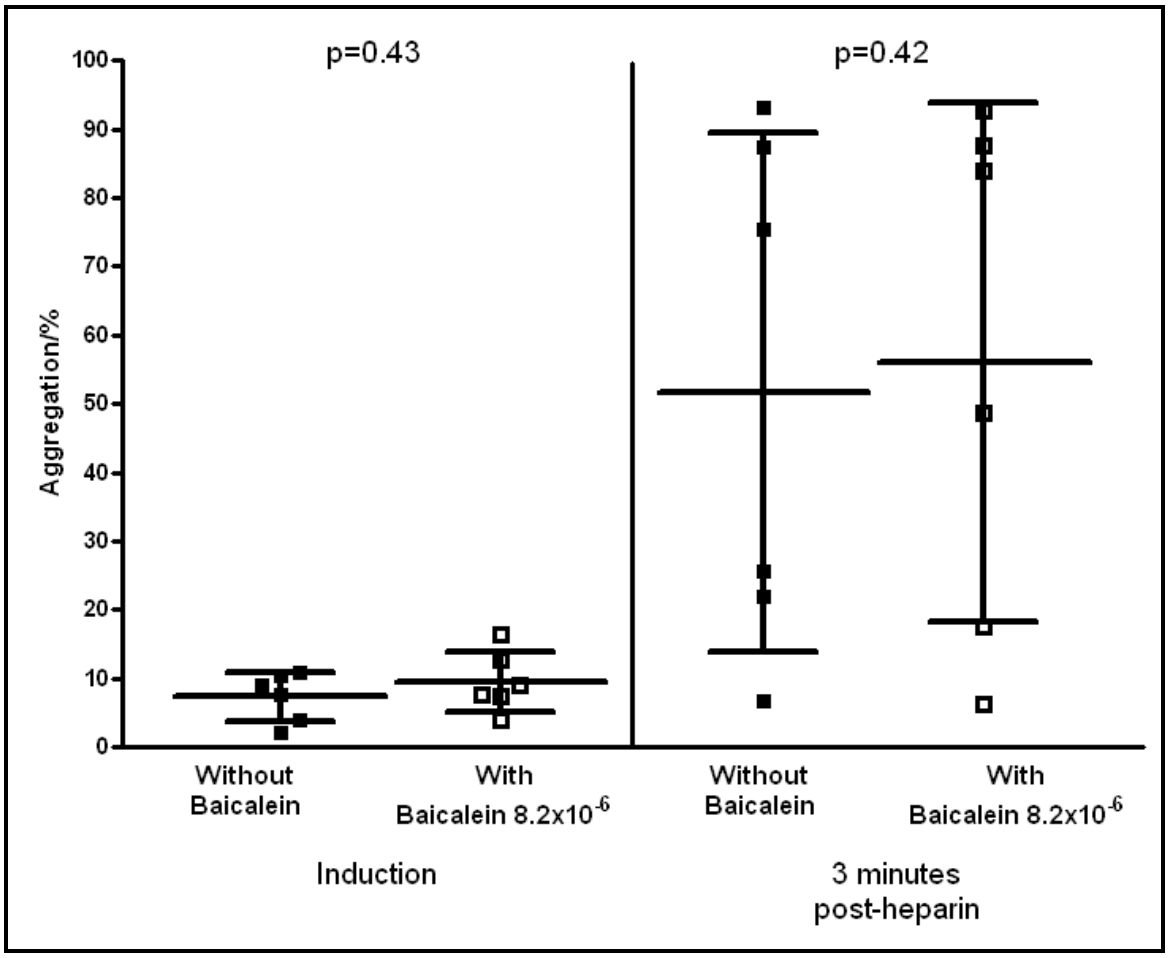

Figure 46 Platelet response to 4×10^{-3}moll^{-1} AA in 11 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with baicalein 8.2×10^{-6}moll^{-1}. Horizontal bar represents mean (±SD). Five patients who exhibited greater than 20% platelet aggregation to AA at induction have been excluded

Five of the 11 patients exhibited greater than 20% aggregation to AA at induction, suggestive of "aspirin failure",[205] and when these subjects were excluded from analysis, the results were not statistically significant. The mean aggregation response to AA at induction was 7.4±3.6%, which was not reduced with baicalein (9.5±4.4%, p=0.43). The rise in aggregation response after heparinisation to 51.7±37.8% was significant (p=0.03), but this was unaffected by incubation with baicalein, at 56.1±37.8% (p=0.42, *Figure 46*).

4.4.5.3 Combination aspirin and baicalein

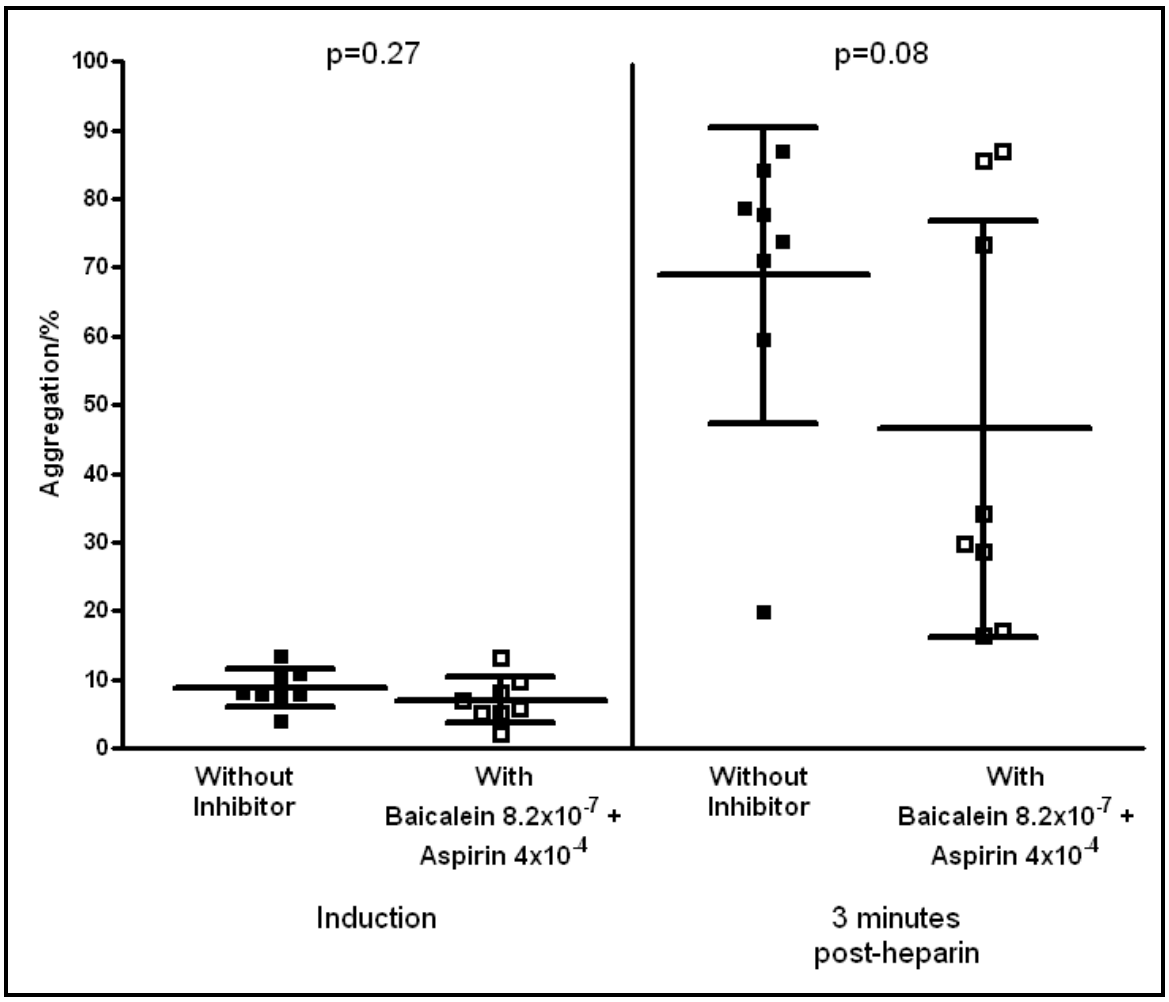

Figure 47 Platelet response to 4×10^{-3}moll^{-1} AA in 11 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with baicalein 8.2×10^{-6}moll^{-1}. Horizontal bar represents mean (±SD)

To simultaneously inhibit both the COX-1 pathway and the 12-LOX pathway, PRP from patients undergoing CEA was also incubated with 4×10^{-4}moll^{-1} aspirin and 8.2×10^{-7}moll^{-1} baicalein simultaneously in 8 patients.

At induction of anaesthesia, mean aggregation to AA was low at 8.8±2.8%. This rose significantly to 68.9±21.5% after heparinisation (p=0.0002). There was no statistically significant change in the aggregatory response to AA at induction of anaesthesia when the PRP was incubated with aspirin and baicalein, at 7.0±3.4% (p=0.27). There was a non-significant reduction in the platelet aggregatory response to AA after heparinisation when the PRP was incubated with aspirin and baicalein, to 46.5±30.3% (p=0.08, *Figure 47*).

4.4.5.4 Thromboxane receptor antagonist (SQ 29548)

In 12 subjects undergoing CEA, the PRP was incubated with the thromboxane receptor antagonist SQ 29548 at a concentration of $1.9 \times 10^{-6} moll^{-1}$. In these patients at induction of anaesthesia, the mean platelet aggregatory response to AA was 15.6±13.9%, which rose significantly to 64.4±21.5% after heparinisation (p=0.0003). When the induction PRP was incubated with SQ 29548, the mean aggregation did not significantly change, at 7.6±3.8% (p=0.08). When the PRP from the post-heparin samples was incubated with SQ 29548, the mean aggregatory response fell significantly to 35.5±28.0% (p=0.04, *Figure 48*).

Two of the patients in this study exhibited greater than 20% aggregation to AA at induction, suggestive of "aspirin failure". When these patients were excluded from analysis, the mean aggregatory response to AA at induction was 10.2±5.0%, rising significantly to 61.0±22.0% after heparinisation (p=0.0002).

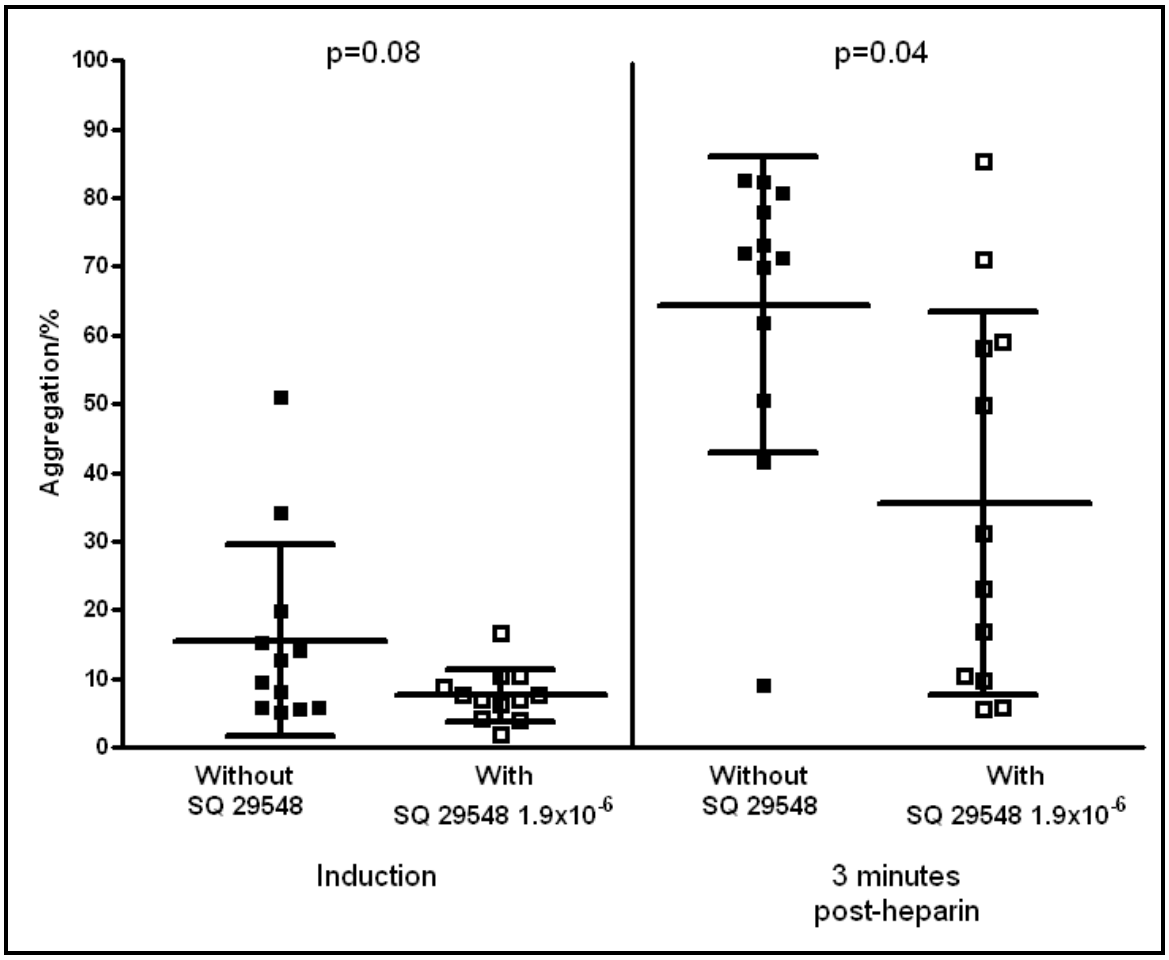

Figure 48 Platelet response to 4×10^{-3}moll^{-1} AA in 12 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with SQ 29548 1.9×10^{-6}moll^{-1}. Horizontal bar represents mean (±SD)

There was no difference in the induction response to AA when incubated with SQ 29548, at 7.3±2.2% (p=0.08). When the post-heparinisation PRP was incubated with SQ 29548, the fall in aggregatory response to AA lost statistical significance at 35.8±28.8% (p=0.11, *Figure 49*).

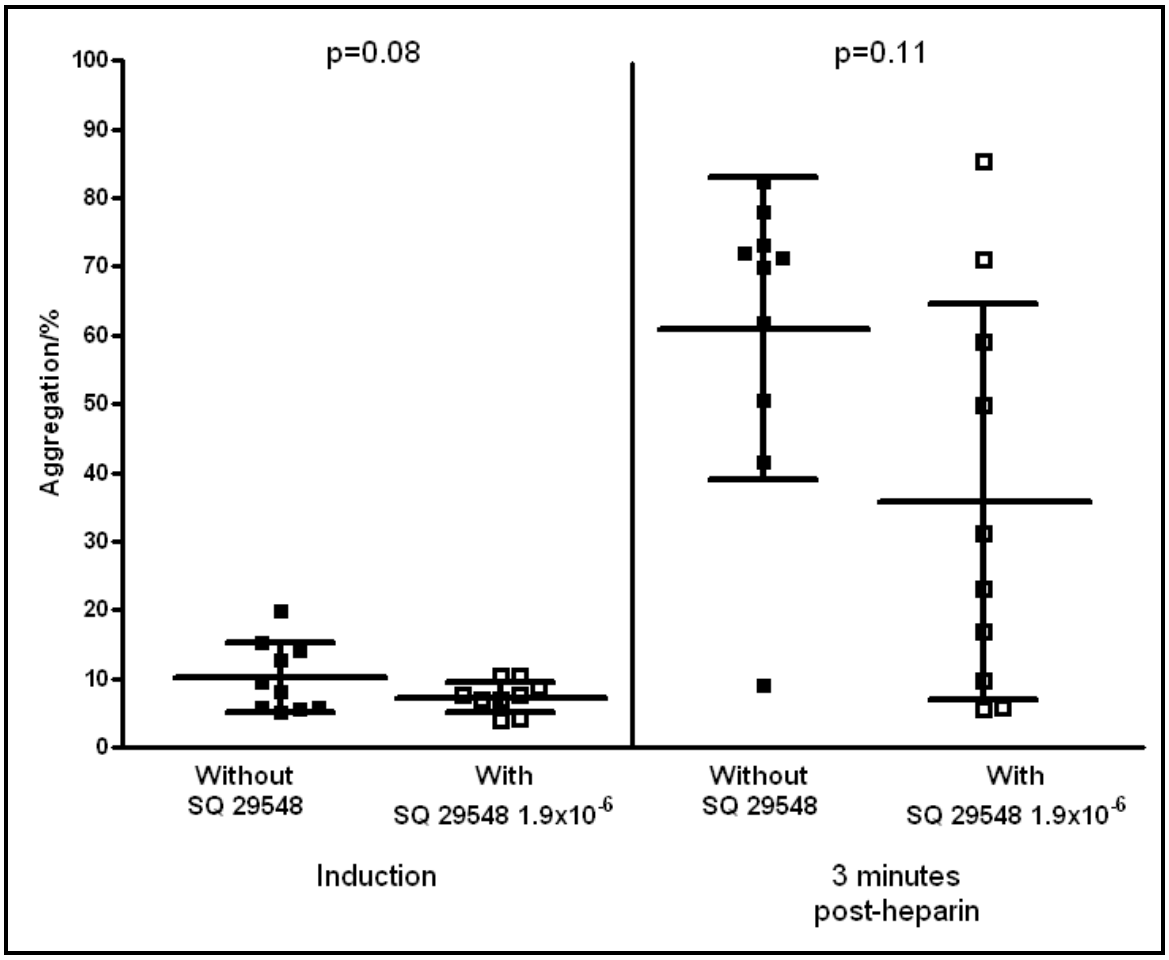

Figure 49 Platelet response to 4×10^{-3}moll^{-1} AA in 10 patients undergoing CEA. Closed squares; aggregation to AA alone; open squares; aggregation to AA in PRP incubated with baicalein 1.9×10^{-6}moll^{-1}. Horizontal bar represents mean (±SD). Five patients who exhibited greater than 20% platelet aggregation to AA at induction have been excluded

4.5 Discussion

The findings from this series of studies confirm that systemic heparinisation causes a transient increase in *ex vivo* platelet aggregation in response to AA, despite adequate aspirination; an apparent "transient aspirin failure".[261,262]

It is perhaps not unexpected that there was an increase in the leucocyte count by 330 minutes after heparinisation in both the UFH and LMWH groups. This is probably explained by the physiological response of the patients to the impact of surgery. Similarly, falls in haemoglobin and erythrocyte counts, which were observed in both groups, were attributable to the coinciding drop in haematocrit, likely due to the intra-operative administration of intravenous fluids.

The success of vascular reconstruction has long relied upon the anticoagulant properties of heparin,[267] and intravenous UFH has traditionally been the drug of choice for the prevention of arterial thromboembolic events. Compared with UFH, LMWH is considered to induce a more stable and predictable anticoagulant dose response, and to have a greater ratio of anti-factor Xa activity to anti-factor IIa activity, which reduces the generation and activation of thrombin.[268] In addition to reducing the incidence of venous thromboembolism,[269] LMWH has previously been shown to be effective in preventing arterial thrombosis.[243]

It is known, however, that while heparin inhibits the enzymatic effects of thrombin, including thrombin-induced platelet aggregation, it can paradoxically potentiate platelet aggregation induced by a range of platelet agonists, including ADP, TRAP, PAF and epinephrine.[244-247] The binding of heparin to platelets is known to increase with increasing molecular weight,[270] and LMWH is less effective than UFH in inducing several pathways of platelet activation.[246,271,272]

The "transient aspirin failure" observed in this study was in patients who had generally been adequately aspirinated, as illustrated by the low mean platelet aggregatory response to AA at induction of anaesthesia. Exclusion of those patients who were observed to be "aspirin resistant" at induction of anaesthesia did not diminish the increase seen in AA-mediated platelet aggregation after heparinisation. Furthermore, the inhibitory effect of aspirin was subsequently regained by the patients' platelets over the period of a few hours, without any further therapy and without any significant change in the platelet count over the course of the procedure and postoperative period. Since the major anti-thrombotic effect of aspirin is irreversible, via the acetylation of platelet COX-1 which inhibits TXA_2 synthesis,[196] and since platelets do not possess a nucleus from which to synthesize new COX-1, this recovery cannot be attributable to restoration of COX-1 activity in the platelets. However, although systemic heparinisation resulted in a significant increase in the *ex vivo* platelet aggregation response to AA, there was no significant difference in this increased response between heparin types. In contrast, the platelets of patients receiving UFH were significantly more responsive to ADP-induced aggregation than the patients who received LMWH. Importantly, this difference persisted into the immediate post-operative period, with the platelets of the patients in the UFH group remaining significantly more responsive to ADP-induced aggregation 330 minutes following heparinisation. This observation is compatible with previous work suggesting that the response to ADP is a significant predictor of embolization risk following CEA.[259]

The proposed importance of the platelet response to ADP is further emphasized by the observation that the platelet responses differed significantly according to the presenting symptom (TIA, stroke, amaurosis fugax or asymptomatic). Patients presenting with TIAs

had the greatest initial response to ADP, followed by patients presenting with strokes (*Figure 40*). Those whose presenting feature was amaurosis fugax actually exhibited the lowest ADP response pre-operatively, although there was no significant difference between their response and the responses of the asymptomatic patients. Although these differences disappeared after heparinisation, this finding supports the theory that the platelet response to ADP has a fundamental relationship to the clinical characteristics of the patient. The platelet response to AA had no relation to the presenting feature of the patients.

When the "aspirin-resistant" subjects were excluded from the analysis, the mean aggregation response to AA at induction was still further reduced with the *ex vivo* addition of aspirin. Whilst the incubation of aspirin with the post-heparin PRP samples did reduce the phenomenon of "transient aspirin failure" from 73.5% in the non-aspirinated group to 58.7% in the aspirinated group, this was not statistically significant. The 12-LOX inhibitor baicalein also reduced the aggregation response amongst the aspirin resistant patients preoperatively, but did not affect the phenomenon of heparin-mediated "transient aspirin failure". In combination with aspirin, baicalein was associated with a reduction in the "transient aspirin failure" from 68.9% to 46.5%, but this did not achieve statistical significance. Given that baicalein alone failed to influence the increase in aggregation to AA following heparinisation, it's probable that the reduction seen in combination with aspirin was mediated purely by COX-1 inhibition, rather than blockade of 12-LOX.

The thromboxane receptor antagonist was used to block the final pathway of AA-mediated platelet aggregation, and SQ 29548 did reduce the aggregation response amongst the initially "aspirin-resistant" patients from a mean of 15.6% to 7.6%, although this was not

statistically significant. Thromboxane receptor antagonism did significantly reduce the phenomenon of heparin-mediated AA aggregation, from 64.4% to 35.5%, although when analysed without the "aspirin resistant" subjects, this significance was lost. However, this also suggests that the change in AA aggregation after heparinisation may be mediated through inhibition of TXA_2 on the platelet.

The statistical significance of the inhibitor results was universally lost when the initially "aspirin-resistant" patients were excluded from the analyses, although the trends in reduction of the phenomenon of heparin-mediated "transient aspirin failure" with aspirin and SQ 29548 remained. There are probably two explanations for this. Firstly, those patients whose platelets aggregated in response to AA at induction of anaesthesia would exhibit less of an increase in aggregation following heparinisation, and the inhibitors would reduce aggregation after heparinisation just as they did beforehand. Secondly, exclusion of the initially "aspirin-resistant" patients from analysis probably reduced the sample sizes to such an extent that statistical significance was lost.

Studies have suggested that between 8% and 45% of the population are aspirin resistant[273-275] and the prevalence in our study population was within this range, with 15 of 65 (23%) defined as aspirin resistant by the arbitrary criterion that they exhibited greater than 20% aggregation to AA.[205] On average, the weight of the resistant patients was 9kg greater than the responding patients (83kg vs. 74kg), but this difference was not significant. One explanation for the observed aspirin resistance might be that there is a weight-dose relationship, and that the relative availability of aspirin in the heavier patients was lower.

This hypothesis is not, however, supported by this data-set, with no correlation observed between platelet response to AA at induction of anaesthesia and patient weight (*Figure 34*).

V

Heparin Activates Platelet 12-LOX – a Study of Platelet Pathways

5.1 Introduction

Aspirin irreversibly inhibits cyclo-oxygenase-1 (COX-1). The platelets of adequately treated individuals are therefore unable to convert arachidonic acid (AA) to the platelet proactive eicosanoid, thromboxane A_2 (TXA_2). For patients undergoing carotid endarterectomy (CEA), peri-operative aspirin treatment reduces the risk of post-operative stroke,[276] probably because the predominant cause is the embolization of platelet-rich material.[95]

Immediately following the administration of heparin during CEA, platelets transiently aggregated in response to AA, before spontaneously reverting to their inhibited state without further aspirin ingestion (*Chapter IV*). A similarly transient increase in platelet aggregation to adenosine diphosphate (ADP) was also observed, and whilst intra-operative anticoagulation with intravenous low molecular weight heparin (LMWH) rather than in the

standard fashion with unfractionated heparin (UFH) did not reduce the phenomenon of transient aspirin resistance, it did significantly diminish this ADP response.

Like COX-1, the platelet enzyme 12-lipoxygenase (12-LOX) also metabolises AA, to 12-hydroxyeicosatretraenoic acid (12-HETE) rather than TXA_2. This eicosanoid has itself been reported to act as a platelet activator, promoting thrombus formation in vivo.[277,278] It was therefore hypothesized that the observed heparin-induced "transient aspirin resistance" might be mediated by the metabolism of AA through this parallel pathway in COX-1-inhbibited platelets. Furthermore, given the known advantages of LMWH over UFH, it was also proposed that there would be less 12-LOX activity in patients heparinised with LMWH rather than UFH during CEA.

5.2 Aims

The aims of this series of experiments were to determine the pathways that were functioning in the platelet in parallel to the transient changes observed in aggregation, and to ascertain whether intra-operative anticoagulation with LMWH rather than UFH would affect platelet pathway activity.

5.3 Materials and Methods

A double-blind randomized controlled trial in consecutive aspirinated patients undergoing CEA was established as described (*Section 4.2*). Fifty-eight patients were included in this

study of platelet pathways; 30 were randomized to receive 5000IU UFH and 28 were randomized to receive 2500IU LMWH intravenously prior to carotid clamping. Plasma and serum were prepared and stored at -70°C for subsequent *en masse* quantification of platelet metabolites.

5.3.1 Blood sampling

Blood samples were taken from an indwelling arterial line into vacutainer tubes (Becton Dickinson, Oxford, UK) with the first 3ml of blood wasted and subsequent samples taken into 0.105M buffered sodium citrate solution for plasma preparation and inert polymer gel/clot activator for serum preparation. The citrated blood was centrifuged at 1500×g for 20 minutes before the supernatant was aspirated and transferred to vials for further spinning on a microfuge at 9300×g for an additional 10 minutes to produce platelet-poor plasma (PPP). Supernatants were aspirated with a Pasteur pipette from the centre, and to within 1-2mm of the buffy coat to avoid aspirating platelets and white cells. The serum tubes were stored upright for 60 minutes to allow full thrombosis of the blood, before being centrifuged at 1500×g for 20 minutes. The serum supernatant was then aspirated and aliquotted. All samples were stored at -70°C. Blood was sampled at two time-points: induction of general anaesthesia; then 3 minutes following systemic heparinisation with either UFH or LMWH.

In addition to plasma and serum prepared directly from collected arterial blood samples PPP was also prepared from the ADP-stimulated platelet aggregometry experiments. Following 10 minutes of aggregometry in response to 3×10^{-6}moll^{-1} ADP (Sigma, Poole,

Dorset, UK) using a PAP4 aggregometer (BioData Corp, Horsham, USA), the samples were microfuged at 9300×g for 10 minutes and the resulting supernatant aspirated from the platelet pellet, before being aliquotted and stored at -70°C. The ADP-stimulated plasma was used in preference to the AA-stimulated samples because of the cross-reactivity of the latter in the kits used to measure platelet metabolites.

5.3.2 Measurement of platelet metabolites

Platelet metabolites were assayed in the stored plasma, serum and ADP-stimulated PPP samples. Enzyme-linked immunoassay kits (ELISAs, Assay Designs, Michigan, USA) were used to determine the concentrations of the platelet metabolites TXB_2 (stable product of COX-1 activity) and 12-HETE (product of 12-LOX activity). The assay procedures were carried out as per the manufacturer's instructions, but briefly, the ELISA kits for both TXB_2 and 12-HETE were similar competitive immunoassays, using goat anti-rabbit IgG in the plate wells. The assays used a polyclonal antibody to the metabolites to bind them in a competitive manner. After a simultaneous incubation at room temperature the excess reagents were washed off the plate and the substrate was added. The enzyme reaction was stopped after a short incubation time, and the yellow colour generated was read on a microplate reader at 405nm. The intensity of the bound yellow colour was inversely proportional to the concentration of the metabolite in the sample.

Initial assay plates were performed on samples taken from selected patients who exhibited a very high platelet aggregation response after heparinisation, and from very low responding patients in order to construct concentration curves from which the optimum sample

dilutions could then be calculated. To construct concentration curves, pre- and post-heparinisation samples were assayed neat and at serial dilutions of 1 in 10, 1 in 100, 1 in 1000 and 1 in 10,000.

5.4 Results

Data were analysed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and Graphpad Prism version 4.03 for Windows (Graphpad Software Inc., La Jolla, California, USA). Paired continuous data were analysed with the use of a 2-tailed paired *t*-test, with non-paired observations analysed with a 2-tailed Mann-Whitney U test. Multiple continuous observations were analysed with a Kruskal-Wallis test. Categorical variables were analysed with chi-square tests. Data are presented as mean ± SD. Probability values <0.05 were considered statistically significant.

5.4.1 Demographics

Platelet metabolites were measured in the plasma, serum and post-ADP stimulated plasma of 58 (30 UFH, 28 LMWH; *Table 11*) patients undergoing CEA. The groups were generally matched, although a significantly greater proportion of patients in the LMWH group presented with TIA compared to the UFH group. Furthermore, the mean age difference of 5 years in favour of the LMWH patients also reached statistical significance.

Variable	**LMWH (n=28)**	**UFH (n=30)**	**p value**
Age/years	69±9.0	64±9.0	*0.04*
Sex			
Male	18 (64%)	21 (70%)	0.64
Female	10 (36%)	9 (30%)	
Weight/kg	78±16.0	75±13.6	0.44
Hypertension	23 (82%)	21 (70%)	0.28
Diabetes	5 (18%)	5 (17%)	0.91
Current smoker	8 (29%)	10 (33%)	0.70
Ex-smoker	15 (54%)	19 (63%)	0.45
Non-smoker	5 (18%)	1 (3%)	0.07
Presentation			
Asymptomatic	5 (18%)	11 (37%)	0.11
Stroke	5 (18%)	8 (27%)	0.42
Transient ischaemic attack	13 (46%)	5 (17%)	*0.01*
Amaurosis fugax	5 (18%)	6 (20%)	0.84
Mean carotid stenosis	79±8.6%	77±8.1%	0.36

Table 11 Characteristics of 58 patients undergoing CEA in whom plasma, serum and post-ADP induced platelet-poor plasma (PPP) was analysed for platelet metabolites TXB_2 and 12-HETE

5.4.2 Platelet metabolites

5.4.2.1 TXB_2

5.4.2.1.1 Plasma TXB_2

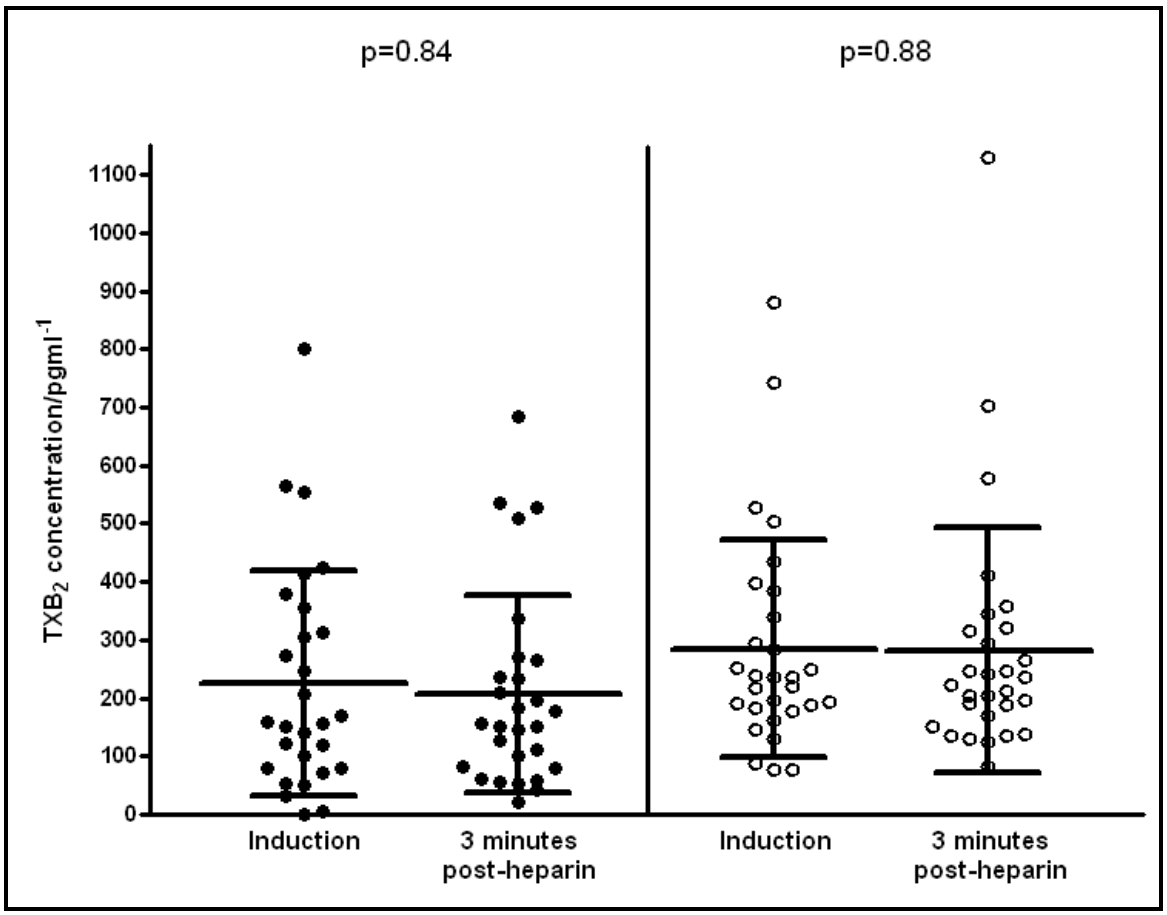

Figure 50 Concentrations of TXB_2 in plasma from 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

Based on the initial dilution studies with samples from high- and low-heparin responding patients, induction and post-heparin plasma TXB_2 concentrations were assayed in undiluted samples.

The mean plasma concentration of TXB_2 at induction of anaesthesia was 226±206pgml^{-1} in the LMWH group. This did not significantly alter following the administration of intravenous LMWH, dropping marginally to 206±170pgml^{-1} (p=0.84). Similarly, the mean concentration of TXB_2 in the UFH group at induction was 284±187pgml^{-1}, which did not significantly change after heparinisation, to 282±211pgml^{-1} (p=0.88, *Figure 50*).

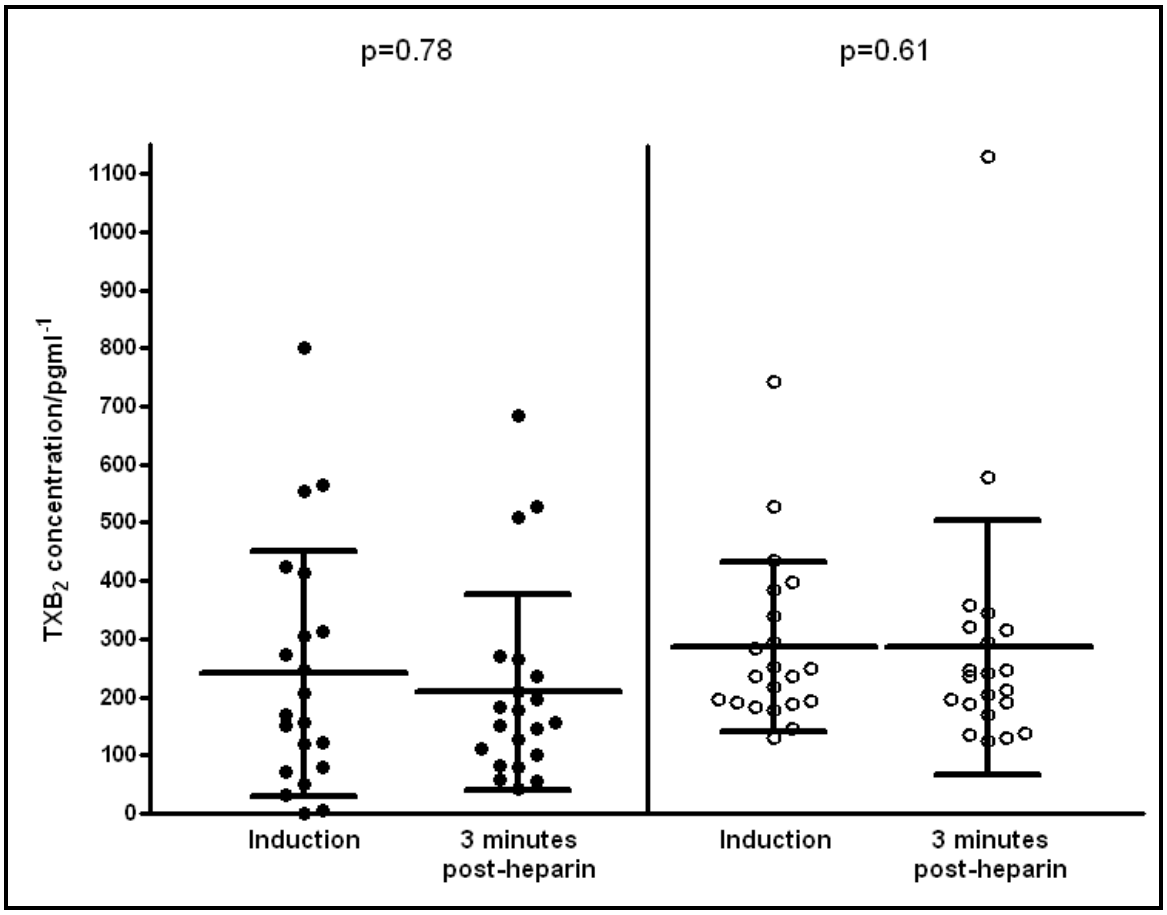

Figure 51 Concentrations of TXB_2 in plasma from 43 patients undergoing CEA. Closed circles; LMWH group (n=21); open circles; UFH group (n=22), horizontal bar represents mean (±SD). 15 patients who exhibited greater than 20% platelet aggregation at induction have been excluded

At induction the TXB_2 generation was statistically the same in both groups (p=0.10). However, the TXB_2 concentration after heparinisation was significantly lower in the LMWH group (p=0.03).

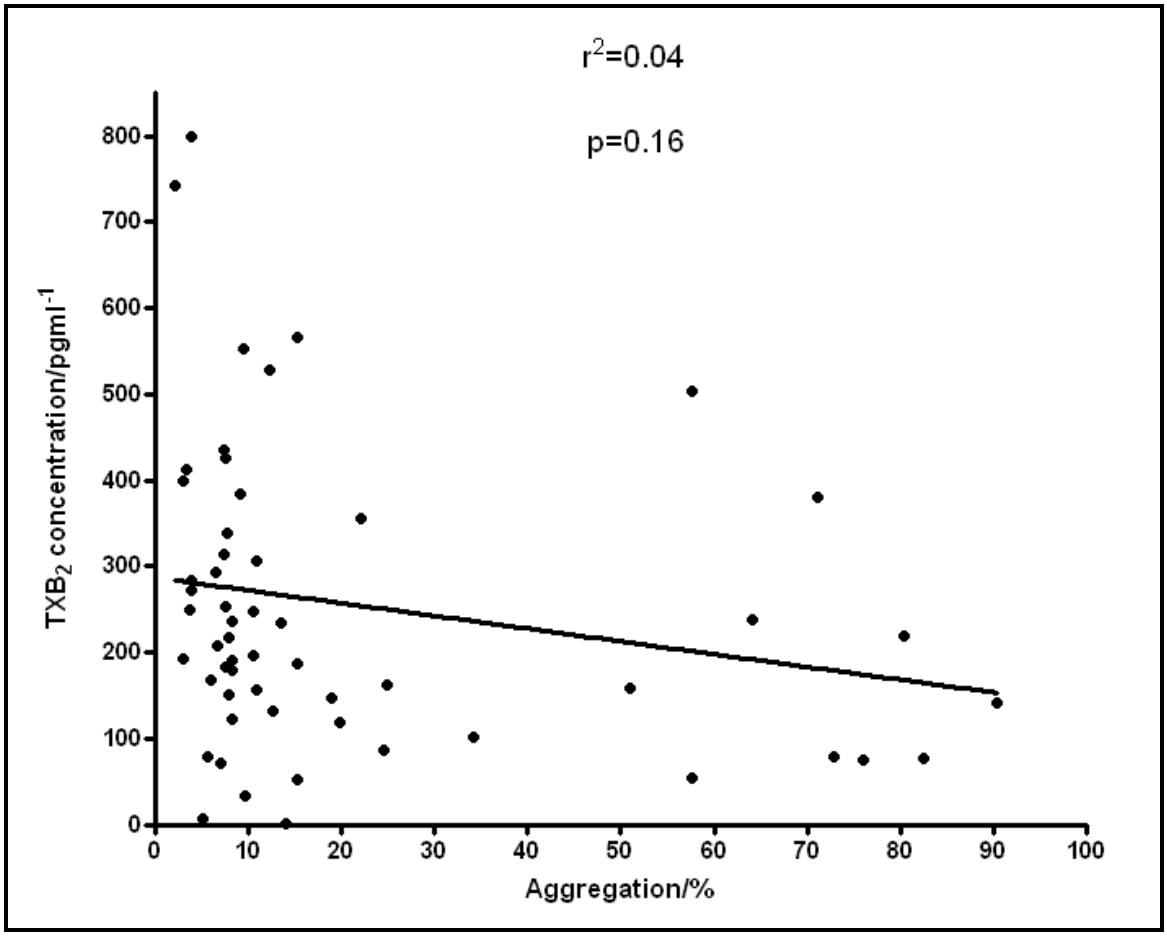

Figure 52 Plasma TXB_2 concentration in 58 patients undergoing CEA against platelet aggregation to 4.0×10^{-3}moll^{-1} AA at induction of anaesthesia

Platelet aggregation at induction of anaesthesia to 4.0×10^{-3}moll^{-1} AA was greater than 20% in 15 of the 58 patients (7 LMWH, 8 UFH, total 26%), indicative of aspirin failure.[205] In order to assess any potential impact of this on the results, the plasma TXB_2 concentrations were also analysed with these individuals excluded (*Figure 51*). However, there was no

correlation between the plasma concentration of TXB_2 and the magnitude of platelet aggregation to $4.0\times10^{-3}moll^{-1}$ AA at induction of anaesthesia (r^2=0.04, p=0.16, *Figure 52*). With the "aspirin resistant" patients excluded, the mean plasma concentration of TXB_2 in the LMWH group at induction of anaesthesia was 241±211$pgml^{-1}$ and in the UFH was 286±146$pgml^{-1}$ (p=0.17).

After heparinisation, there remained no significant change in the plasma concentration of TXB_2 in the LMWH group, to 209±169$pgml^{-1}$ (p=0.78) nor in the UFH group, to 286±219$pgml^{-1}$ (p=0.61). The mean plasma concentration following heparinisation remained significantly greater in the UFH group than in the LMWH group (p=0.03).

5.4.2.1.2 Serum TXB_2

Serum TXB_2 concentrations were assayed in neat serum. Figure 53 shows the serum concentrations of TXB_2 in the LMWH and UFH groups at induction of anaesthesia and 3 minutes post-heparinisation. At induction, the serum concentration was significantly lower in the LMWH group at 3135±4183$pgml^{-1}$ than in the UFH group, at 6527±6774$pgml^{-1}$ (p=0.004). After heparinisation, the serum TXB_2 in the LMWH group dropped to 2219±4304$pgml^{-1}$ (p=0.07) and that in the UFH group dropped significantly to 2820±3715$pgml^{-1}$ (p=0.002). The serum concentration of TXB_2 in the LMWH group was significantly lower after heparinisation than it was in the UFH group (p=0.01).

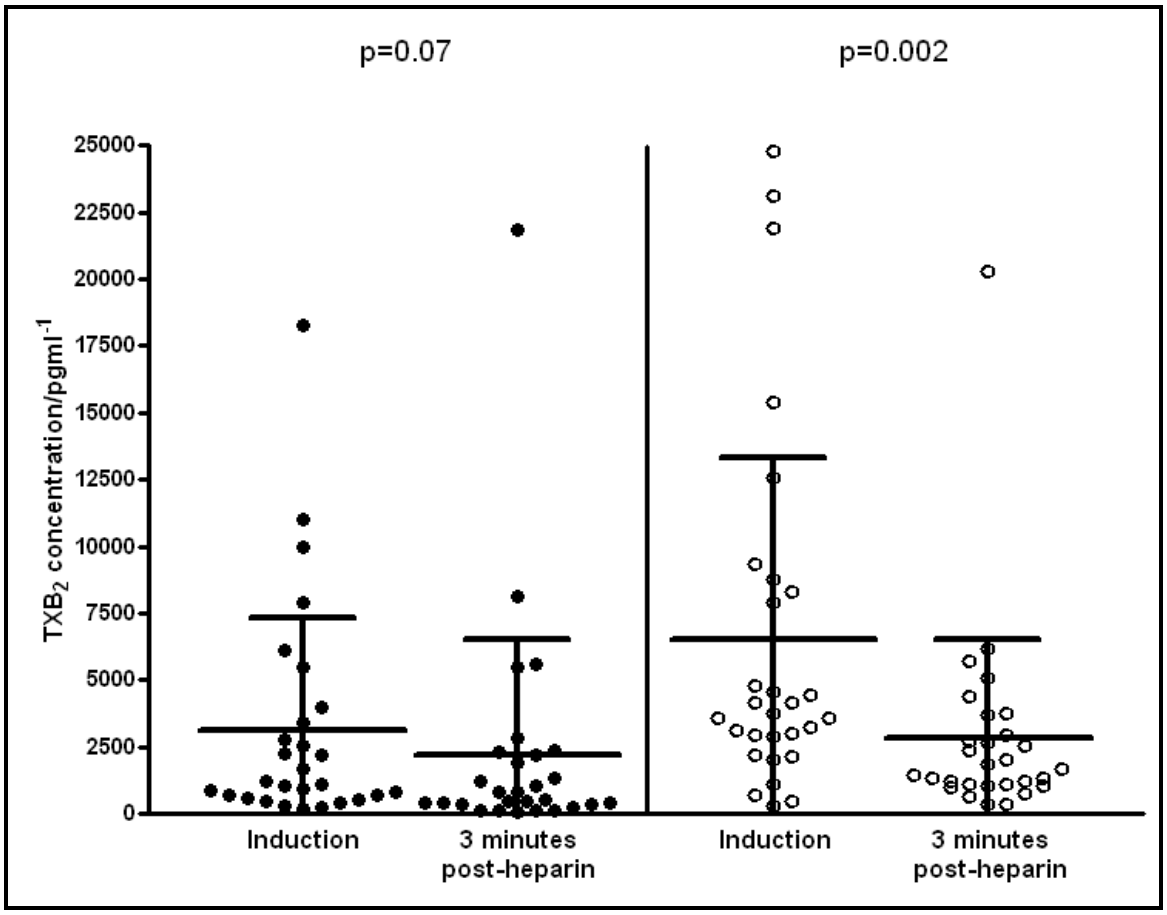

Figure 53 Concentrations of TXB_2 in serum from 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

Although there was no correlation between the serum concentration of TXB_2 at induction and the corresponding platelet aggregatory response to 4.0×10^{-3}moll^{-1} AA (r^2=0.0002, p=0.92 *Figure 54*), for completeness the serum concentrations were also analysed without the patients who exhibited greater than 20% platelet aggregation to 4.0×10^{-3}moll^{-1} AA at induction of anaesthesia (the "aspirin resistant" patients, *Figure 55*).

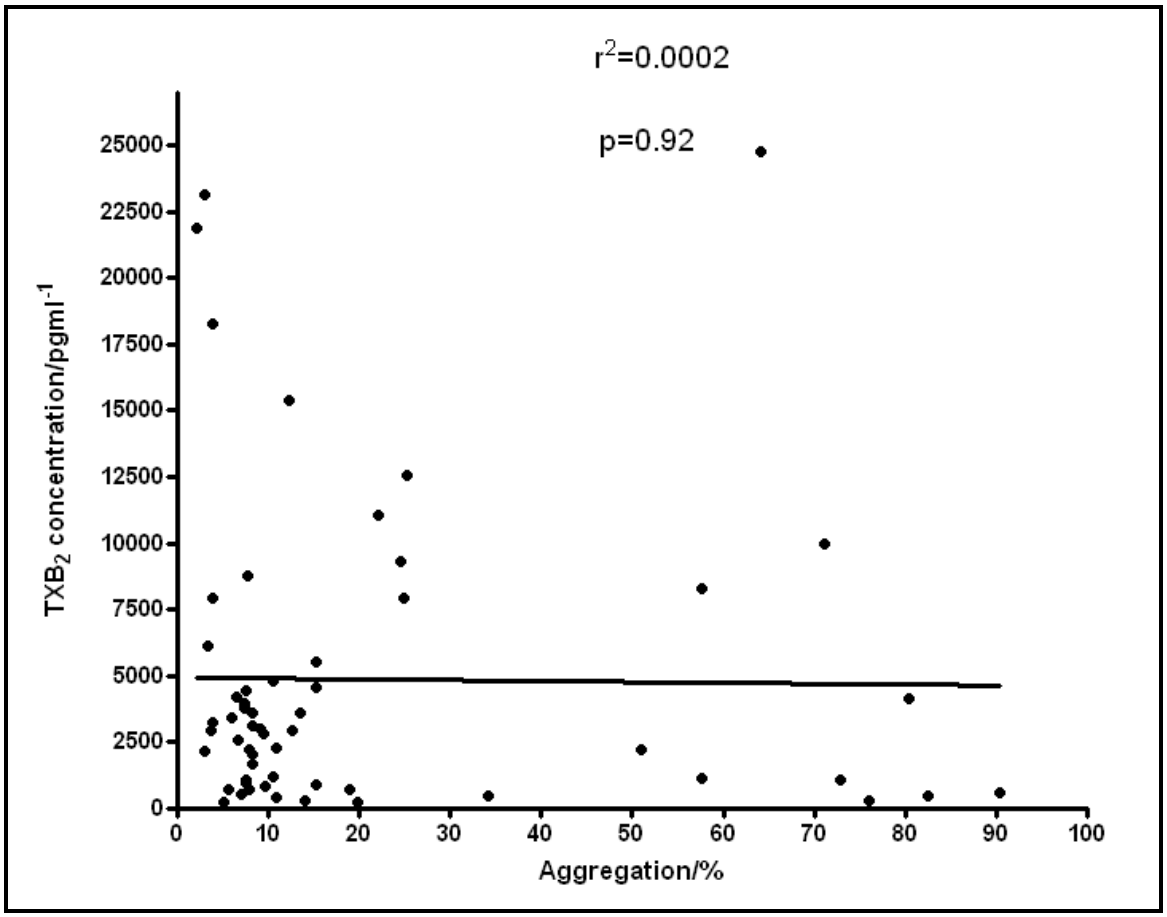

Figure 54 Serum TXB_2 concentration at induction of anaesthesia in 58 patients undergoing CEA against platelet aggregation to 4.0×10^{-3}moll^{-1} AA at induction of anaesthesia

At induction of anaesthesia, the mean serum concentration of TXB_2 in the LMWH group was 2923±4120pgml^{-1} which dropped to 1448±2014pgml^{-1} after heparinisation (p=0.08). The mean serum TXB_2 concentration in the UFH group at induction was 5785±6358pgml^{-1} which was significantly greater than in the LMWH group at this stage. Following heparinisation, the mean serum TXB_2 concentration in the UFH group dropped significantly to 2931±4173pgml^{-1} (p=0.004). The post-heparinisation serum concentration of TXB_2 was significantly lower in the LMWH group than in the UFH group (p=0.004).

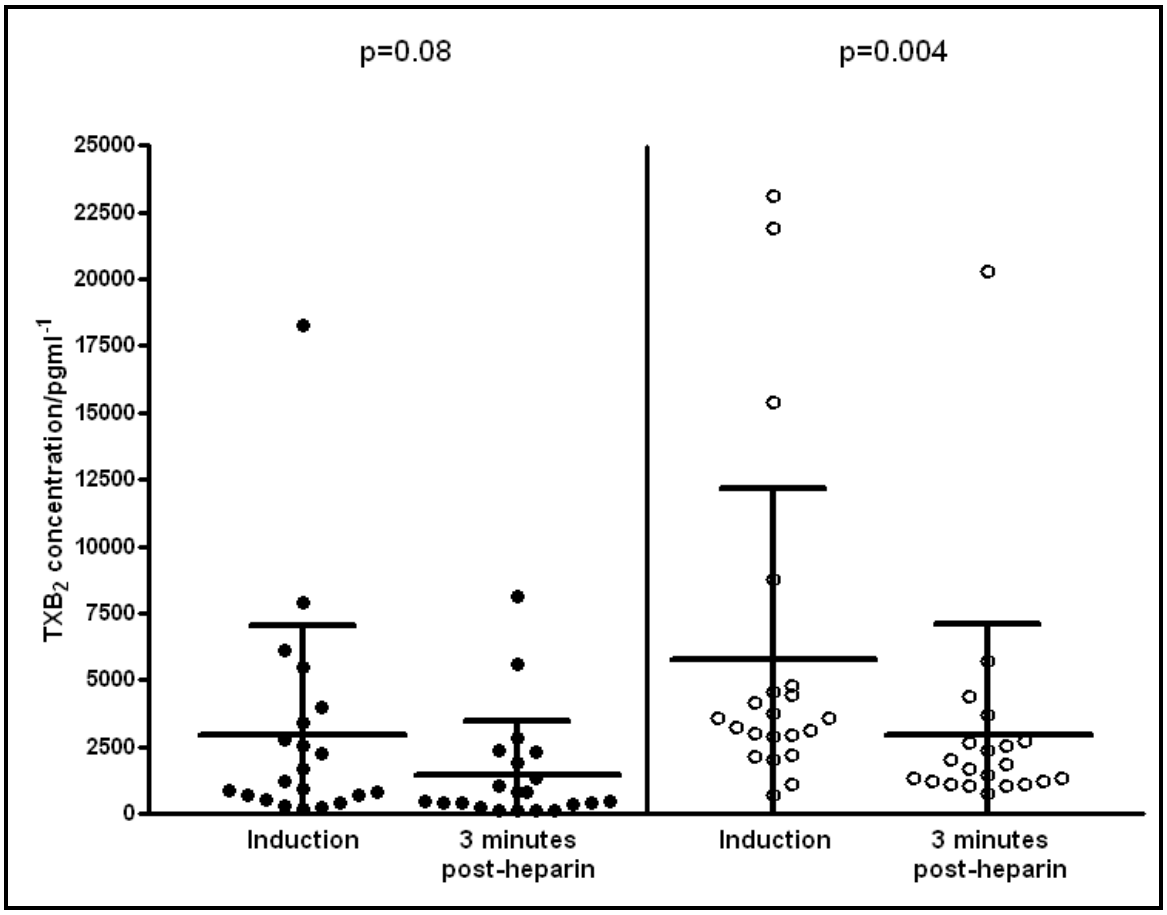

Figure 55 Concentrations of TXB_2 in serum from 43 patients undergoing CEA. Closed circles; LMWH group (n=21); open circles; UFH group (n=22), horizontal bar represents mean (±SD). 15 patients who exhibited greater than 20% platelet aggregation at induction have been excluded

5.4.2.1.3 TXB_2 generation on clotting

Measurement of both plasma and serum metabolite concentrations facilitated an assessment of the actual metabolite generation during the process of clotting; the plasma having come from anticoagulated blood (collected in citrate), and the serum having come from thrombosed blood (collected in gel tubes). Subtracting the concentration of metabolite in the plasma, from the concentration in the serum, gave a pure value for the metabolite generated during the process of thrombosis (*Figure 56*).

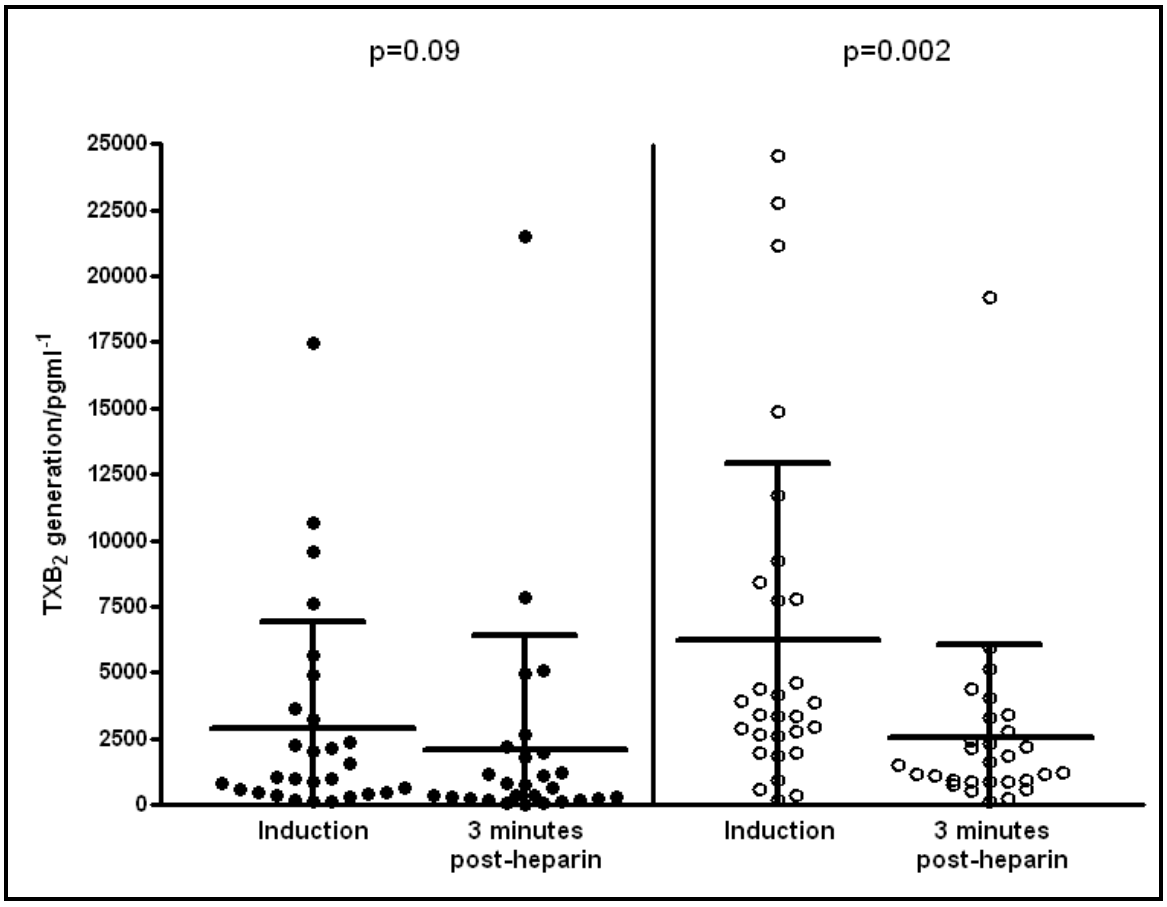

Figure 56 Generation of TXB_2 on clotting in 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

The mean generation of TXB_2 in the LMWH group at induction of anaesthesia was 2908±4040pgml^{-1}, which was not significantly altered by heparinisation, to 2013±4239pgml^{-1} (p=0.09). The generation of TXB_2 in the UFH group at induction was significantly greater than in the LMWH group, at 6243±6672pgml^{-1} (p=0.004). This dropped significantly following heparinisation to 2538±3526pgml^{-1} (p=0.002). After heparinisation, the generation of TXB_2 was significantly lower in the LMWH group than in the UFH group (p=0.02).

5.4.2.1.4 TXB_2 generation after ADP aggregation

PRP was stimulated with 3.3×10^{-6}moll^{-1} ADP for 10 minutes of aggregometry, before PPP was prepared from these samples and stored. The platelet metabolites were then subsequently assayed. Given that the metabolites had already been assayed in the plasma samples, it was possible to subtract these values from the ADP-stimulated PPP to calculate how much metabolite was generated by the platelets alone after *in vitro* stimulation with ADP, thus excluding possible contributions from extra-platelet sources.

Initial assay plates were run to determine the optimum sample dilutions, resulting in the TXB_2 samples being run neat at both time points (induction of anaesthesia and post-heparinisation). Figure 57 shows the "net" generation of TXB_2 in response to 3.3×10^{-6}moll^{-1} ADP. In the LMWH group, the "net" generation of TXB_2 in response to ADP at induction was 60±265pgml^{-1}, which did not significantly alter after heparinisation, at 88±260pgml^{-1} (p=0.65).

The mean net generation of TXB_2 in the UFH group at induction of anaesthesia was 1992±5991pgml^{-1}, which dropped to 1054±3394pgml^{-1} following heparinisation (p=0.47). The means at each time-point were not significantly different between the two heparin types (p=0.75 at induction, p=0.38 after heparinisation), with the standard deviations in the UFH group widened by 3 high-TXB_2-producing outliers, who generated large concentrations of TXB_2 consistently before and after heparinisation (*Figure 57*, arrowed).

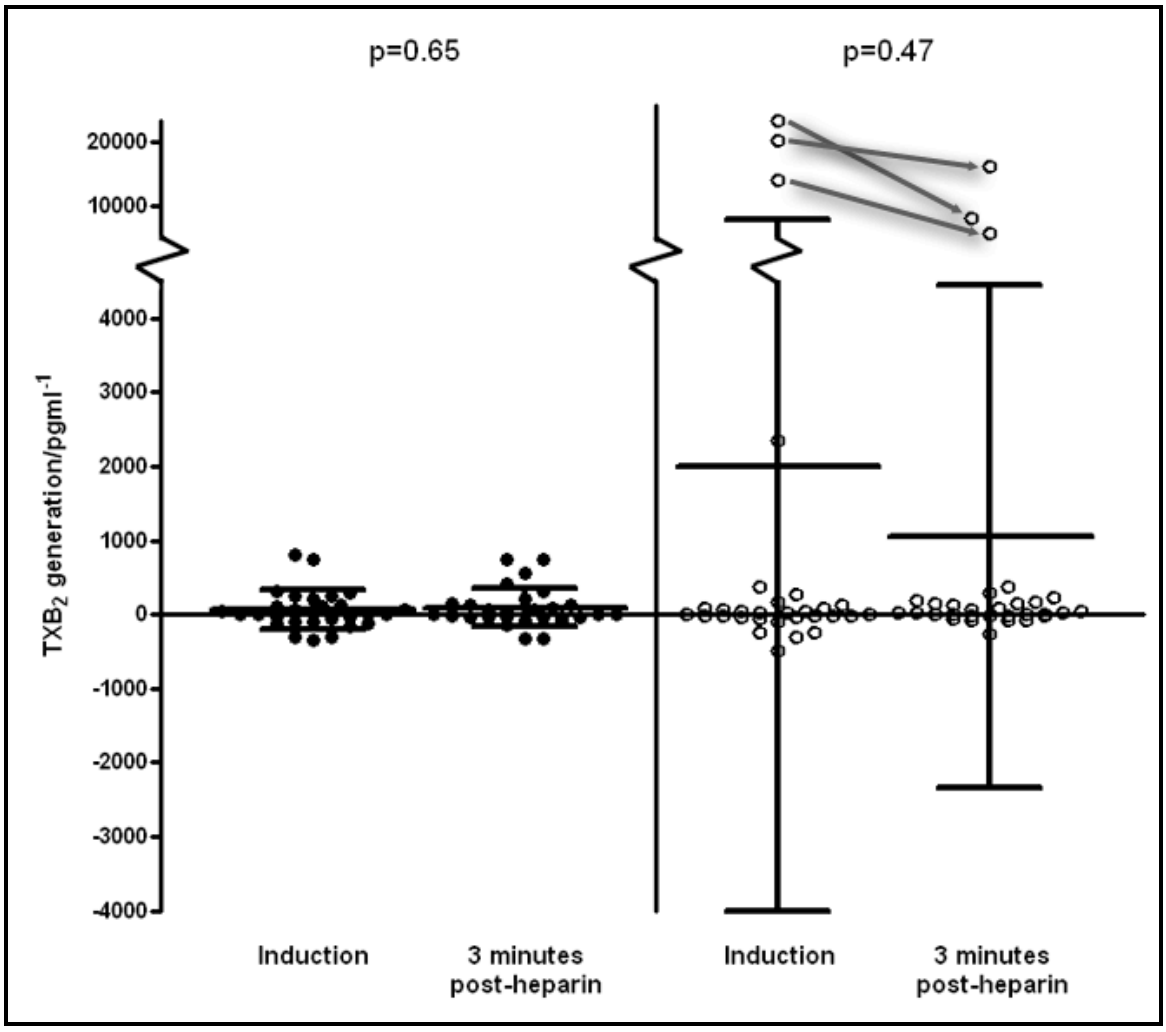

Figure 57 Generation of TXB_2 from platelets stimulated with ADP in 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD). Grey arrows indicate 3 outliers in the UFH group, consistent before and after heparinisation

5.4.2.1.5 Analysis of potential confounding of TXB_2 by presenting symptom

Because of the statistical differences between the LMWH and UFH groups in terms of presenting symptom status (*Table 11*), the series of TXB_2 studies were all re-analysed looking specifically at presenting status to ascertain whether this had influenced the results (*Figure 58*).

5.4.2.1.5.1 Plasma TXB_2 concentration according to presentation

For the plasma concentration of TXB_2 the presenting feature made a significant difference to the results (*Figure 58*, A). The mean plasma concentration of TXB_2 at induction of anaesthesia was highest in the asymptomatic patients, at 354±226pgml^{-1}, followed by the amaurosis fugax patients at 283±180pgml^{-1} (p=0.50), the stroke patients at 251±190pgml^{-1} (p=0.15) with the TIA patients lowest at 162±122pgml^{-1} (p=0.003). After heparinisation, the mean plasma concentration of TXB_2 did not significantly alter in the asymptomatic patients, at 340±284pgml^{-1} (p=0.63). The mean concentration in the amaurosis fugax patients was 257±168pgml^{-1}, which was not significantly different from the induction level (p=0.69) or from the post-heparinisation level in the asymptomatic patients (p=0.72). The post-heparinisation concentration of TXB_2 in the stroke patients was 233±120pgml^{-1}, not significantly different from either the induction concentration (p=1.00) or from the post-heparinisation asymptomatic patients (p=0.41). The mean plasma concentration of TXB_2 in the TIA patients did not significantly alter after heparinisation, to 167±125pgml^{-1} (p=0.89), but was significantly lower than the concentration in the asymptomatic patients (p=0.02).

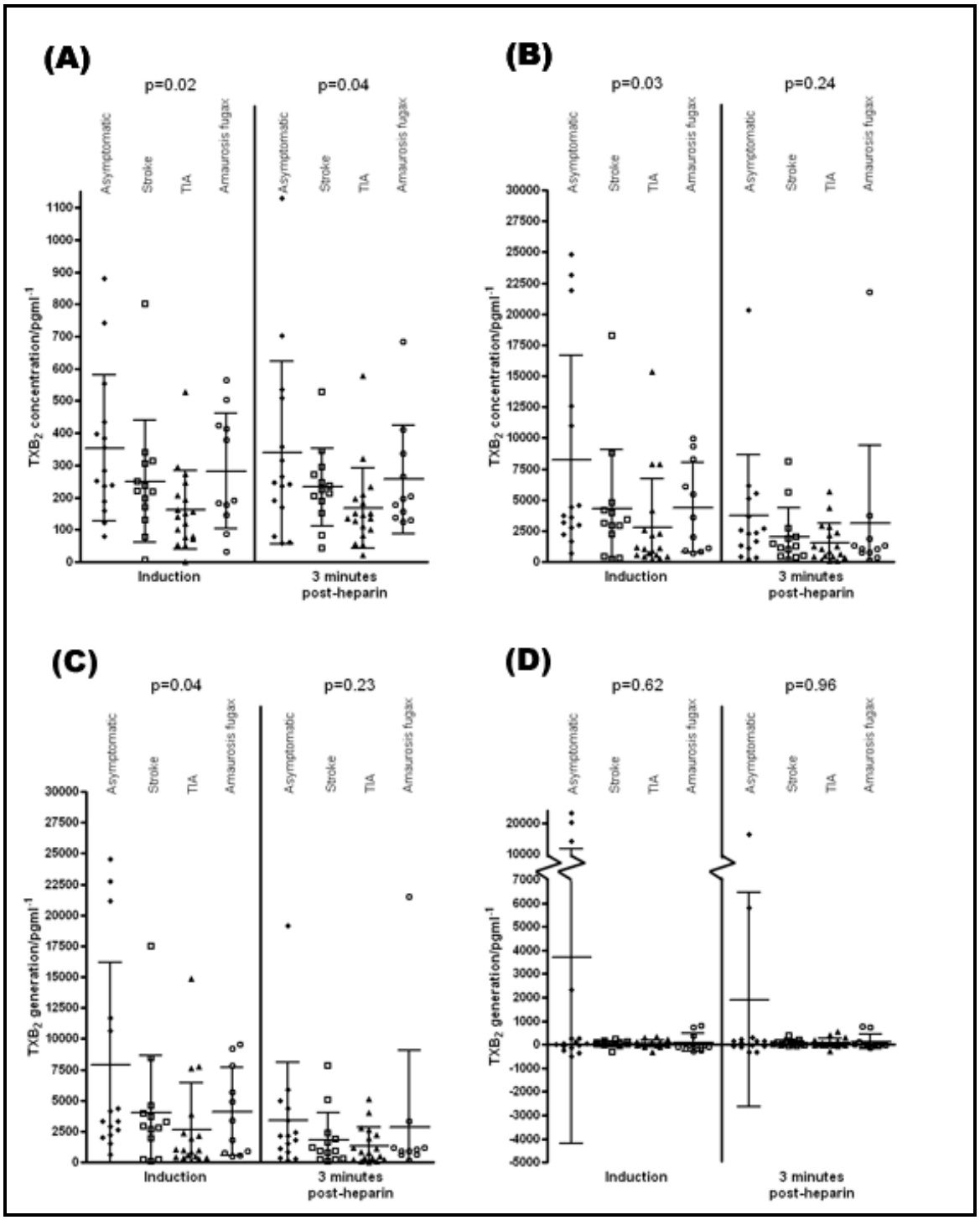

Figure 58 Results of the TXB_2 studies, plotted according to the symptoms at presentation in 58 patients undergoing CEA. Closed diamonds; Asymptomatic patients (n=16); open squares; Stroke patients (n=13); closed triangles; TIA patients (n=18); open circles; Amaourosis fugax patients (n=11). (A) plasma concentration, (B) serum concentration, (C) TXB_2 generation on clotting, (D) TXB_2 generation on activation with ADP. Horizontal bar represents mean (±SD)

5.4.2.1.5.2 Serum TXB_2 concentration according to presentation

Presenting symptom also affected the serum concentration of TXB_2 at induction of anaesthesia, but not after heparinisation (*Figure 58*, B). Again, the highest mean serum concentration of TXB_2 was demonstrated in the asymptomatic patients, at 8226±8442pgml^{-1}. The amaurosis fugax patients had a mean serum concentration of TXB_2 of 4394±3611pgml^{-1} (p=0.30), and the stroke patients 4265±4779pgml^{-1} (p=0.20). The TIA patients demonstrated a significantly lower mean serum concentration of TXB_2 at 2771±3937pgml^{-1} (p=0.003). Following heparinisation, there was no significant difference in the mean serum concentration of TXB_2 according to presenting symptom (p=0.24).

5.4.2.1.5.3 Generation of TXB_2 on clotting according to presentation

The differences seen in the plasma and serum concentrations of TXB_2 were predictably reflected in the levels of TXB_2 generated on clotting, given the calculation to achieve this figure (serum concentration – plasma concentration, *Figure 58*, C).

At induction, the highest generation of TXB_2 was again observed in the asymptomatic patients, at 7872±8348pgml^{-1}. The generation in the amaurosis fugax patients was 4111±3546pgml^{-1} (p=0.30), followed by the stroke patients at 4015±4609pgml^{-1} (p=0.23). The generation of TXB_2 on clotting in the TIA patients was significantly lower than the asymptomatic patients, at 2609±3835pgml^{-1} (p=0.003). Following heparinisation, the presenting features of the patients had no influence on the generation of TXB_2 on clotting (p=0.23).

5.4.2.1.5.4 TXB_2 generation after ADP activation according to presentation

Figure 58, D illustrates the generation of TXB_2 after platelet activation with ADP. The presenting symptom did not influence the plasma generation of TXB_2 either at induction of anaesthesia (p=0.62) or after heparinisation (p=0.96).

5.4.2.2 12-HETE

5.4.2.2.1 Plasma 12-HETE

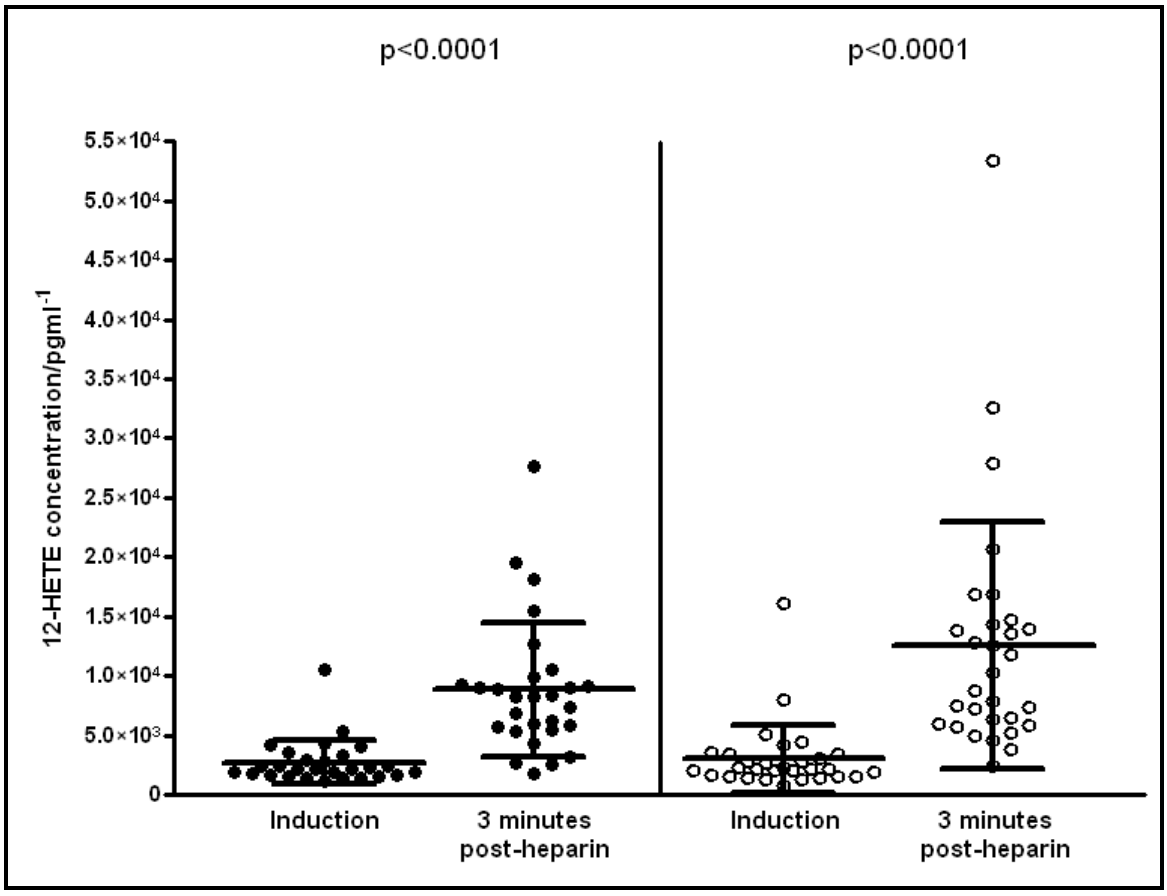

Figure 59 Concentrations of 12-HETE in plasma from 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

Based on studies to determine the optimum dilutions, induction samples were run neat, and post-heparinisation samples were assayed at 1 in 5 dilution. The mean plasma concentrations of 12-HETE at induction of anaesthesia were similar, at 2730±1862pgml^{-1} in the LMWH group and 3045±2862pgml^{-1} in the UFH group (p=0.87). Following heparinisation with either LMWH or UFH, these both increased significantly to 8836±5633pgml^{-1} in the LMWH group (p<0.0001), and to 12544±10357pgml^{-1} in the UFH group (p<0.0001). However, although the post-heparinisation plasma level of 12-HETE was greatest in the UFH group, this was not statistically significant (p=0.18, *Figure 59*).

5.4.2.2.2 Serum 12-HETE

The serum concentrations of 12-HETE were assayed at 1 in 100 dilution for the induction samples, and 1 in 20 dilution for the post-heparinisation samples. The mean serum concentration of 12-HETE at induction was 708728±599006 pgml^{-1} in the LMWH group versus 568686±560095pgml^{-1} in the UFH group (p=0.31). After heparinisation, the serum 12-HETE concentration in the LMWH group dropped significantly to152130±188991pgml^{-1} (p<0.0001) and to 45274±29771pgml^{-1} in the UFH group (p<0.0001). This drop in serum 12-HETE concentration in the UFH group was significantly greater than in the LMWH group (p=0.01, *Figure 60*).

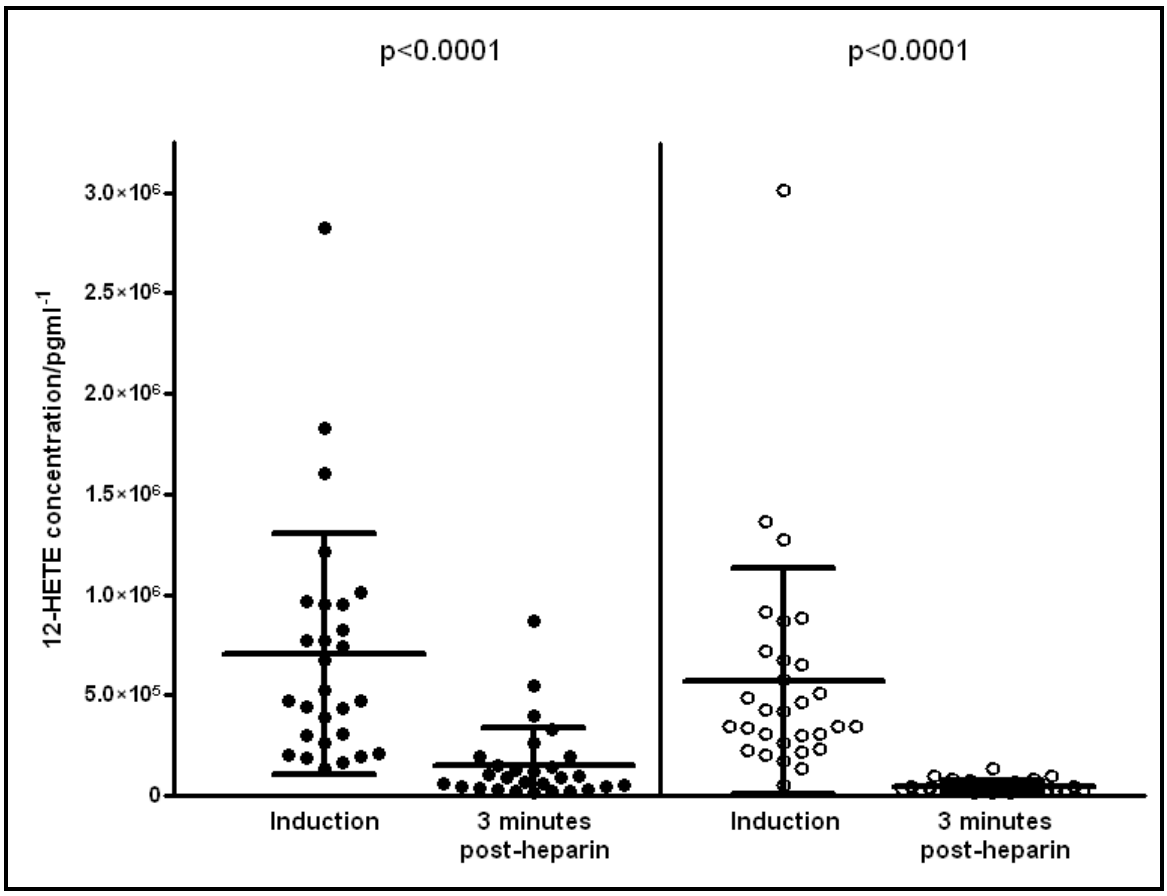

Figure 60 Concentrations of 12-HETE in serum from 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

5.4.2.2.3 12-HETE generation on clotting

The assaying of 12-HETE in both plasma (from anticoagulated blood) and serum (from clotted samples) allowed the former to be subtracted from the latter to give a value for the amount of 12-HETE generation during the process of whole blood clotting (*Figure 61*). At induction of anaesthesia in the LMWH group the mean concentration of 12-HETE generated by thrombosis was 705997±598881pgml^{-1}, which fell significantly following heparinisation, to 143294±187947pgml^{-1} ($p<0.0001$).

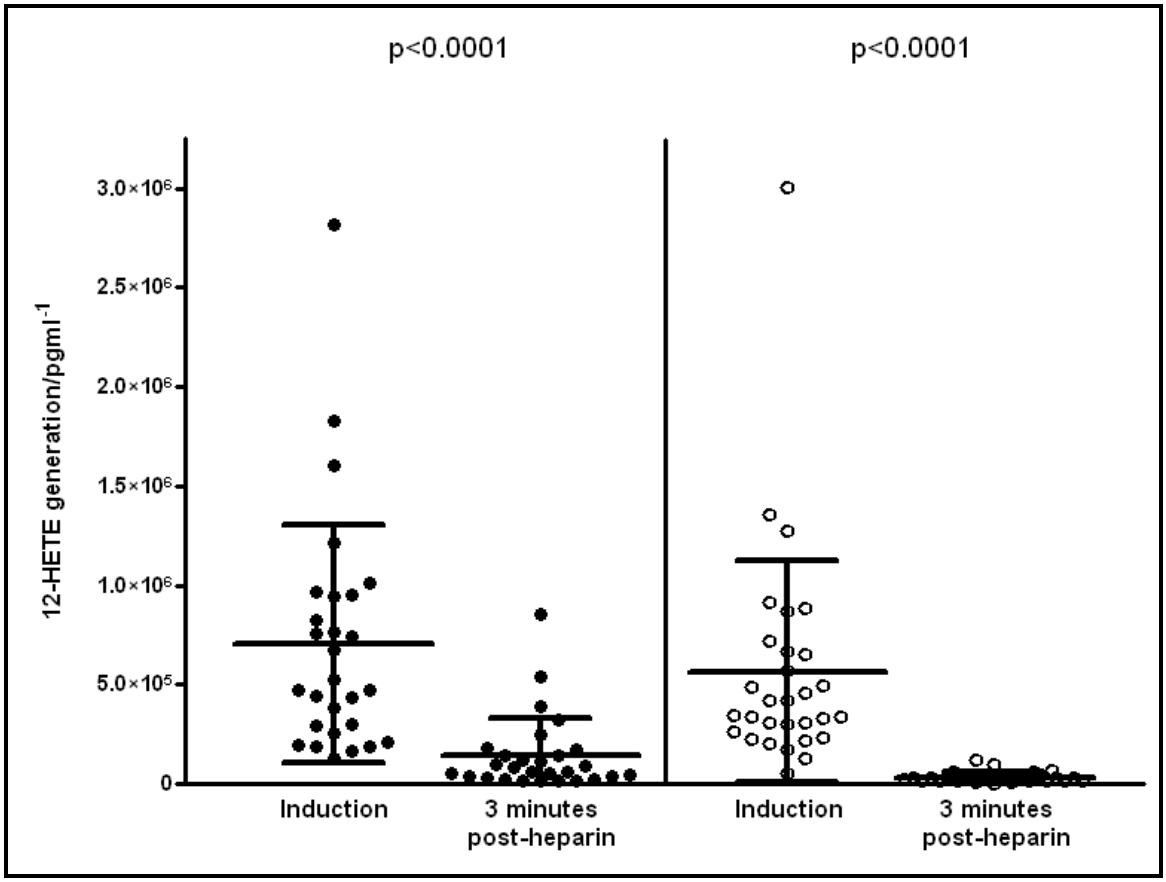

Figure 61 Generation of 12-HETE on clotting in 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

Similarly, the generation of 12-HETE on thrombosis in the UFH group was high at induction of anaesthesia, at 565640±560088pgml^{-1}, dropping significantly to a mean of 32730±26487pgml^{-1} ($p<0.0001$) after heparinisation. The induction levels were not significantly different between the two groups ($p=0.31$), but the drop in the generation of 12-HETE on thrombus formation was significantly greater in the UFH group after the administration of heparin ($p=0.0002$).

5.4.2.2.4 12-HETE generation after ADP aggregation

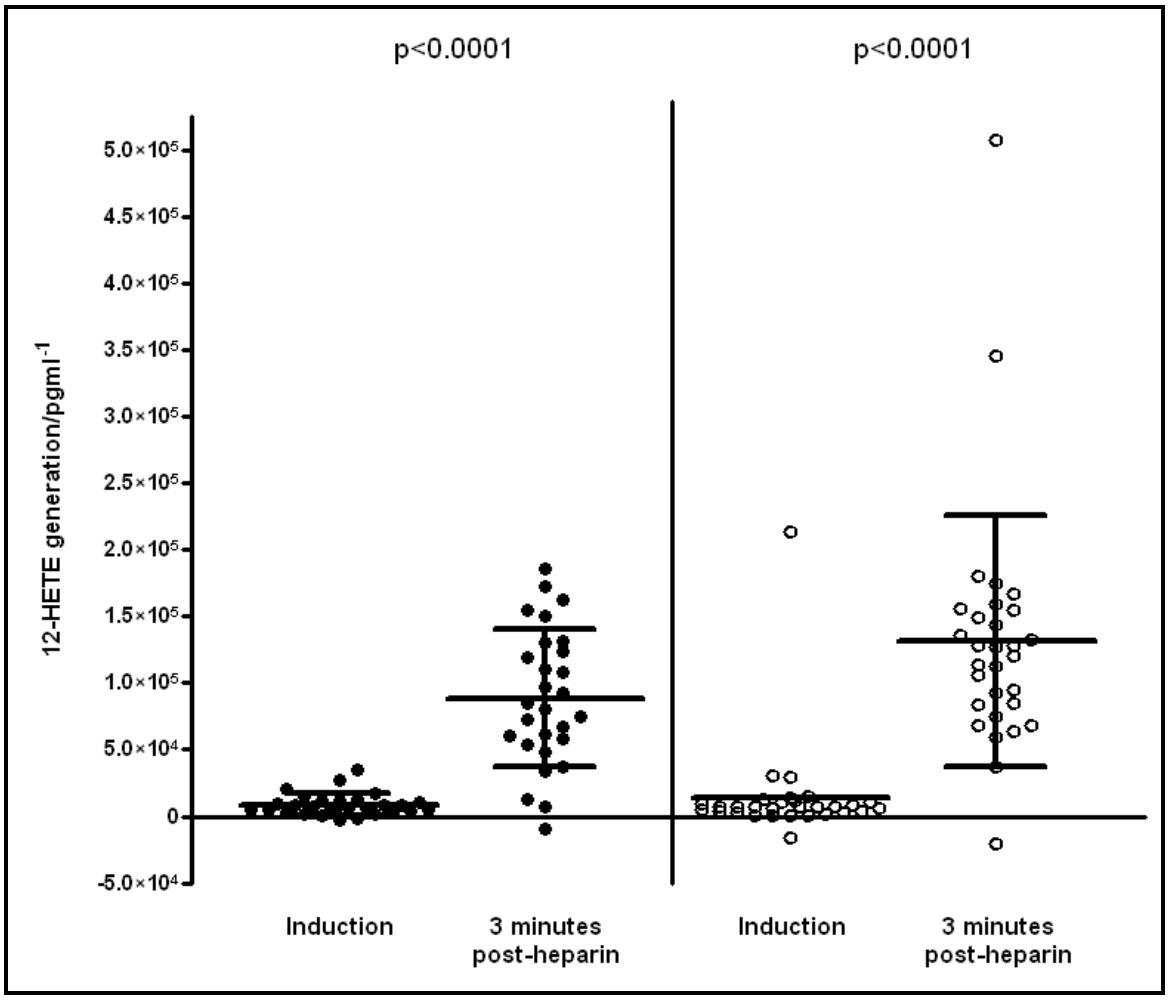

Figure 62 Generation of 12-HETE from platelets stimulated with ADP in 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD)

As previously stated, initial assays determined the optimum sample dilutions. Post-ADP plasma samples from induction of anaesthesia were assayed at 1 in 5 dilution, and post-ADP samples from the heparinised samples were assayed at 1 in 50 dilution. At induction of anaesthesia, the mean "net" generation of 12-HETE on aggregation with 3.3×10^{-6}moll^{-1} ADP was 8852±8269pgml^{-1} in the LMWH group, versus 14362±38557pgml^{-1} in the UFH

group (p=0.89). The levels rose significantly after heparinisation; to 88687±51145pgml^{-1} in the LMWH group (p<0.0001) and nearly tenfold to 131660±94197pgml^{-1} in the UFH group (p<0.0001). The post-heparinisation generation of 12-HETE after stimulation with ADP was significantly greater in the UFH group than in the LMWH group (p=0.04, Figure 62).

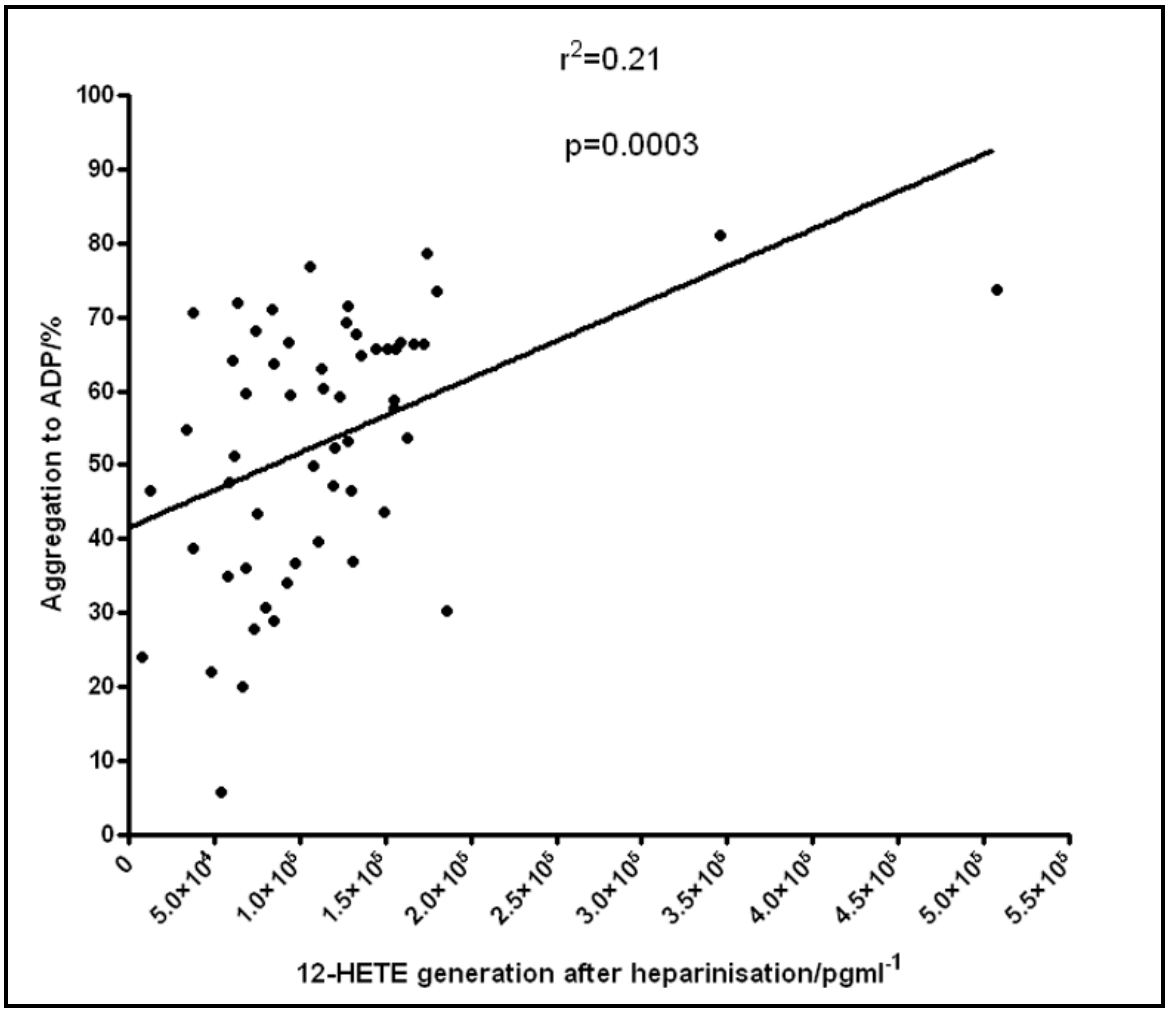

Figure 63 Correlation between platelet aggregation to 3.3×10^{-6}moll^{-1} ADP after heparinisation and "net" plasma generation of 12-HETE in 58 patients undergoing CEA

Figure 63 and Figure 64 demonstrate that there was a significant correlation among the population as a whole (both LMWH and UFH patients) between the aggregation response

to 3.3×10^{-6}moll^{-1} ADP after heparinisation (with either LMWH or UFH) and the generation of 12-HETE from the ADP-stimulated platelets (r^2=0.21, p=0.0003).

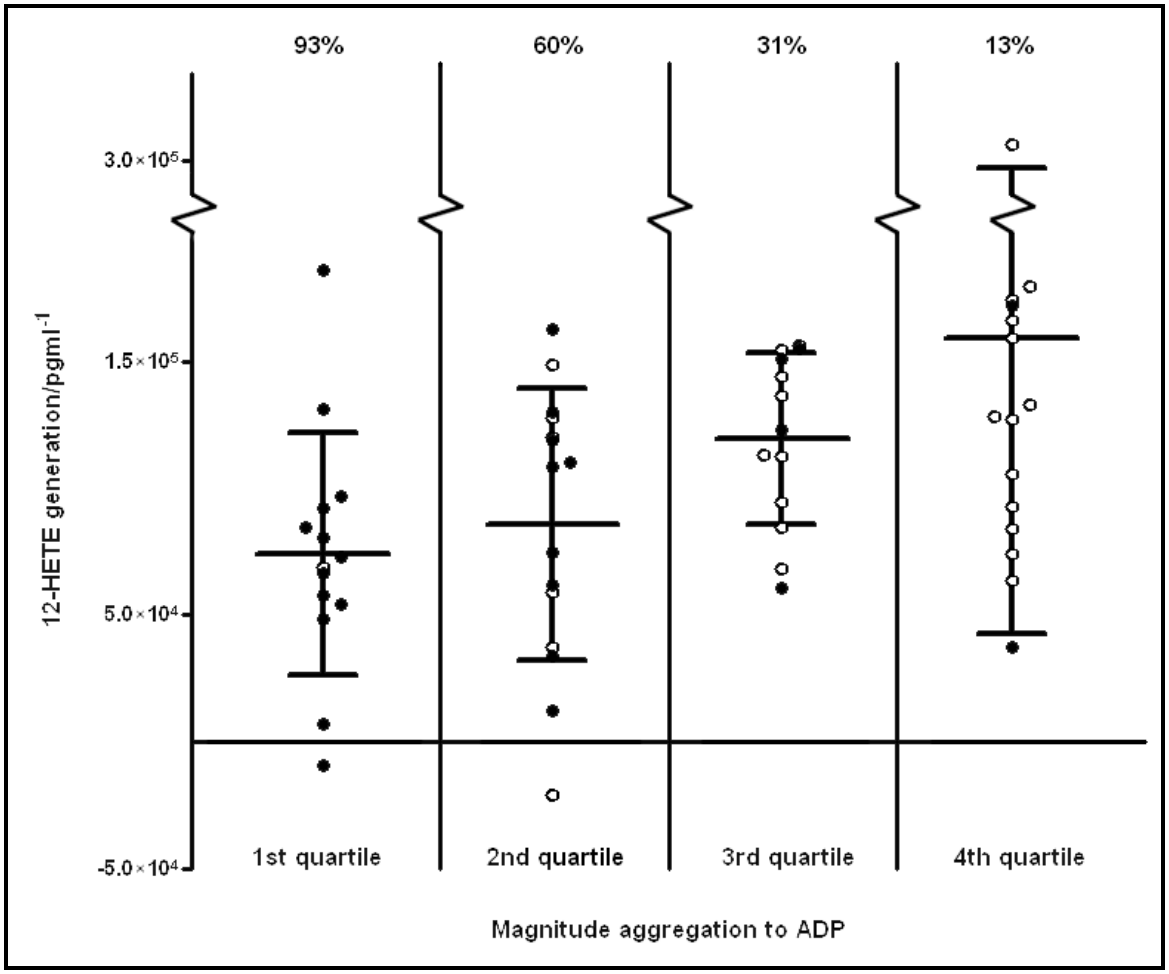

Figure 64 "Net" plasma generation of 12-HETE after platelet aggregation in response to 3.3×10^{-6}moll^{-1} ADP after heparinisation versus corresponding platelet aggregation in quartiles of magnitude in 58 patients undergoing CEA. Closed circles; LMWH group (n=28); open circles; UFH group (n=30), horizontal bar represents mean (±SD). Percentage figures refer to the proportion of that quartile who were randomised to LMWH

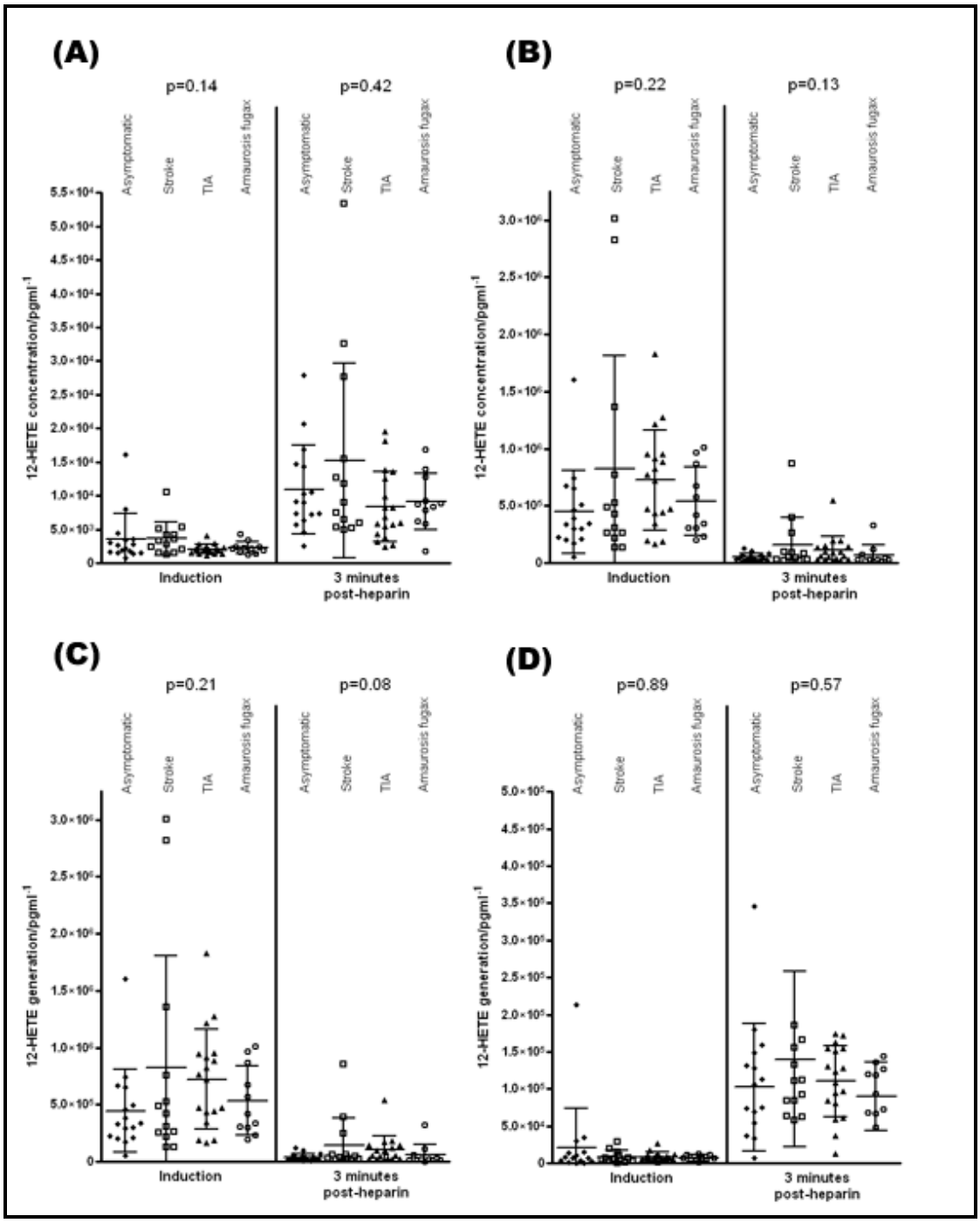

Figure 65 Results of the 12-HETE studies, plotted according to the symptoms at presentation in 58 patients undergoing CEA. Closed diamonds; Asymptomatic patients (n=16); open squares; Stroke patients (n=13); closed triangles; TIA patients (n=18); open circles; Amaourosis fugax patients (n=11). (A) plasma concentration, (B) serum concentration, (C) 12-HETE generation on clotting, (D) 12-HETE generation on activation with ADP. Horizontal bar represents mean (±SD)

The platelets aggregating in the highest (4^{th}) quartile of magnitude generated significantly higher amounts of 12-HETE than the platelets in the 1^{st} quartile (159571±116609pgml^{-1} versus 74201±47723pgml^{-1}, p=0.007). Furthermore, the proportion of patients who had received LMWH in each quartile gradually decreased from 93% in the 1^{st} quartile to 13% in the upper quartile (*Figure 64*).

5.4.2.2.5 Analysis of potential confounding of 12-HETE by presenting symptom

Figure 65 illustrates the analysis of the 12-HETE studies taking into account the differences between the LMWH and UFH groups in symptom status (Table 11). Contrary to the TXB_2 studies, the results of the 12-HETE assays were uniformly unaffected by the presenting symptom of the patients.

5.5 Discussion

AA is metabolised via two pathways in the platelet – mediated by the enzymes COX-1 or 12-LOX - to the eicosanoids TXB_2 and 12-HETE respectively.[279] In the series of studies presented here, there was no increase in the plasma concentration of TXB_2 after heparinisation with either UFH or LMWH. As the detectable end-product of metabolism of AA via the COX-1 pathway, this implies that platelet COX-1 was no more active after heparinisation than before, despite the observed increases in platelet aggregation. Furthermore, when platelets were directly stimulated *ex vivo* with ADP addition to PRP, there was still no increase in the production of TXB_2 in either the UFH or LMWH groups,

which would have been expected were COX-1 inhibition somehow being transiently overcome.

There were significant increases observed in the plasma concentrations of the 12-LOX product 12-HETE after the administration of both LMWH and UFH, with similar rises in both heparin types. Direct stimulation *ex vivo* of PRP with ADP also resulted in significant platelet secretion of 12-HETE following heparinisation, which was greater in the platelets that had been exposed to UFH. The implication is that UFH and LMWH both lead to activation of platelet 12-LOX and the subsequent secretion of 12-HETE. Moreover, UFH effected greater 12-LOX activation than LMWH, and crucially there was a significant correlation between the post-heparinisation platelet aggregatory response to ADP and the 12-LOX activity, suggesting that the platelets which exhibited the greatest aggregation response to ADP following heparinisation also had the most 12-LOX activity. Of note, the proportion of patients who had received LMWH rather than UFH systematically decreased from 93% in the first (low) quartile of ADP aggregation, to 13% in the upper quartile, of highest aggregation response.

The results of the serum assays for both metabolites are seemingly incompatible with the plasma studies – in both the TXB_2 and 12-HETE studies, the serum levels of metabolite dropped after heparinisation. Consequently, the calculated "generation" of TXB_2 and 12-HETE on clotting (the plasma concentration, "unclotted blood", subtracted from the serum concentration, "clotted blood") was also significantly reduced after heparinisation. But this is explained by the fact that the post-heparin samples were prepared from anticoagulated

blood; they would not be expected to undergo as aggressive a thrombosis as the pre-heparin (unanticoagulated) samples. In retrospect, it was predictable that the post-heparin samples would generate less of the metabolites of platelet activation because of the intra-operative anticoagulation with heparin. It is, however, noteworthy that the patients who received UFH displayed significantly greater reductions in their "thrombosis-generation" of both TXB_2 and 12-HETE after heparinisation than the patients who received LMWH, perhaps suggesting a more aggressive anticoagulation by the intravenous UFH than by the LMWH.

The failure of TXB_2 to increase after heparinisation suggests that the increases observed in platelet aggregation to AA and ADP were not mediated by a true reversal of the aspirin inhibition of the COX-1 pathway. Could the phenomenon of "transient aspirin resistance" actually have been mediated by activation of the 12-LOX pathway? Whilst the COX-1 pathway, and its irreversible inhibition by aspirin, is well understood, relatively little is known about the physiological actions of 12-HETE or the clinical significance of 12-LOX. Currently it is unclear whether 12-HETE plays an active role in mediating thrombus formation *in vivo*. However, there is evidence to suggest that 12-HETE can act as a platelet activator,[280-282] and that inhibition of 12-HETE is associated with a decrease in platelet adhesion independent of COX-1 inhibition.[283] There is also evidence to link 12-HETE with cardiovascular disease: it has been demonstrated to act as a vasoconstrictor in small renal arteries;[284] and its secretion by platelets from patients with hypertension is higher than in normotensive control subjects.[285] In an animal model of coronary artery thrombosis, flow variations characteristic of human acute coronary syndromes were associated with both increased concentrations of plasma and intra-platelet 12-HETE. Interestingly, the

pharmacological inhibition of 12-LOX reduced these flow variations, with a corresponding reduction in the 12-HETE concentration.[280] It has also been postulated that the potential role played by certain bacteria in the development of atherosclerosis may be mediated by 12-LOX. *Chlamydia pneumoniae* stimulates platelet aggregation, which is not affected by COX-1 inhibition, but is significantly reduced by 12-LOX inhibitors.[278] In addition, urease from *Bacillus pasteurii* also promotes platelet aggregation requiring AA and again, whilst COX-1 inhibition fails to reduce the level of enhanced platelet aggregation, inhibition of 12-LOX does reduce it, suggesting products of the 12-LOX pathway are integral to the effect.[286]

In this series of studies, there were two potentially confounding issues, and the data were re-analysed to investigate these. Firstly, some patients were classed as "aspirin resistant" at induction of anaesthesia based on the magnitude of their platelet response to AA.[205] Secondly, there were a significantly greater proportion of patients in the LMWH group whose presenting event was a TIA. There was no correlation between the generation of TXB_2 and the magnitude of aggregation response to AA at induction of anaesthesia, suggesting that the increased platelet activity wasn't necessarily driven by increased COX-1 pathway activity. Furthermore, excluding these high AA-aggregating patients did not affect the general trends observed with the TXB_2 studies.

The TIA patients had the lowest concentrations of plasma TXB_2 pre- and post-heparinisation, and the lowest serum concentration of TXB_2 at induction of anaesthesia. Therefore, it is possible that the LMWH levels of TXB_2 may have been lowered by the fact

that this group was proportionally made up of more TIA patients. This may explain why the serum concentration of TXB_2 (and consequently the TXB_2 generated on clotting) was significantly lower in the LMWH group at induction of anaesthesia. The sample sizes are probably too small to draw any conclusions from the observation that patients presenting with TIA seem to exhibit the least COX-1 activity, with the asymptomatic patients displaying the greatest COX-1 activity (as evidenced by the higher TXB_2 concentrations), but this warrants further investigation. The differences between the LMWH and UFH groups in terms of presenting symptoms did not influence the results of the 12-HETE experiments.

VI

Post-operative Embolization is Reduced by Low Molecular Weight Heparin

6.1 Introduction

The early detection of technical error using the dual modalities of intra-operative transcranial Doppler monitoring (TCD) and completion angioscopy has virtually abolished intra-operative stroke during CEA. Although post-operative stroke can be the result of hyperperfusion or haemorrhage, the most common cause is early post-operative carotid thrombosis (POCT).[98,100] Stroke due to POCT is preceded by 1-2 hours of increasing embolization, which is detectable by extending the use of TCD into the early post-operative period.[126,256] As it is demonstrable prior to the clinical manifestation, the progression onto stroke can be prevented by early implementation of treatment. Until the drug's withdrawal, the local protocol in Leicester was to start an incremental dose infusion of Dextran,[287] although the demand for this has been all but eliminated by the subsequent routine policy of pre-CEA ADP-inhibition with clopidogrel 75mg.[288]

That increasing embolization is predictive of post-operative stroke is of additional importance in the study of CEA-related complications, since the measurement of embolization can be used as a surrogate outcome measure for stroke risk.[126,256] Given the relatively low incidence of post-operative stroke, the impact of an intervention can be assessed by studying much smaller cohorts of patients.

In Chapter IV it was demonstrated that there was a significant increase in the platelet aggregatory response to AA and ADP following heparinisation during CEA, and that the response to ADP was significantly greater in those patients who had received UFH rather than LMWH. Chapter V's study of the platelet pathways showed that this phenomenon did not appear to be mediated by a reversal of aspirin's inhibition of the COX-1 pathway, as there were no changes in the generation of TXB_2. Increases in the generation of 12-HETE implicated the poorly understood 12-LOX pathway as one potential explanation. Given the integral role played by platelets in POCT,[100] it was hypothesized that the reduction in platelet activation observed with the use of LMWH instead of UFH as the pre-clamping anticoagulant might be associated with a reduction in rates of post-operative embolization.

6.2 Aims

The aim of this study was to investigate whether the substitution of intravenous LMWH in place of UFH for pre-clamp anticoagulation during CEA would result in lower post-operative embolization.

6.3 Materials and Methods

6.3.1 Study design

A double-blind randomized controlled trial in consecutive patients scheduled for CEA was established, as already described (*Section 4*.2). Prior to carotid clamping patients were anticoagulated either in our standard fashion, with 5000IU UFH or with 2500IU LMWH intravenously. Two hundred and twenty nine patients were initially recruited. Forty six exclusions meant that 183 patients were randomized, with 91 receiving UFH and 92 receiving LMWH (*Figure 66*).

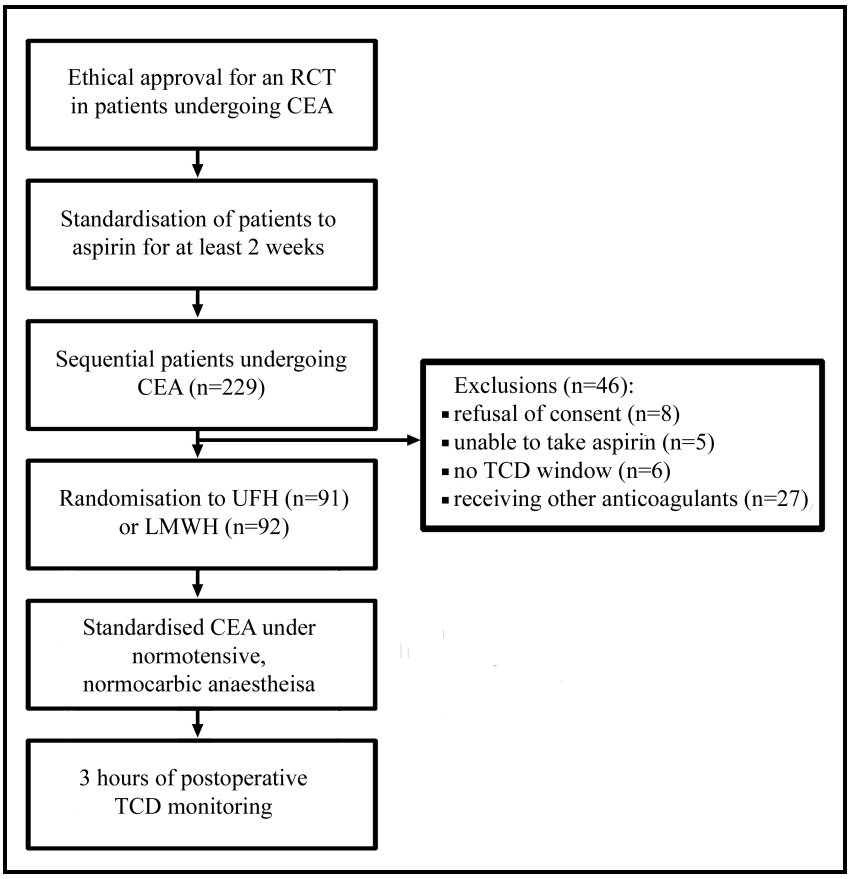

Figure 66 Study design and flow and recruitment of patients

6.3.2 Operation and intra-operative monitoring

All patients underwent a standardized CEA with the use of normotensive, normocarbic general anaesthesia, and the routine placement of a Pruitt-Inahara intra-luminal shunt. There were six consultant surgeons either performing the CEA or supervising a trainee. Independent pharmacists dispensed either 5000IU UFH or 2500IU LMWH, and systemic heparinisation was administered intravenously by the anaesthetist 4 minutes prior to carotid clamping and insertion of the shunt. The surgeon was blinded to the type of heparin used. Continuous TCD monitoring of the blood flow velocity in the ipsilateral middle cerebral artery was performed for the duration of the operation. All TCD waveform data were stored on digital audiotape for blinded offline analysis by the same vascular technician. Post-operative embolization was quantified with the use of standardized consensus criteria.[289] The carotid arteriotomy was closed with a Dacron patch (W. L. Gore, Flagstaff, Arizona, USA) in all cases, and completion angioscopy was performed in patients prior to restoration of flow.[95]

6.3.3 Post-operative monitoring

All patients were monitored for three hours post-operatively with TCD. Using pre-existing local criteria, any patient with more than 25 emboli in any 10 minute period was given a 30ml intravenous bolus of Dextran (Pharmacia Ltd, Milton Keynes, United Kingdom), followed by an infusion starting at 20mlhour^{-1} and then systematically increased until the rate of embolization diminished. Thereafter, Dextran was continued for 12 hours.

6.3.4 Bleeding tendency

The time from restoration of blood flow to removal of the surgical drapes was used to estimate the time required to secure haemostasis, and acted as a surrogate measure of excess bleeding.

6.3.5 Statistical analysis

Analysis was with SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA); discrete data analysed with the use of contingency tables (Fisher's exact and chi-square tests); and continuous data analysed with the use of a 2-tailed Mann-Whitney *U* test. Data are presented as mean ± SD. Probability values <0.05 were considered statistically significant.

Whilst 50% of patients will have one or more emboli detected in the early post-operative period, only 5% will develop sustained embolization,[108] making meaningful statistical analysis not straightforward. In order to be able to demonstrate a difference between the two groups, as in previous work,[260] an arbitrary distinction between "low embolization" and "high embolization" was identified. For this work, it was decided that the upper quartile would be used as a cut-off.

6.4 Results

6.4.1 Demographics

Variable	*UFH (n=91)*	*LMWH (n=92)*	*p value*
Age/years	67±10.3	70±8.3	0.13
Sex			
Male	70(77%)	69(75%)	0.76
Female	21(23%)	23(25%)	
Weight/kg	76±12.7	80±14.2	0.64
Hypertension	69(76%)	75(82%)	0.35
Diabetes	14(15%)	22(24%)	0.15
Current smoker	20(22%)	25(27%)	0.41
Presentation			
Asymptomatic	17(19%)	13(14%)	0.41
Stroke	24(28%)	24(26%)	0.97
TIA	34(37%)	43(47%)	0.20
Amaurosis fugax	16(18%)	12(13%)	0.40
Mean carotid stenosis/%	77±8	80±8	0.29

Table 12 Demographic breakdown of study participants

Of the 229 patients undergoing CEA during the study period 46 (21%) were excluded (*Figure 66*). The 183 (79%) remaining patients were randomized to either LMWH (n=92) or UFH (n=91). The two groups were demographically matched (*Table* 12).

6.4.2 Post-operative embolization

Figure 67 shows the patients ranked according to the number of post-operative emboli they experienced.

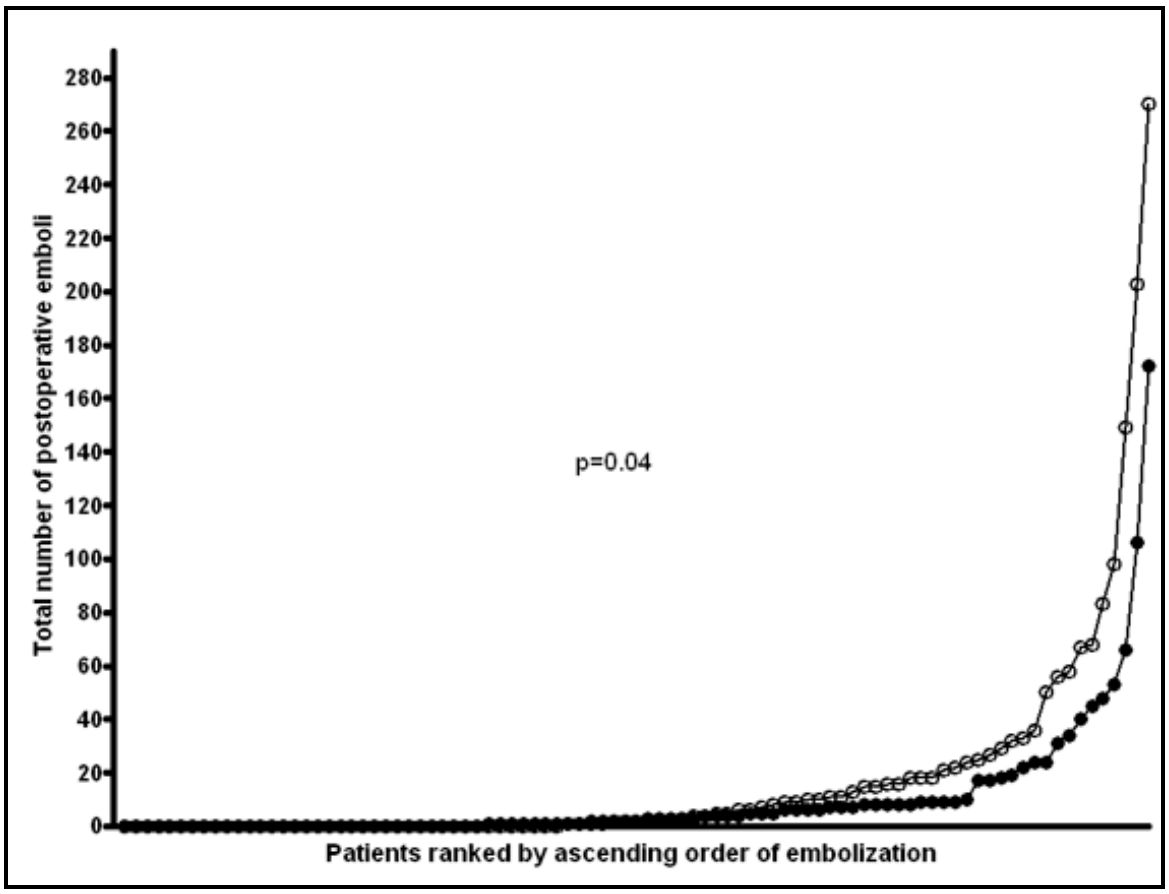

Figure 67 183 patients ranked according to the magnitude of post-operative embolization after CEA. Closed circles; LMWH group (n=92); open circles; UFH group (n=91)

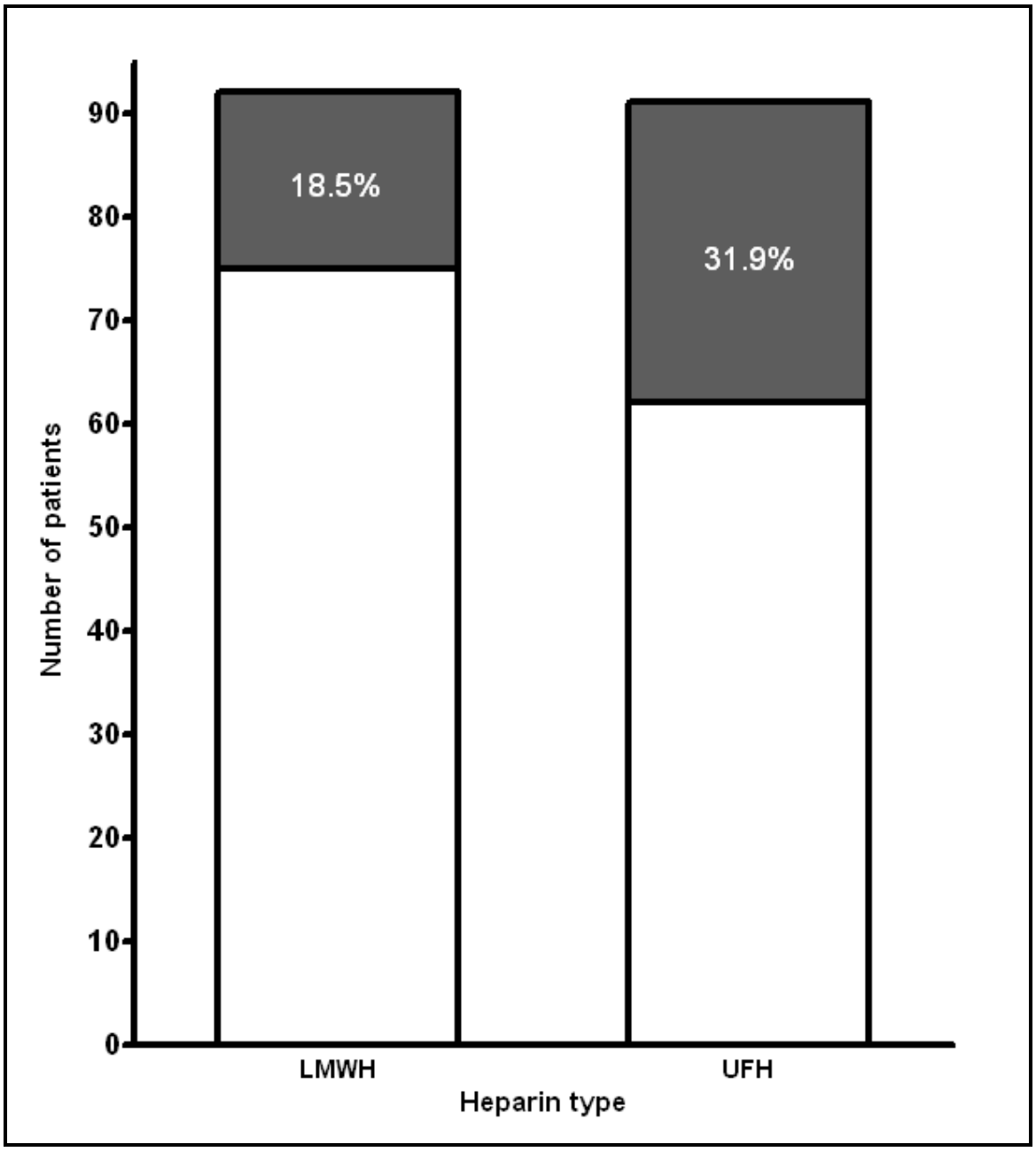

Figure 68 Proportion of patients randomized to 5000IU UFH (n=91) or 2500IU LMWH (n=92) intravenously prior to carotid clamping experiencing high-rate embolization (shaded bars), compared to those who did not (open bars)

The mean number of post-operative emboli was 12 in the LMWH group, versus 18 in the UFH group (p=0.247), but patients randomised to UFH were found to be twice as likely to be "higher" embolizers (29 of 91; 31.9%) than those randomised to LMWH (17 of 92;

18.5%, *Figure 67 & Figure 68*). This equates to an odds ratio of 2.06; 95% CI, 1.04 to 4.10; p=0.04. Figure 3 illustrates the total number of patients in each group, showing the proportion experiencing "high embolization" (shaded bars).

Two patients (2.2%) in the LMWH group received Dextran therapy to control high-grade embolization, whereas four patients (4.4%) in the UFH group received Dextran (p>0.05).

6.4.3 Peri-operative haemostatic function

One (1.1%) patient in each group required re-exploration for post-operative bleeding. The length of time taken from flow restoration to skin closure was used as an indirect marker of haemostasis, and was not significantly different between the two groups: the median time from restoration of flow to removal of the drapes was 23±8.5 minutes in the LMWH group and 24±7.5 minutes in the UFH group (p=0.18, *Figure 69*).

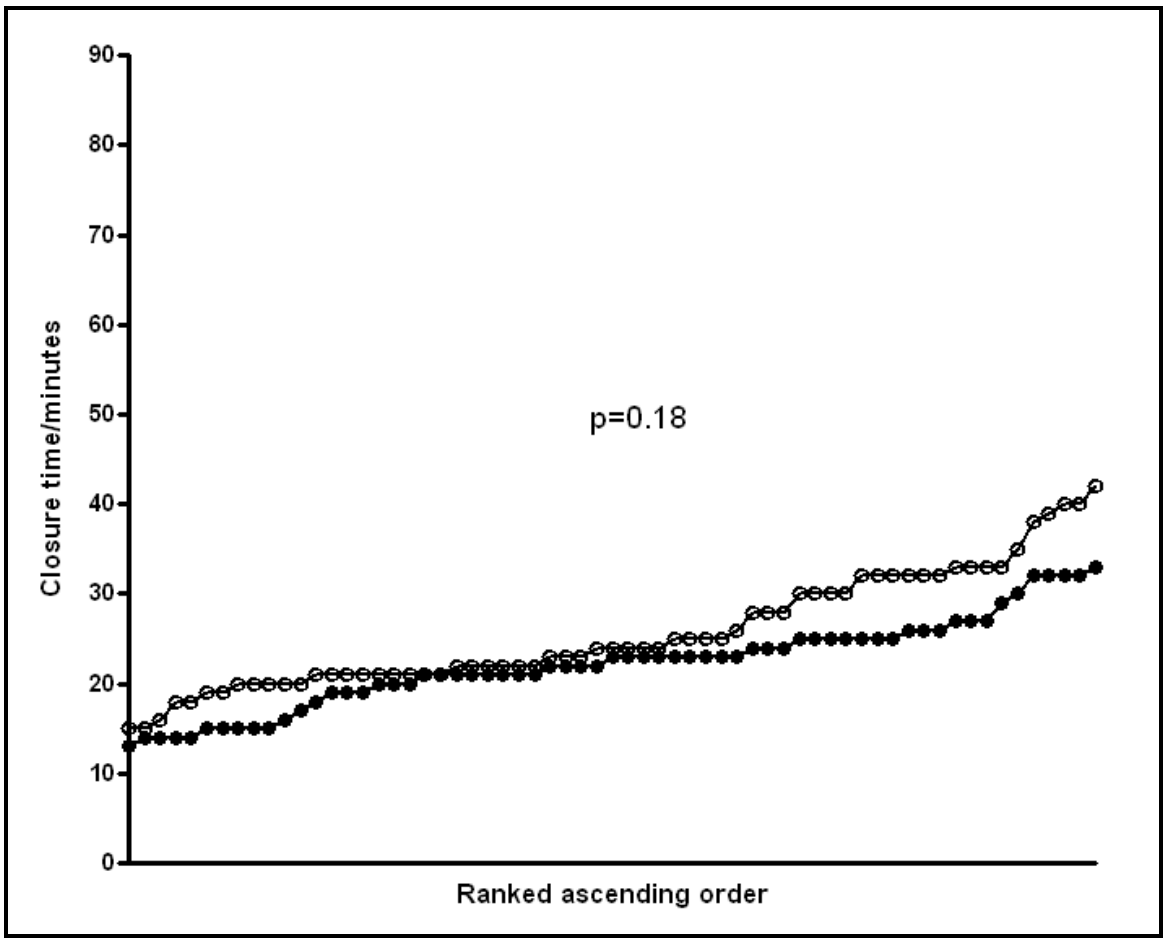

Figure 69 Time taken from restoration of flow to removal of the surgical drapes, an indirect measure of the time required to achieve haemostasis, in 183 patients undergoing CEA. Closed circles; LMWH group (n=92); open circles; UFH group (n=91)

6.4.4 Post-operative embolization and platelet aggregation

There were no correlations between the post-operative embolization rate and the platelet aggregatory response to AA at induction (p=0.35), 3 minutes post-heparinisation (p=0.09), 120 minutes post-heparinisation (p=0.60) and 330 minutes post-heparinisation (p=0.22). Similarly, the post-operative embolization rate did not correlate with the platelet aggregatory response to ADP at induction (p=0.42), 3 minutes post-heparinisation

(p=0.13), 120 minutes post-heparinisation (p=0.15) or 330 minutes post-heparinisation (p=0.19).

6.4.5 Post-operative embolization and generation of platelet metabolites

6.4.5.1 TXB_2 concentrations at induction of anaesthesia

Figure 70 shows how post-operative embolization correlated with the four TXB_2 concentrations at induction of anaesthesia. There was no correlation between plasma TXB_2 at induction of anaesthesia and post-operative embolization (p=0.21), but significant correlations existed between post-operative embolization and serum TXB_2 (p=0.001), generation of TXB_2 on clotting (p=0.001) and the generation of TXB_2 from platelets activated by ADP (p=0.006).

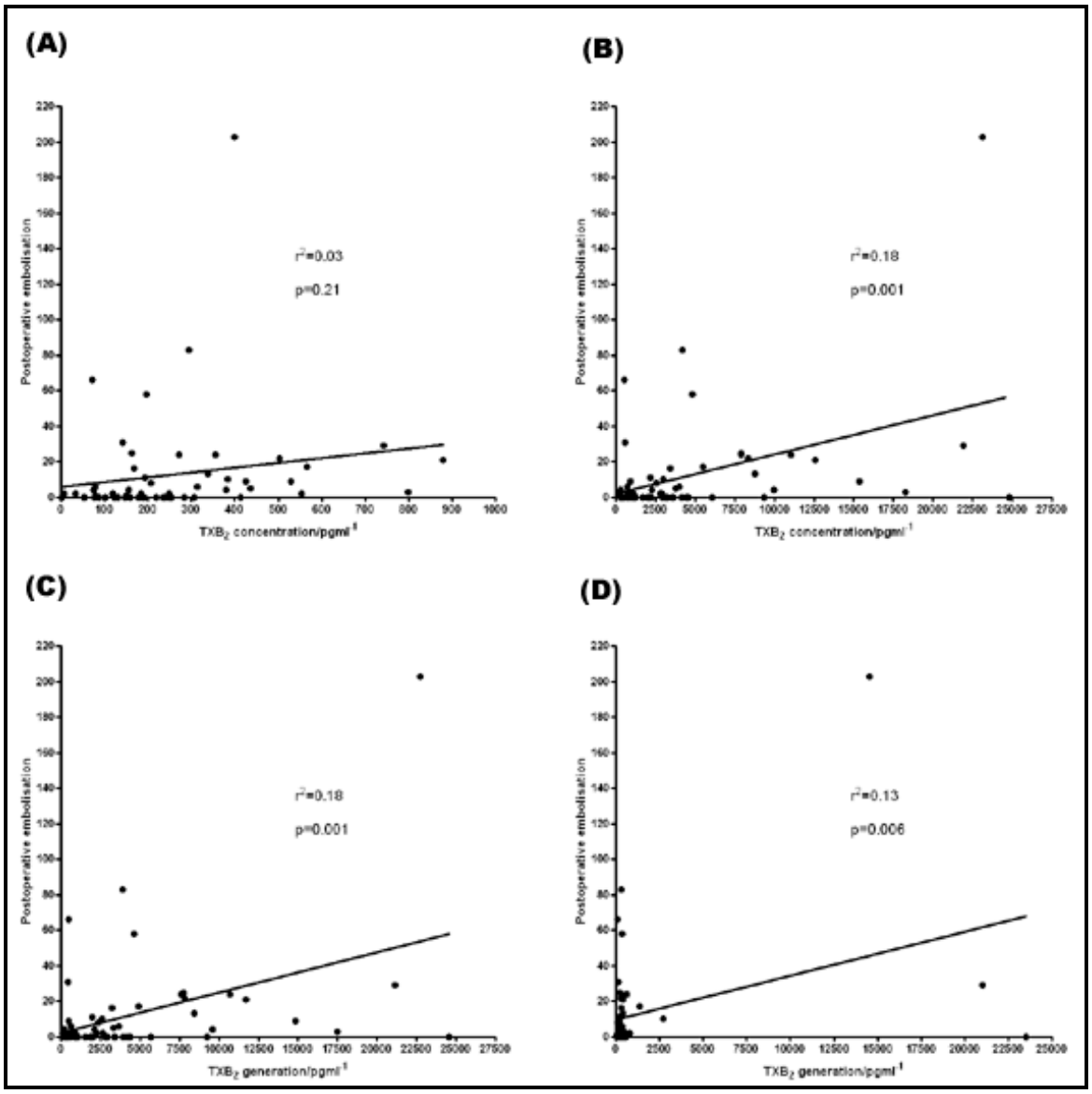

Figure 70 Correlation of post-operative embolization and TXB_2 concentration at induction of anaesthesia; (A) plasma concentration, (B) serum concentration, (C) generation on clotting, (D) generation on activation with ADP

6.4.5.2 TXB_2 concentrations 3 minutes after heparinisation

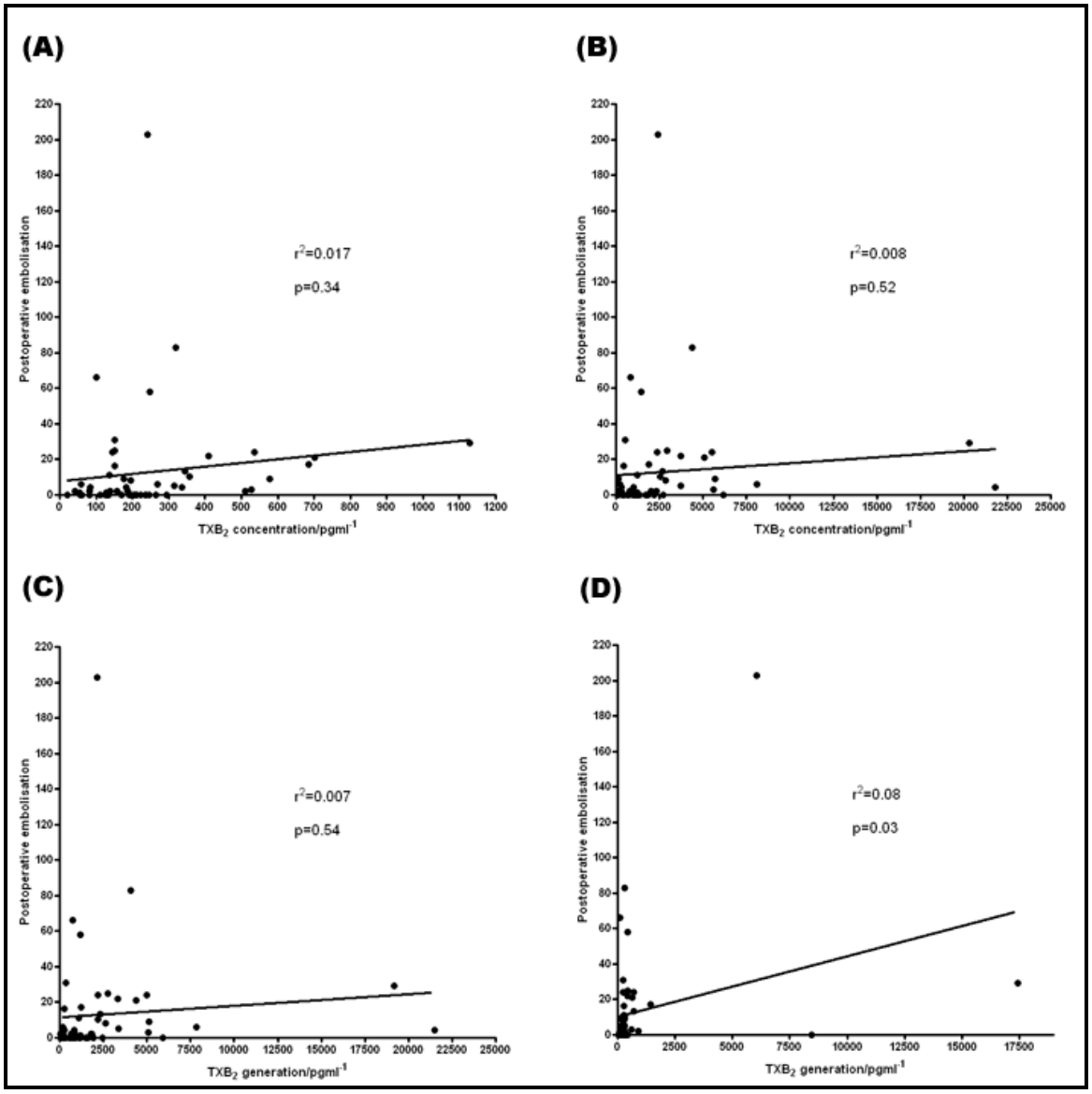

Figure 71 Correlation of post-operative embolization and TXB_2 concentration 3 minutes post-heparinisation; (A) plasma concentration, (B) serum concentration, (C) generation on clotting, (D) generation on activation with ADP

There was generally less correlation between the post-heparinisation concentrations of TXB_2 and post-operative embolization (*Figure 71*). There was no correlation between the plasma TXB_2 (p=0.34), serum TXB_2 (p=0.52) and TXB_2 generation on clotting (p=0.54)

following heparinisation and post-operative embolization. There was, however, a correlation between the generation of TXB_2 after platelet activation with ADP and post-operative embolization (p=0.03).

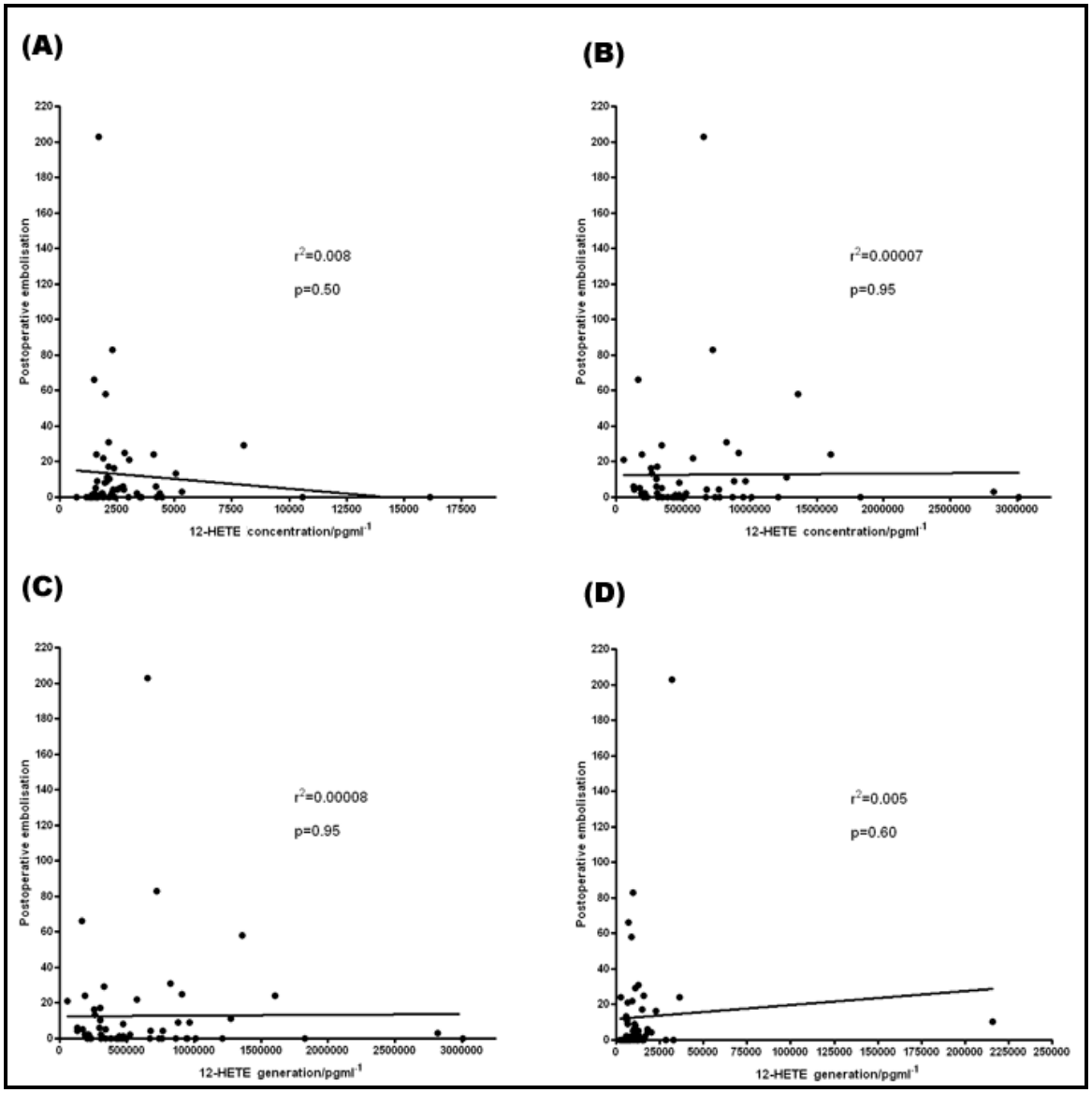

Figure 72 Correlation of post-operative embolization and 12-HETE concentration at induction of anaesthesia; (A) plasma concentration, (B) serum concentration, (C) generation on clotting, (D) generation on activation with ADP

6.4.5.3 12-HETE concentrations at induction of anaesthesia

Figure 72 demonstrates that there were no correlations observed between post-operative embolization and the concentrations of 12-HETE at induction of anaesthesia for plasma (p=0.50), serum (p=0.95), generation on clotting (p=0.95) or platelet generation after activation with ADP (p=0.60).

6.4.5.4 12-HETE concentrations 3 minutes after heparinisation

Following heparinisation there were no significant correlations between post-operative embolization and the concentrations of 12-HETE in plasma (p=0.76), serum (p=0.66) or on clotting (p=0.65). There was, however, a significant correlation between post-operative embolization and the platelet generation of 12-HETE following ADP activation (p=0.02, *Figure 73*).

6.4.6 Peri-operative morbidity and mortality

The 30-day disabling stroke or death rate was 2.2% (2 patients) in both the LMWH and UFH groups. One patient in the UFH group suffered an acute, intra-operative carotid artery dissection and a second patient died following a post-operative intra-cerebral haemorrhage, most likely related to a post-operative hyperperfusion syndrome on day 11. In the LMWH group, one patient suffered an intra-operative stroke due to embolic occlusion of the ipsilateral middle cerebral artery following shunt insertion. The second patient died following a myocardial infarction on post-operative day 5. None of these patients

experienced high embolization post-operatively. Complications were evenly distributed amongst the six consultant surgeons.

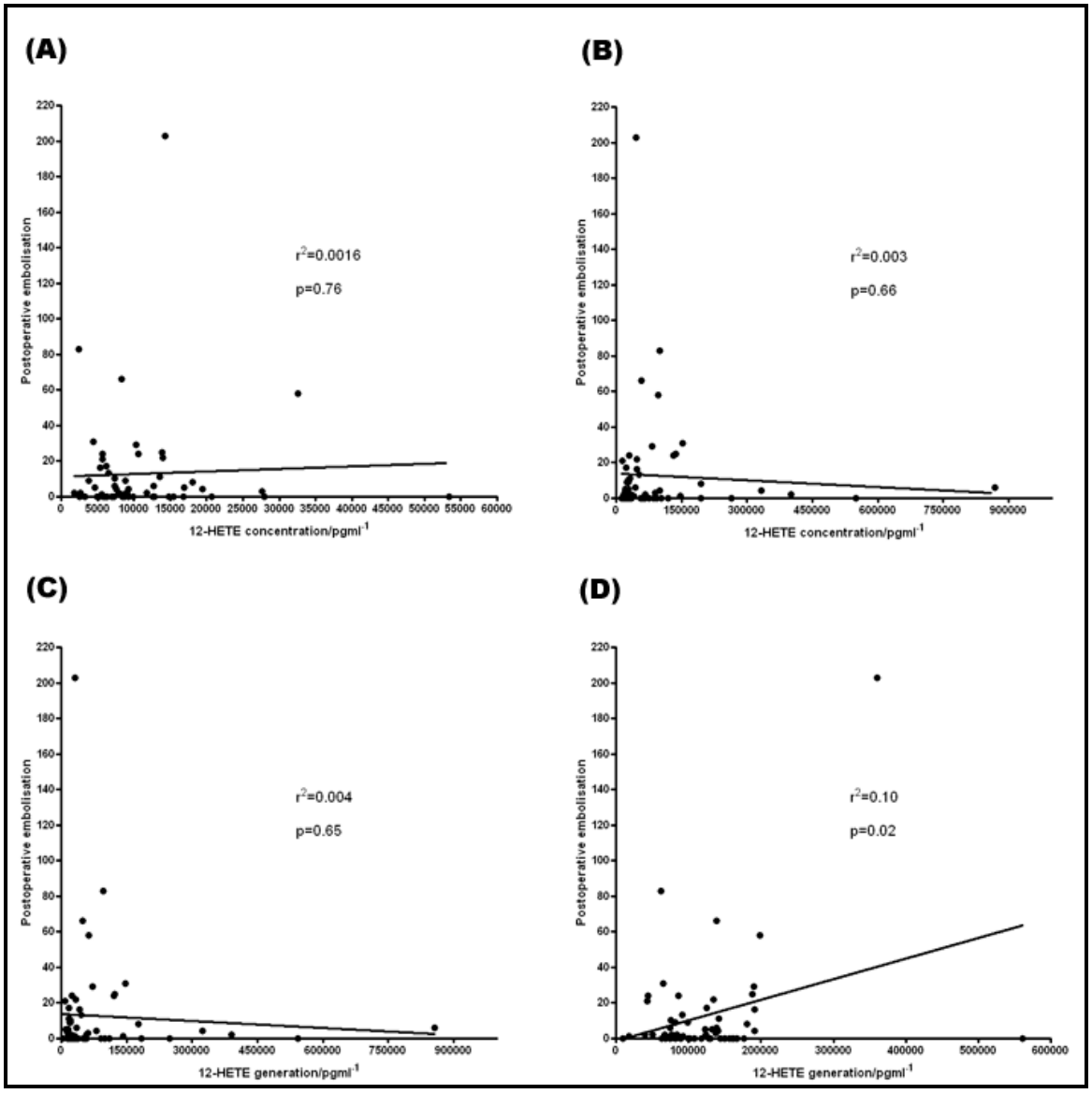

Figure 73 Correlation of post-operative embolization and 12-HETE concentration 3 minutes post-heparinisation; (A) plasma concentration, (B) serum concentration, (C) generation on clotting, (D) generation on activation with ADP

6.5 Discussion

Post-operative embolization has a well-proven relationship to post-operative cerebral ischaemic events, and has been shown to be an effective surrogate outcome measure for stroke risk.[126,256] By using post-operative embolization as an end-point an intervention can be assessed – in this case the effect of the different heparins - on a smaller cohort than would be required using the clinical end-point of stroke.

The difference in post-operative embolization, and by association, CEA-related stroke risk, was small. Nonetheless, those patients who received LMWH rather than UFH prior to carotid clamping were half as likely to experience high-rate post-operative embolization. There were positive correlations between the magnitude of post-operative embolization and the pre-operative concentrations of TXB_2, suggesting a link between platelet activity and post-operative embolization. It is interesting that the higher the pre-operative concentration of TXB_2, the higher the post-operative embolization rate. However, the associations were weak, and it is possible they were affected by a single high-embolizing outlier. Certainly no correlation was demonstrated between the magnitude of post-operative embolization and the platelet aggregatory response to AA and ADP.

This study has shown that intra-operative anticoagulation with LMWH (2500IU dalteparin) rather than UFH (5000IU heparin) was associated with a significant (twofold) reduction in higher rate embolization following CEA, without an increase in bleeding. The thromboembolic material that leads to POCT is invariably platelet-rich.[95,100] However,

while anti-platelet therapy has been shown to reduce the risk of post-operative stroke,[276] embolization does still occur despite adequate aspirin therapy.[259] Given that post-operative embolization is an effective surrogate marker for stroke risk, the results of this study might imply that LMWH produced a lower level of POCT than did UFH. Taken with the findings already reported on the effects of heparin on platelet activity, it is possible that part of the effect on post-operative embolization was mediated by the varying platelet effects of the two heparin types. Definitive confirmation of this association would probably require larger studies, and the introduction of LMWH for use in this setting would require a re-licensing of the drug.

VII

A Study of Potential Mechanisms for Heparin-Platelet Interaction During CEA

7.1 Introduction

The platelets of patients undergoing CEA demonstrate a significant increase in aggregation to AA and ADP following intra-operative heparinisation, via a COX-1/aspirin-independent mechanism, possibly associated with the 12-LOX pathway and the release of 12-HETE. A number of hypotheses were proposed to explain how heparin might mediate these effects.

7.1.1 The lipase hypothesis

It is unclear why heparin should seem to drive platelet AA metabolism through the 12-LOX pathway, but there is some evidence that human platelets can be stimulated to produce 12-HETE in the presence of lipase.[290,291] Lipoprotein lipase (LPL) mediates the hydrolysis of triglyceride rich lipoproteins, and is located at the surface of the vascular endothelium in extra-hepatic tissues bound to heparin sulphate proteoglycans (HSPG).[292] Because

synthetic heparins have chemical structures similar to that of HSPG they display affinity for LPL. Heparin releases LPL from its endothelial ligand, heparan sulphate, to form heparin-lipase complexes in the plasma. This was first demonstrated in 1943 with the finding that immediately after heparin administration, the degree of postprandial lipaemia was markedly decreased.[293] This is known as the "clearing" reaction, which results from the release of LPL from peripheral tissues with a temporary increase in lipolysis. More recent studies of this reaction have actually revealed a biphasic response to heparin administration. The first phase occurs within 5 minutes post-heparin (UFH and LMWH) and is similar to the "clearing" reaction, with increased lipolysis and subsequent drop in plasma triglyceride levels which then declines (gradually with UFH and rapidly with LMWH) over the first 30 minutes. A second phase, when the activity of LPL and subsequently the catabolism of triglyceride rich lipoproteins is markedly reduced, leads to temporary hyperlipidaemia.[294,295]

Taking into account these known aspects of lipase metabolism, and the potential interaction with platelet-derived 12-HETE, it was hypothesized that the increase in platelet-derived 12-HETE observed in our study population might be associated with an increase in plasma lipase activity after heparinisation.

7.1.2 Heparin antibodies

Given the systemic nature of atherosclerosis, it is not unusual for patients undergoing CEA to have undergone vascular interventions previously. As in CEA, the risk of arterial

thrombosis during other vascular procedures is frequently reduced by using intravenous heparin. Roughly 20% of patients presenting for vascular surgical procedures either already have pre-existing heparin-induced antibodies, or subsequently develop them.[239] About 17% of patients treated with UFH, and 8% of patients treated with LMWH will form antibodies against complexes of platelet factor 4 (PF4) and heparin.[225,235] Whilst most patients who form anti-heparin-PF4 antibodies suffer no clinical consequences, in as many as 20% of patients the antibody complexes are functionally active, triggering platelet activation.

It was therefore hypothesized that the increase in platelet activation following heparinisation might be mediated by an immune reaction involving heparin antibody complexes.

7.1.3 Anti-factor Xa activity

Heparins work by inducing a conformational change in the antithrombin III molecule, converting it into a high affinity inhibitor of the clotting factors IIa, IXa, Xa, XIa and XIIa.[224] UFH inactivates IIa and Xa in equal proportions, whereas LMWH binds preferentially to Xa, with a Xa:IIa ratio of 4:1, giving a more targeted and predictable anticoagulant response.[225]

Predictably, there is an inverse relationship between measures of platelet activity and anti-FXa activity – platelets exhibit less contractile force in the presence of greater anti-FXa activity and there is less thrombin generation.[296] Given that these studies were assessing

differences in responses to either UFH or LMWH, it was hypothesized that there might be differences in the anti-FXa activity between the two groups.

7.2 Aims

This series of studies was aimed at investigating possible causes for the platelet-heparin interactions that had been observed, with focus on lipase activity, heparin antibodies and anti-factor Xa activity.

7.3 Materials and Methods

7.3.1 Study design

The double-blind randomized controlled trial was established as already described (*Section 4*.2). Of the 183 patients undergoing elective CEA who were randomized to receive either standard 5000IU UFH or 2500IU LMWH intravenously prior to carotid clamping, plasma lipase activity was determined in 40 patients at induction of anaesthesia and 3 minutes after heparinisation. The presence of heparin-PF4 antibody complexes was determined in serum from 77 patients, and anti-FXa activity was assayed in 74 patients 3 minutes after heparinisation, and 120 minutes after heparinisation.

7.3.2 Blood sampling

Blood samples were taken from an indwelling arterial line into vacutainer tubes (Becton Dickinson, Oxford, UK) with the first 3ml of blood wasted and subsequent samples taken into 0.105M buffered sodium citrate solution for plasma preparation and inert polymer gel/clot activator for serum preparation. Citrated blood was centrifuged at 1500×g for 20 minutes before the supernatant from this was aspirated and aliquotted to be stored at -70°C prior to batch analysis. Serum tubes were stored upright for 60 minutes to allow full thrombosis of the blood, before being centrifuged at 1500×g for 20 minutes. The serum supernatant was then aspirated and aliquotted to be stored at -70°C.

7.3.3 Plasma lipase activity

A commercial kit (Confluolip Continuous Fluorometric Lipase Test, Progen Biotechnik GMBH, Heidelberg, Germany) was used to determine the lipase activity in the stored plasma samples from induction of anaesthesia, and 3 minutes post-heparinisation in 40 individuals. The manufacturer's instructions were adapted so that the samples could be run on a fluorometer. The principle of the test was that a lipase substrate has a fluorescence which is "quenched" by a trinitrophenyl group.[297,298] On contact with active lipase from the plasma sample, the "quencher" is hydrolysed and the resulting fluorescence can then be detected and quantified using a flourometer. The kinetic increase in fluorescence intensity at 37°C is proportional to lipase activity. Post-heparin plasma was diluted 1 in 10 as per the manufacturer's instructions, and temporal fluorescence was measured at 342nm excitation and 400nm emission.

7.3.4 Heparin antibodies

The presence of heparin-PF4 antibody complexes was determined in pre-operative serum from 77 patients undergoing CEA, using a commercial ELISA kit (PF4 Enhanced, GTI Diagnostics, Waukesha, USA). The ELISA procedure involved adding patient serum to microwells coated with PF4 complexed to polyvinyl sulphate (PVS). If an antibody recognising a site on PF4:PVS was present, binding occurred. Unbound antibodies were then washed away. An alkaline phosphatase labelled anti-human globulin reagent (anti-IgG/A/M) was added to the wells and incubated. The unbound anti-IgG/A/M was washed away and the substrate p-nitrophenyl phosphate (PNPP) was added. After 30 minutes, the reaction was stopped by sodium hydroxide, and the optical density of the colour that developed was measured on a spectrophotometer. Where serum was positive for heparin-PF4 antibodies, a confirmatory procedure was conducted. This involved re-assaying the serum in the presence of heparin to a final concentration of $100IUml^{-1}$. Inhibition of a positive reaction by 50% or more in the presence of excess heparin was considered confirmatory for heparin-dependent antibody. The procedure was carried out according to the manufacturer's instructions.

7.3.5 Plasma anti-FXa activity

Anti-FXa activity samples were run on an automated blood coagulation analyser (CA-1500, Sysmex Corp., Hyogo, Japan). In principle, the method involved incubating the plasma samples with an excess of ATIII and FXa. When both FXa and ATIII are present in excess, the rate of FXa inhibition is directly proportional to the heparin concentration.[299,300] *In vitro* inhibition of FXa by the ATIII-heparin complex took place, with residual FXa being

inversely proportional to the level of heparin in the sample. This residual quantity of FXa was determined by the rate of hydrolysis of a chromogenic substrate. The FXa cleaved the chromogenic substrate and the rate of peptide nucleic acid (pNA) release (colour formation) was measured at 405nm, and was inversely proportional to the concentration of heparin in the sample.

The anti-FXa activity was measured in plasma from samples from 74 patients undergoing CEA, at 3 minutes after heparinisation and 120 minutes post-heparinisation.

7.3.6 Statistical analysis

SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for data analysis, with discrete data analysed with the use of contingency tables (Fisher's exact and chi-square tests), and continuous data analysed with the use of a 2-tailed Mann-Whitney *U* test. Data are presented as mean ± SD. Probability values <0.05 were considered statistically significant.

7.4 Results

7.4.1 Plasma lipase activity

7.4.1.1 Demographics

Table 13 shows the demographic breakdown of participants in the lipase arm of the study. The UFH and LMWH groups were well-matched.

Variable	***UFH (n=18)***	***LMWH (n=22)***	***p value***
Age/years	63±10.0	68±8.9	0.14
Sex			
Male	15(83%)	15(68%)	0.46
Female	3(17%)	7(32%)	
Weight/kg	76±8.0	78±13.7	0.62
Hypertension	13(72%)	21(96%)	0.07
Diabetes	4(22%)	7(32%)	0.72
Current smoker	4(22%)	9(41%)	0.18
Presentation			
Asymptomatic	6(33%)	5(23%)	0.50
Stroke	4(22%)	3(14%)	0.68
TIA	5(28%)	10(46%)	0.33
Amaurosis fugax	3(17%)	4(18%)	1.00
Mean carotid stenosis/%	78±6.4	77±8.3	0.62

Table 13 Demographic breakdown of study participants

7.4.1.2 Plasma lipase activity

At induction of anaesthesia, the plasma lipase activity was similarly low in both the LMWH and UFH groups, at 0.28±0.03pmolml^{-1}min^{-1} in the LMWH group and 0.28±0.05pmolml^{-1}min^{-1} in the UFH group (p=0.47, *Figure 74*).

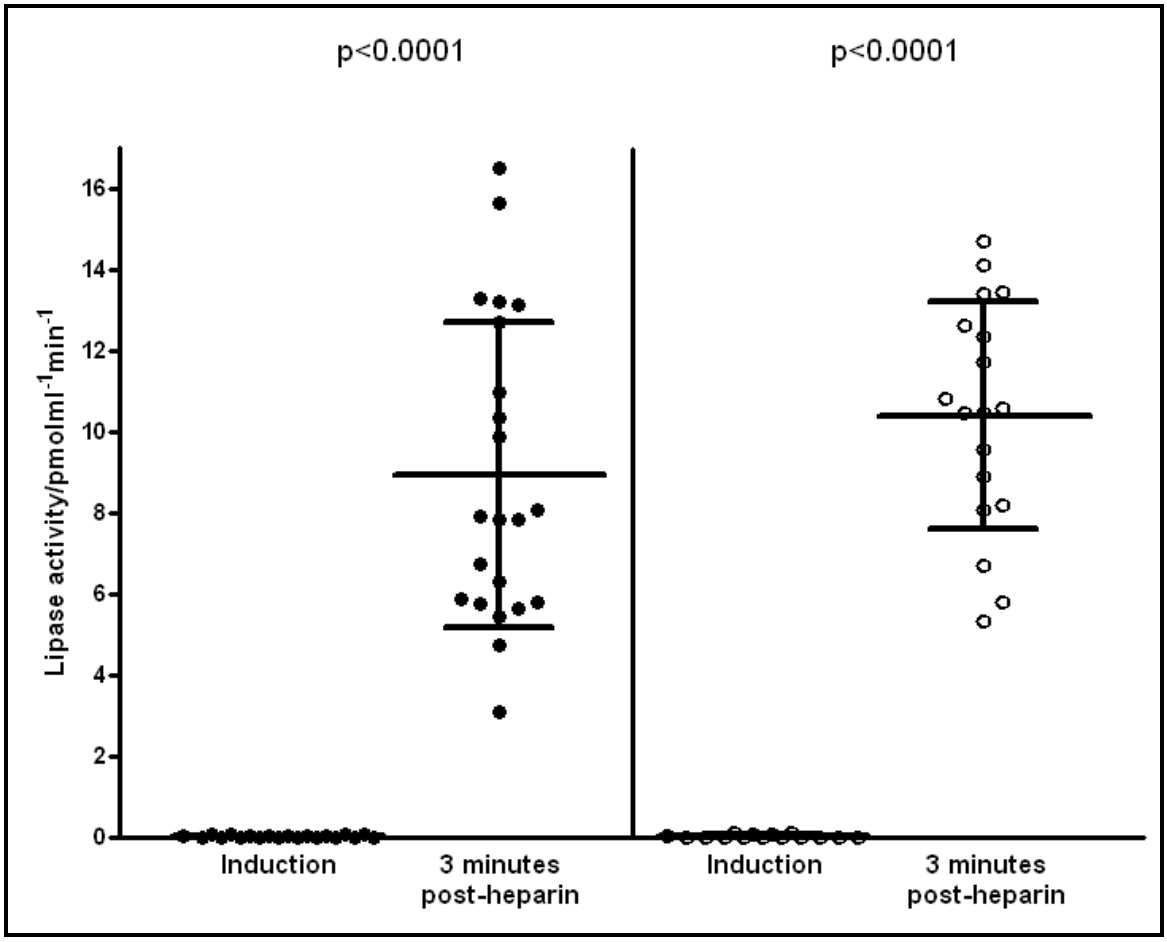

Figure 74 Activity of plasma lipase in 40 patients undergoing CEA. Closed circles; LMWH group (n=22); open circles; UFH group (n=18), horizontal bar represents mean (±SD)

Following heparinisation, the lipase activity increased significantly in both groups, to 8.9±3.8pmolml^{-1}min^{-1} in the LMWH group (p<0.0001) and to 10.4±2.8pmolml^{-1}min^{-1} in

the UFH group ($p<0.0001$). There was, however, no significant difference in the increase between the two heparin types ($p=0.12$).

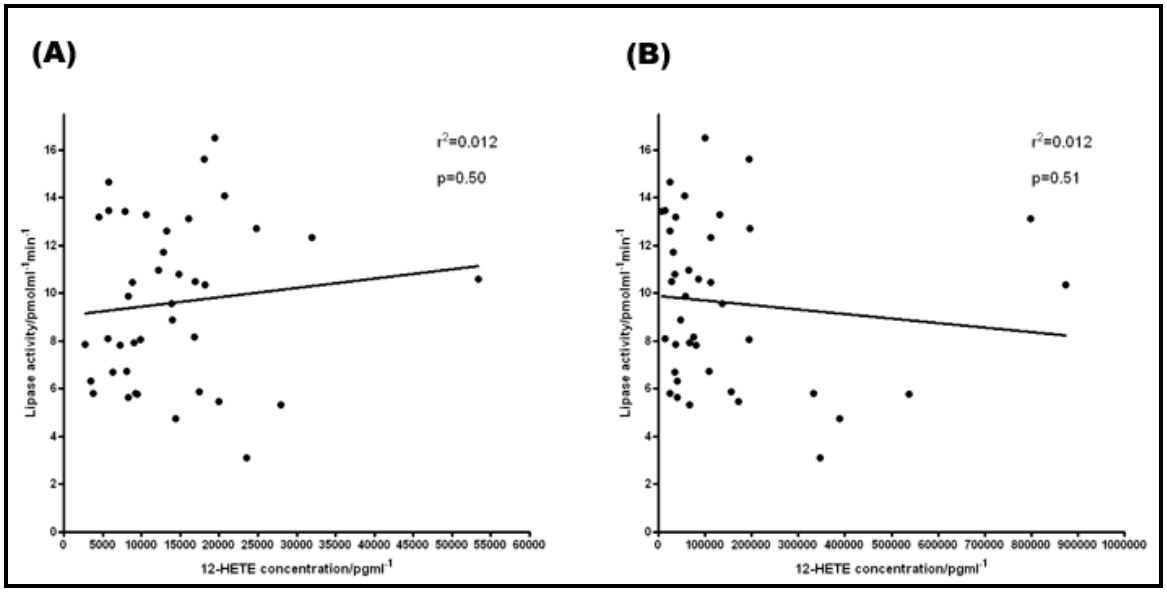

Figure 75 Plasma lipase activity after heparinisation with either LMWH or UFH against corresponding 12-HETE concentration in plasma (A) and serum (B) in 40 patients undergoing CEA

Analysis did not reveal any correlations between plasma lipase activity following heparinisation and any of the markers of platelet activity (aggregation to AA and ADP, generation of platelet metabolites). In particular, no correlation between plasma lipase activity and 12-HETE generation was observed (*Figure 75*).

7.4.2 Heparin antibodies

7.4.2.1 Demographics

Variable	*UFH (n=35)*	*LMWH (n=42)*	*p value*
Age/years	64±9.6	68±9.1	0.08
Sex			
Male	26(74%)	26(62%)	0.33
Female	9(26%)	16(38%)	
Weight/kg	74±13.1	77±15.6	0.44
Hypertension	24(69%)	37(88%)	0.05
Diabetes	5(14%)	10(24%)	0.39
Current smoker	11(31%)	13(31%)	0.31
Presentation			
Asymptomatic	10(29%)	8(19%)	0.42
Stroke	11(31%)	8(19%)	0.29
TIA	8(23%)	18(43%)	0.09
Amaurosis fugax	6(17%)	8(19%)	1.00
Mean carotid stenosis/%	78±7.9	79±8.6	0.51

Table 14 Demographic breakdown of study participants

7.4.2.2 Heparin antibody assessment

Heparin-PF4 antibodies were assayed in the stored pre-operative serum of 77 patients undergoing CEA. Table 14 demonstrates that the UFH and LMWH groups were reasonably well-matched.

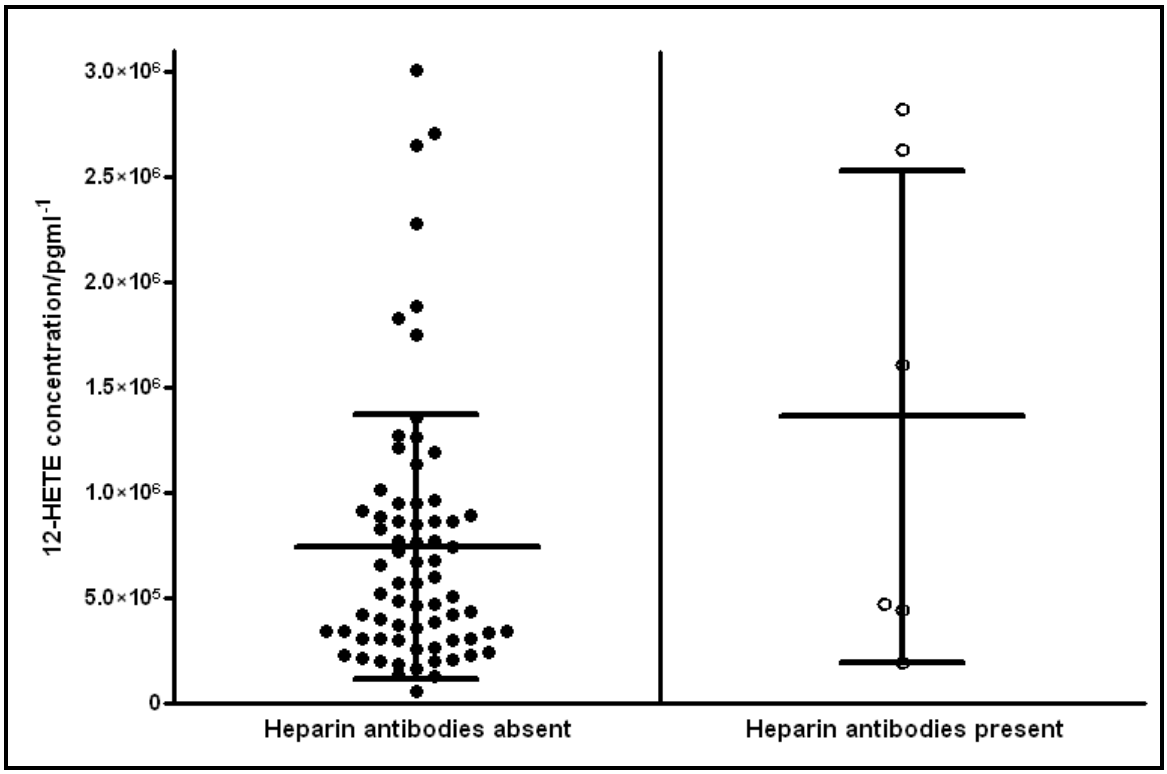

Figure 76 Serum concentration of 12-HETE at induction of anaesthetic in 77 patients undergoing CEA, plotted by the presence or absence of serum heparin-PF4 antibodies. Closed circles; no antibodies (n=71); open circles; antibodies present (n=6), horizontal bar represents mean (±SD)

Six (7.8%) patients exhibited heparin antibodies in the serum samples from induction of anaesthesia, 5 of who went on to receive LMWH during their operation. With such a low proportion of patients positive for the antibodies, it was predictable that any positive correlations with the platelet activity or metabolites would be weak. The only relationship

demonstrated was between the presence of heparin antibodies, and the serum concentration of 12-HETE at induction of anaesthesia ($p=0.04$, $r^2=0.06$). The mean concentration of serum 12-HETE at induction of anaesthesia was $744404\pm626231 pgml^{-1}$ in those patients who were not positive for heparin antibodies, but was two times higher in those who were positive, at $1362500\pm1167030 pgml^{-1}$. The difference here was not, however, statistically significant ($p=0.95$, *Figure 76*).

7.4.3 Anti-FXa activity

The anti-factor Xa activity was assayed in the plasma of 74 patients undergoing CEA (40 in the LMWH group, 34 in the UFH group). The characteristics of the study participants are detailed in Table 15. The two groups were reasonably well-matched.

Immediately following heparinisation, there was a significant difference between the mean anti-FXa activities in the LMWH and UFH groups, with the levels at $0.552\pm0.179 IUml^{-1}$ and $1.466\pm0.372 IUml^{-1}$ respectively ($p<0.0001$). At 120 minutes following heparinisation, the anti-FXa activity was significantly reduced in both groups, at $0.286\pm0.090 IUml^{-1}$ in the LMWH group ($p<0.0001$) and $0.444\pm IUml^{-1}$ in the UFH group ($p<0.0001$). There remained significantly more anti-FXa activity in the UFH group at this time-point ($p<0.0001$, *Figure 77*).

Patient weight significantly affected the magnitude of anti-FXa activity at 3 minutes post-heparinisation regardless of heparin type administered ($p=0.04$ and $p=0.0003$ for LMWH and UFH respectively).

Variable	***UFH (n=34)***	***LMWH (n=40)***	***p value***
Age/years	64±9.7	68±9.3	0.06
Sex			
Male	25(74%)	24(60%)	0.32
Female	9(26%)	16(40%)	
Weight/kg	74±15.0	76±15.0	0.48
Hypertension	23(68%)	35(88%)	0.05
Diabetes	4(12%)	9(23%)	0.36
Current smoker	11(32%)	12(30%)	0.79
Presentation			
Asymptomatic	9(27%)	8(20%)	0.59
Stroke	11(32%)	8(20%)	0.29
TIA	8(24%)	17(43%)	0.14
Amaurosis fugax	6(18%)	7(18%)	1.00
Mean carotid stenosis/%	78±7.9	79±8.3	0.57

Table 15 Demographic breakdown of study participants

At 120 minutes post-heparinisation, there was no longer a correlation between anti-FXa activity and patient weight in the LMWH group (p=0.17), but there remained an association between anti-FXa activity and patient weight in the UFH group (p=0.006, *Figure 78*).

There was no correlation between the magnitude of post-operative embolization and the degree of anti-FXa activity 3 minutes following heparinisation (p=0.68) or 120 minutes following heparinisation (p=0.53). There was, however, a significant correlation between the anti-FXa activity 3 minutes post-heparinisation and the platelet aggregatory response to ADP at the same time-point (p=0.0003, *Figure 79*). The platelet response to AA did not correlate with anti-FXa activity (p=0.75).

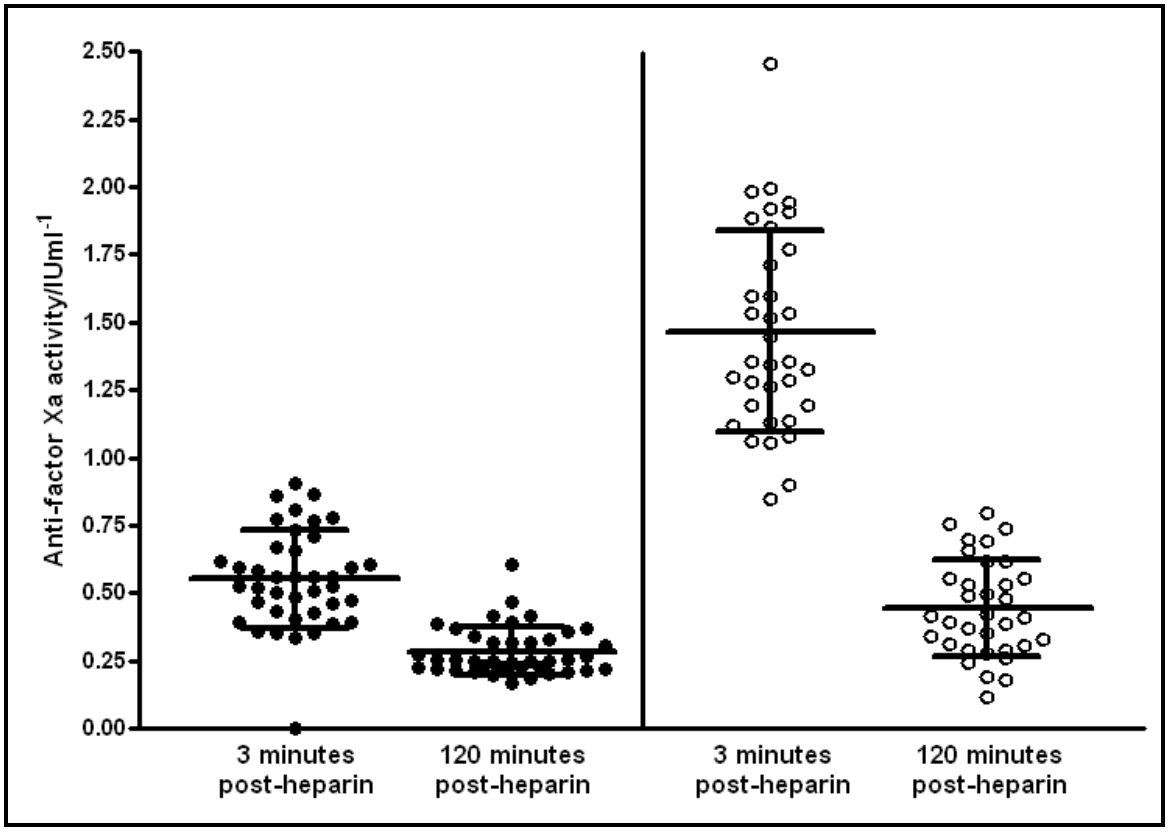

Figure 77 Anti-factor Xa activity in plasma of 74 patients undergoing CEA. Closed circles; LMWH group (n=40); open circles; UFH group (n=34), horizontal bar represents mean (±SD)

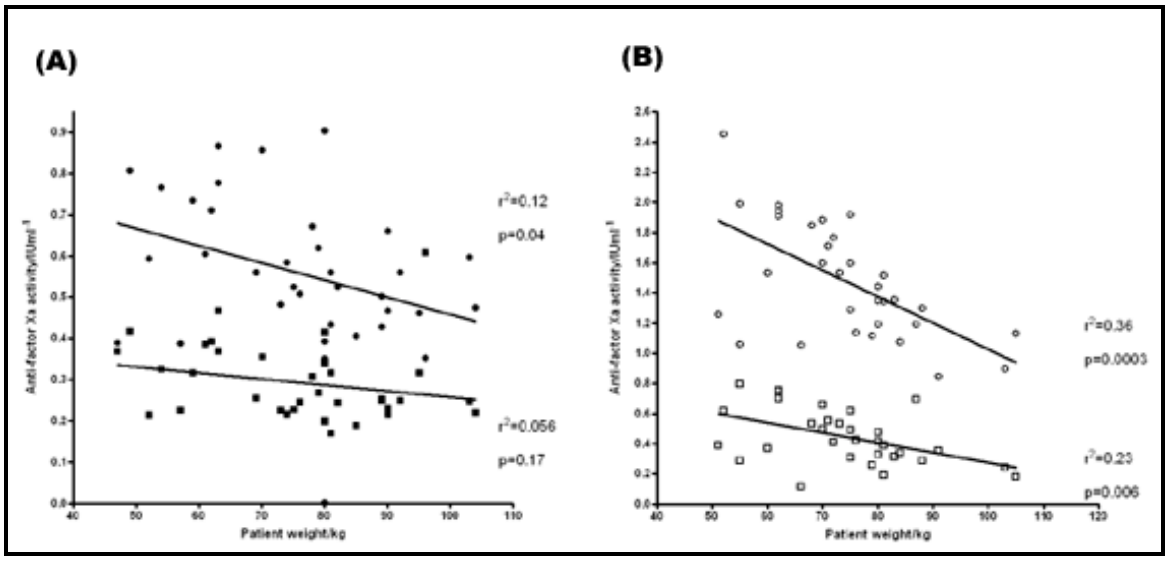

Figure 78 Correlation between anti-FXa activity and patient weight in 74 patients undergoing CEA. Closed symbols; LMWH group (n=40, (A)); open symbols; UFH group (n=34, (B)); circles; 3 minutes post-heparinisation; squares; 120 minutes post-heparinisation

There was no correlation between the concentration of TXB_2 in plasma, serum or plasma after platelet stimulation with ADP and anti-FXa activity 3 minutes after heparinisation (p=0.15, p=0.76 and p=0.38 respectively). However, there were weak correlations between anti-FXa activity and the concentration of 12-HETE in plasma, serum (negative correlation) and plasma from ADP-stimulated platelets at the same time-point (*Figure 80*).

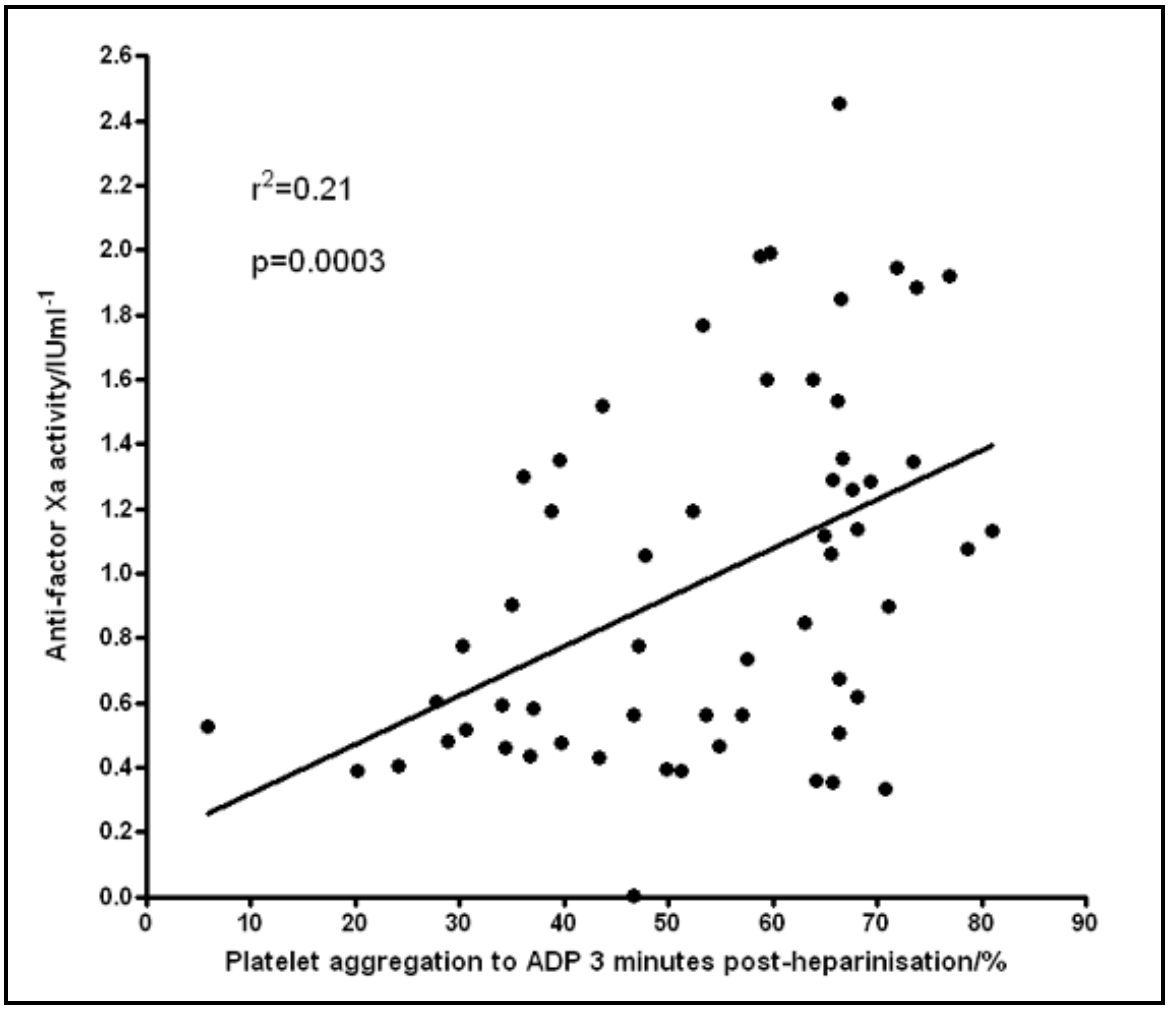

Figure 79 Correlation between anti-FXa activity 3 minutes post-heparinisation and the platelet aggregatory response to ADP at the corresponding time point in 74 patients undergoing CEA

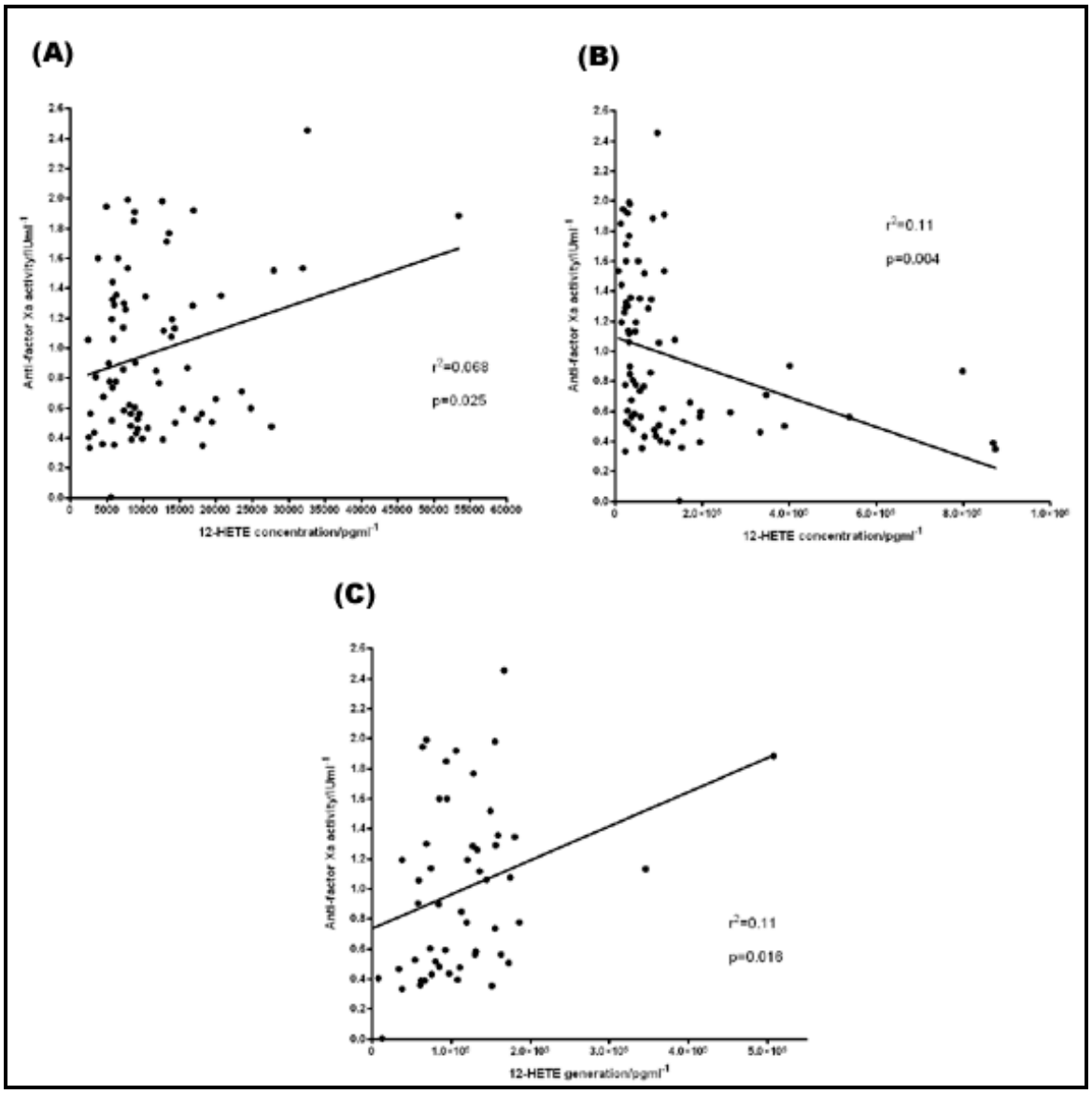

Figure 80 Correlation between anti-FXa activity and plasma 12-HETE concentration (A), serum 12-HETE concentration (B) and plasma generation of 12-HETE from ADP-stimulated platelets (C) in 74 patients undergoing CEA

7.5 Discussion

As anticipated, the lipase activity was low before heparinisation, but rose significantly thereafter. Perhaps most interestingly, the rise in plasma lipase activity was similar after administration of both heparin types; LMWH affected as much plasma lipase activity as

UFH. Both the plasma lipase activity and the generation of 12-HETE increased after heparinisation, although given that there was no correlation observed between lipase activity and 12-HETE concentration, it is difficult to confirm a direct relationship. The hypothesis that heparin increases lipase activity, leading to an increase in 12-HETE secretion has not been proven, and these experiments warrant repeating with greater numbers of patients.

Of 77 patients undergoing CEA, 7.8% exhibited positivity for heparin antibodies, commensurate with published data.[225,235,239] Certainly at this low prevalence, the heparin antibody hypothesis does not explain the phenomenon of transient aspirin resistance. Although there was a weak association observed between the presence of heparin antibodies and serum 12-HETE concentration at induction of anaesthetic, this is likely to be explained by the low sample size. A larger, higher-powered study might provide a more robust answer to this question. However, given the paucity of positive antibody results observed herein, in comparison to the high incidence of increased platelet aggregation and 12-HETE production following heparinisation, it is unlikely that this particular immunological mechanism is responsible. It would be interesting to repeat the studies of heparin antibodies in these patients post-CEA to assess whether the single bolus of intra-operative heparin gave rise to the development of antibodies in any of the patients.

Immediately following heparinisation, there was a difference between the anti-FXa activity in the two heparin groups, with activity significantly lower in the LMWH group than in the UFH group. This difference persisted at 120 minutes post-heparinisation, with the UFH patients significantly more anticoagulated than the LMWH patients at this point. There

was a relation between the platelet response to ADP after heparinisation and the corresponding degree of anti-FXa activity. The greater the degree of heparinisation (as measured by anti-FXa activity), the greater the platelet response to ADP. This provides further evidence that the increase in platelet response following heparinisation is directly related to an effect of the heparin itself. Moreover, the plasma concentration of 12-HETE and the plasma generation of 12-HETE from ADP-stimulated platelets also correlated with the degree of anti-FXa activity.

Given the apparent relation between heparin and increased platelet activity, the question arises of how much heparin is required? The rationale for pre-clamp heparinisation is in the prevention of "stump" thrombosis in the proximal vessel whilst it is clamped. In this series of studies there was significantly less anti-FXa activity in those patients who received LMWH rather than UFH. But those who received LMWH demonstrated less platelet interaction and lower post-operative embolization, in association with reduced 12-LOX activity and 12-HETE generation. Yet there was no evidence of stump thrombosis in any of the patients studied, suggesting that all were adequately anticoagulated for the purposes of carotid clamping. Furthermore, in those patients who received UFH, there remained significant anti-FXa effect at 120 minutes after administration, long after the need for arterial anticoagulation. It could be argued that the UFH group were "over-heparinised"; less heparinisation was not associated with clinical complications, and greater heparinisation was associated with increased platelet reactivity and post-operative embolization.

Anti-FXa activity showed a significant negative correlation with the patients' weight, with the degree of anticoagulation being inversely proportional to patient weight. This is perhaps intuitive, and might suggest that a more targeted approach to anticoagulation during CEA replace the current, universal approach, with weight-adjustment taken into consideration. It might even be contended that the approach to intra-operative anticoagulation for the prevention of arterial "stump" thrombosis during CEA be re-evaluated. As new anticoagulant drugs are developed, it might be possible to switch to a combination of better anti-platelet therapy (such as pre-operative clopidogrel) and, for example, a direct thrombin inhibitor, such as bivalirudin. Initially employed in patients who were intolerant of heparin, the hirudins are increasingly being assessed for use in a wider clinical setting, with some evidence that they cause less platelet interaction and may be associated with less thrombotic complications than heparin.[301-303] This warrants further assessment in the setting of vascular interventions.

VIII

Summary

Carotid endarterectomy (CEA) is of proven benefit in the prevention of stroke for patients with moderate and severe carotid stenosis.[17,79,80,83] The paradox is that the very intervention aimed at averting stroke in the long-term, does itself pose an upfront procedural risk. If, hypothetically, there was no chance of stroke associated with CEA then the relative reduction in the long-term stroke risk would increase from 45% to 75%.[95] The phased research programme in Leicester, one aspect of which this body of work is a continuation, has aimed to systematically investigate and introduce techniques to reduce the risk associated with CEA.

Locally, the implementation of intra-operative monitoring and quality control procedures saw the intra-operative stroke risk drop to 0%.[253] Yet, these measures did not alter the incidence of post-operative stroke, and attention turned to the likely integral role played by platelets. It was shown that patients' "thromboembolic" potential after CEA was related to their platelet sensitivity to ADP,[259] a line of investigation that led to the observation that pre-operative platelet ADP inhibition with clopidogrel significantly reduced post-operative embolization (and by extrapolation, stroke risk).[260] But there was an additional, unexpected finding; immediately following systemic heparinisation with 5000IU unfractionated heparin (UFH), prior to carotid clamping, there was a tenfold increase in platelet aggregation to arachidonic acid (AA),

despite the patients demonstrating adequate aspirin inhibition at the start of the procedure.[262]

Was this increase in heparin-mediated platelet aggregation a reproducible finding? What mechanisms might explain the increase in platelet aggregation? Would LMWH reduce the platelet interaction and effect lower post-operative rates of embolization? These questions were addressed using a double-blind, randomised controlled trial with a total of 183 patients; 91 receiving UFH, and 92 receiving LMWH intravenously prior to carotid clamping. A series of laboratory-based studies were undertaken in sub-groups of this overall population.

Platelet aggregometry was performed in response to AA and ADP (32 LMWH, 33 UFH), from blood sampled at induction of general anaesthesia, then at 3, 120 and 330 minutes post-heparinisation. Following anticoagulation there was a significant increase in the platelet aggregatory response to AA, equivalent with both heparin types. An increase in aggregation after heparinisation with both UFH and LMWH was also observed in response to ADP, but here the increase was significantly greater in those patients who had received UFH. This difference was still present 330 minutes following heparinisation. It was noted that at induction of anaesthesia, the level of platelet aggregation in response to ADP was significantly higher in the patients whose clinical manifestation of carotid artery stenosis was either stroke or transient ischaemic attack (TIA), in comparison to the patients who were asymptomatic or presented with amaurosis fugax. This may suggest that the symptoms produced by a carotid stenosis are in part attributable to the platelet response to ADP.

Platelet-rich-plasma (PRP) was incubated with various inhibitors of platelet pathways, prior to stimulation with AA to identify a mechanistic explanation for the observed phenomenon of "transient aspirin resistance". Inhibition of the COX-1 pathway was tested in 20 patients by the *in vitro* addition of aspirin; this significantly reduced both pre- and post-heparin AA-aggregation. The 12-LOX inhibitor baicalein significantly lowered pre-heparin AA-aggregation in 26 patients, but 12-LOX inhibition after heparinisation did not significantly reduce the augmented AA-response. The thromboxane receptor antagonist SQ 29548 did not significantly alter the pre-heparin platelet response to AA, but platelets from heparinised individuals exhibited significantly less AA-mediated aggregation after incubation with the TRA. This implies that the increases seen in platelet aggregation following heparinisation may indeed be mediated by agonism by TXA_2.

Increased platelet aggregation following heparinisation was further investigated in 58 patients undergoing CEA - the hypothesis being that platelets in which the COX-1 pathway was inhibited by aspirin might be able to metabolize agonists via the alternative 12-LOX pathway. The products of both the COX-1 (thromboxane, TXB_2) and 12-LOX pathways (12-HETE) were assayed. Whilst there was no change in the generation of TXB_2 after heparinisation in either the UFH or LMWH groups, there was a significant increase in the generation of 12-HETE following heparinisation with both UFH and LMWH, which in plasma was not significantly different between the two types. However, the generation of 12-HETE from platelets stimulated *in vitro* with ADP was significantly greater in the UFH group, with a significant correlation between aggregation to ADP and 12-HETE generation.

These findings appeared to be echoed clinically; there was a significant difference in post-operative embolization rates between those patients who received UFH and those who received LMWH. Patients who received the standard anticoagulation were twice as likely to be "high embolizers" than those who received LMWH. Whilst there were no correlations observed between the platelet aggregatory response and the magnitude of post-operative embolization, a relationship was observed between the rate of post-operative embolization and the *in vitro* generation of 12-HETE from platelets that had been aggregated in response to ADP.

Heparinisation clearly induced an increase in the platelet aggregatory response to both AA and ADP, which may have been due to preferential metabolism of the agonists through the 12-LOX pathway. But why should heparin interact with platelets in this way? Supplementary laboratory studies showed that whilst heparinisation caused a significant increase in plasma lipase activity, this was equivalent between the two heparin types, and there was no correlation between plasma lipase activity and 12-LOX function. Heparin antibodies, whilst detected in the serum of some of the study participants, weren't prevalent enough to explain the phenomenon either.

The degree of heparin-induced anticoagulation was measured *en masse* retrospectively by determining anti-factor Xa activity in stored plasma samples. The anti-factor Xa activity 3 minutes after heparinisation was significantly greater in the plasma of those patients who had received standard UFH than those who had received LMWH, which persisted at 120 minutes. Furthermore, there was an inverse relationship between the patients' weight and the anti-factor Xa activity. There was a strong association between

the platelet aggregatory response to ADP immediately following heparinisation and the anti-factor Xa activity – the greater the degree of anticoagulation, the greater the platelet aggregatory response to ADP.

This body of work lends weight to the emerging hypothesis that the behaviour of patients' platelets is integral to their "thromboembolic potential", and the platelet response to the physiological agonist ADP appears to be of particular importance. The findings from this series of studies would strongly support the exploration of alternative regimes of peri-operative anticoagulation, which could ultimately result in the abandonment of heparin in this role.

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IX

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