# Embolisation and Stroke Risk in Carotid Artery Disease: The role of Carotid Plaque Volume and Resistance to Platelet Inhibitory Therapy

**A thesis submitted in fulfilment of the requirements for the degree of Master of Philosophy in the faculty of Medical and Human Sciences**

**2015**

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# I. Abstract


1 INTRODUCTION - The indication of surgery in carotid artery disease is based on symptom status and the severity of carotid artery stenosis as measured by duplex ultrasound. The severity of stenosis itself is a poor predictor of stroke risk. An accurate predictor of stroke risk in carotid artery disease (CAD) must be identified to allow optimum clinical management.

2 AIMS - This study aimed to investigate risk factors proposed for stroke in CAD. We aimed to determine whether cerebral emboli are associated with the degree of carotid stenosis, carotid plaque volume (CPV) or resistance to platelet inhibitory therapy and to identify any circulating biomarkers that predict stroke risk in order to contribute to the development an accurate risk prediction model for CAD patients.

3 METHODS – Patients scheduled to undergo carotid endarterectomy (CEA) were recruited from the vascular department of The University Hospital of South Manchester (UHSM). Multiplate aggregometry of fresh venous blood was undertaken in 90 patients to detect platelet response to Aspirin or Clopidogrel. Transcranial Doppler ultrasound (TCD) of the middle cerebral artery was used to investigate the presence of microemboli. The severity of carotid artery stenosis was measured using duplex ultrasound preoperatively. The carotid plaque volume (CPV) of the endarterectomy specimen was measured using a modified Archimedes technique. Metabolomic analysis of plasma samples was used to identify any biomarkers present at levels significantly different between asymptomatic and symptomatic groups.

4 RESULTS – Mean residual platelet aggregation despite antiplatelet therapy was 28.5 ± 2.8 AU in asymptomatic patients compared with 55.5 ± 3 AU in symptomatic patients (p=0.01). Resistant patients had more frequent MES at a mean rate of 6.6 ± 2.8 /hour compared with 1.8 ± 1.7 /hour in non-resistant patients (p=0.003). There was a significant association between the frequency of MES and residual platelet aggregation (p=0.002). No correlation between severity of stenosis and MES could be identified (p=0.7) but CPV significantly related to MES (p=0.03). No significant plasma biomarker was identified but metabolomic investigation of plasma proved to be a successful method of analysis.

5 CONCLUSIONS - It is clear that carotid stenosis alone is a poor predictor of stroke risk in CAD. Antiplatelet resistance and CPV were associated with symptom status and cerebral emboli in CAD and may therefore predict stroke risk. Further research is needed to identify significant plasma biomarkers.

# II. Figures

# III. Abbreviations

| | |
|---|---|
| CAD | Carotid artery disease |
| CEA | Carotid endarterectomy |
| UK | United Kingdom |
| AF | Amaurosis fugax |
| CVA | Cerebrovascular accident |
| TIA | Transient ischaemic attack |
| TCD | Transcranial doppler ultrasound |
| MCA | Middle cerebral artery |
| MES | Microembolic signal |
| ADP | Adenosine diphosphate |
| AA | Arachadonic acid |
| CPV | Carotid plaque volume |
| AU | Aggregation units |
| SPSS | Statistical package for the social sciences |
| SOP | Standard operating protocol |
| CADET | Centre for advanced discovery and experimental therapeutics |

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## VI. Acknowledgements

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# 1. INTRODUCTION

## 1.1 Introduction to stroke

Cerebrovascular accident (CVA) or "stroke" currently follows myocardial infarction and all cancers as the third leading cause of death in the western world (1). In addition to significant mortality rates CVA is also associated with serious and permanent disability in those whom it affects, significantly impacting on the patients quality of life and leading to lifelong challenges for both the patient themselves and their family members (2). With an average of 150,000 strokes occurring each year in the UK alone, equating to roughly one person being effected every five minutes, insights into stroke prevention clearly represent a key research focus to improve the health of the nation (3).

Strokes are categorised by causative mechanism and are therefore classed as being either ischaemic or haemorrhagic in nature (4) *(Fig 1).*

Haemorrhagic stroke occurs following the rupture of a cerebral blood vessel, commonly following trauma or rupture of a cerebral aneurism, and currently account for around 13% of all strokes (5). Haemorrhagic stroke is further sub categorised according to the anatomical location of the vessel *(fig 2)* (5).

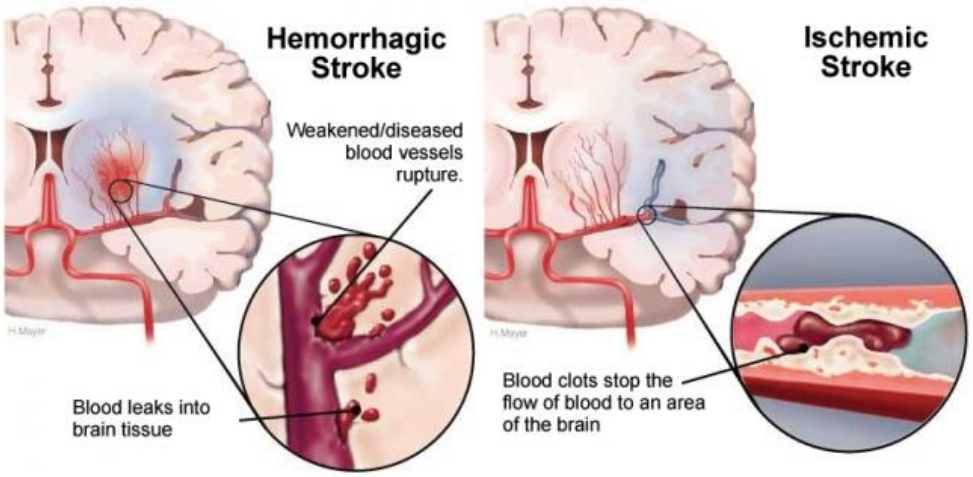


*Fig 1: Image to show the differing aetiologies of haemorrhagic (left) and ischaemic (right) stroke. Ischaemic stroke occurs when blood flow to the brain is interrupted via thrombus formation or embolus. Haemorrhagic stroke occurs when a cerebral blood vessel ruptures interrupting blood supply to the distal area. (4)*

Subdural haemorrhage and subarachnoid haemorrhage both occur when the rupture occurs outside of the brain tissue itself on the surface of the brain and leads to blood accumulating within the cranial cavity, which subsequently leads to increased intracranial pressure and neural tissue damage (6). Intracerebral haemorrhage occurs when the ruptured vessel is located within the brain tissue itself, allowing leaking blood to subsequently infiltrate the brain tissue leading to neural damage. (6).

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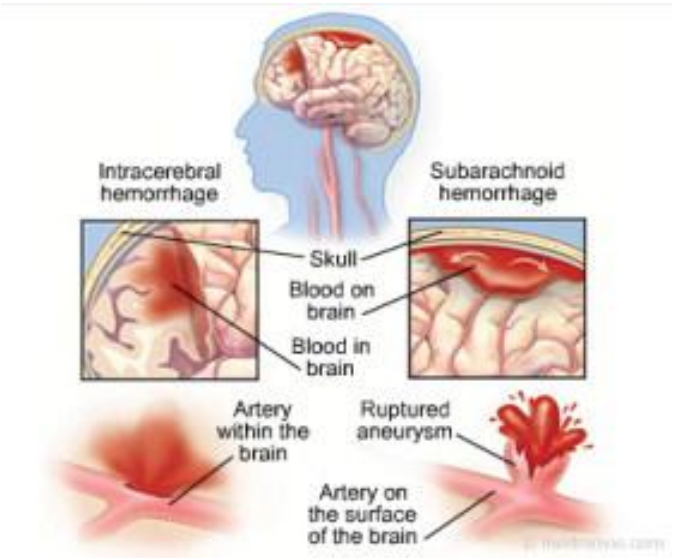



*Fig 2: Image to show the differing aetiologies of subarachnoid and intracerebral haemorrhage. Intracerebral haemorrhage occurs when the ruptured blood vessel is located within the brain tissue whereas subarachnoid haemorrhage occurs when the ruptured vessel is located on the surface of the brain below the arachnoid layer (4).*

Ischaemic stroke occurs following the blockage of a cerebral blood vessel and is the most common form of stroke, with a prevalence of 87% (6). Ischaemic stroke most commonly occurs due to cerebral thromboembolism, the source of this embolic material is widely varied with atrial fibrillation, paroxysmal embolism, coagulopathies and carotid artery disease all known to be causative agents *(fig 3)* (6). Ischaemic stroke can also follow thrombus formation within a cerebral vessel, known as cerebral thrombosis, although this is known to be a less common causative mechanism (6).

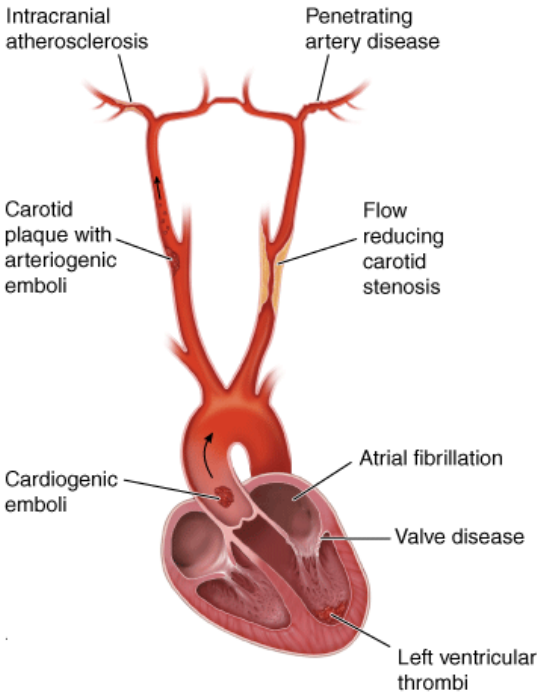



*Fig 3: Image to show the varied sources of thrombus which can embolise the brain and cause ischaemic stroke. Atrial fibrillation, paroxysmal embolism, coagulopathies and carotid artery disease all known to be causative agents (6).*

## 1.2 Carotid artery disease as a cause of stroke

### *1.2.1 Pathophysiology of Ischemic stroke in carotid artery disease*

Ischaemic stroke is currently thought to directly contribute to around 11% of all deaths in the western world, in the United Kingdom (UK) alone this therefore equates to 53,000 deaths per year which can be attributed to Ischaemic stroke.(7). Ischaemic stroke is also a leading cause of premature mortality and the single largest cause of long-term disability seen in our population, affecting around 400,000 stroke survivors in the UK each year (8). Carotid artery disease (CAD) is

directly responsible for around 30% of all ischemic strokes and is therefore the cause of up to 45,000 ischaemic strokes per year in the UK (9).

CAD is characterised by the formation of atherosclerotic plaques within the internal carotid arteries (10). Risk factors for the development of CAD include, increasing age, male sex, obesity, tobacco smoking, diabetes, alcohol consumption and hypertension (10). Atherosclerotic plaque development is a well characterised process which passes through a series of defined steps (11) *(Fig 4).*

Firstly, endothelial damage within a blood vessel in combination with pathologically high circulating cholesterol levels or abnormal cholesterol handling mechanisms leads to the deposition of cholesterol within the vascular intima (11). The endothelial damage also activates an inflammatory process which leads to the migration and invasion of macrophages into the vascular intima. These macrophages subsequently ingest cholesterol and become known as “foam cells” (11). The subsequent accumulation of foam cells leads to the growth of a “fatty streak” within the vessel wall which can continue to increase in size and encroach into the vessel lumen (12). As the developing plaque progress and further invades the vessel lumen its presence can alter laminar flow leading to the activation of clotting factors, thus causing thrombus formation and the risk of subsequent thromboembolism (12). Plaque architecture can be highly unstable and as such prone to rupture, this can lead to thrombosis, throboembolism and occlusion of the vessel (11,12).

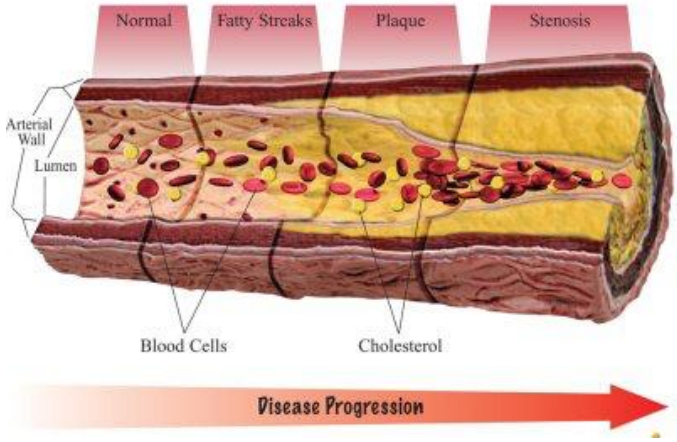



*Fig 4: Graphic image depicting the progressive development of an atherosclerotic plaque as seen in CAD. Damage to the endothelium leads to the deposition of cholesterol within the intima causing fatty streak development. Macrophages then ingest the cholesterol and develop into foam cells. The plaque progress and encroaches the vessel lumen, its presence can lead to the activation of clotting factors, leading to thrombus formation (11).*

The mechanism of ischaemic strokes in CAD is therefore due to embolism of plaque or thrombus material (12). This occurs when the thrombus which has formed around atherosclerotic plaque breaks free from the vessel wall or plaque products are released into the circulation following plaque rupture. (13). Due to the anatomical location of the carotid arteries and their role as the primary supplier of oxygenated blood to the brain, once released into the systemic circulation the thrombus or plaque fragments are taken on a pathway through the

cerebral vessels (13). If these thrombus fragments are large enough they may then become lodged in small cerebral vessels, interrupting blood flow to the brain and causing infarction and ischaemic stroke (14) *(Fig 5).*

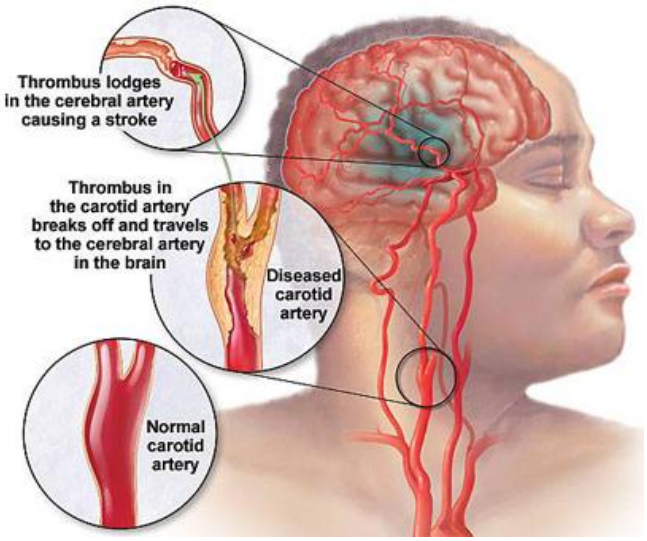



*Fig 5. Image showing how thromboembolism in carotid artery disease can lead to ischaemic stroke. Thrombus formed around an atherosclerotic plaque breaks free or plaque products are released into the circulation following plaque rupture and are taken on a direct pathway to the cerebral circulation. (14).*

### *1.2.2 Thrombus formation and embolisation*

Thrombus formation in CAD occurs due to the disruption of normal physiological conditions caused by the presence of an atherosclerotic

plaque within a vessel wall (14). The set of factors which predispose the development of thrombus were described by the pathologist Rudolf Virchow and are known as “Virchow’s triad” (15). Virchow’s triad is comprised of three pathological mechanisms; endothelial damage, altered blood flow and hypercoagulability. The presence of each of these factors increase the likelihood of thrombus formation and therefore, the acknowledgement of the presence of one or all of these factors in a patient increases their risk of thrombus formation (15).

In carotid artery disease there is an increased potential for thrombus formation as all three factors in Virchow’s triad are commonly present due to the presence of the carotid plaque (16). To elaborate, endothelial damage is both a precursor to the initial development of a carotid plaque and is further propagated by the growth of the lesion within the vessel intima and is therefore already present (17). Altered laminar flow of blood will be experienced within the carotid artery due to the encroachment of the carotid plaque into the vessel lumen, leading to an altered and turbulent flow which increases the likelihood of the interaction of pro thrombotic factors. We therefore, by definition of CAD, will already have two of the factors of the triad present in CAD patients (18). The final factor, hypercoagulability, can result from several risk factors including alcohol intake, obesity and smoking, all of which also predispose to CAD and which are therefore seen commonly in patients with CAD (19). Therefore, it can be seen that a CAD patient is likely to be at increased risk of thrombus formation (19).

On a cellular level, thrombus formation in carotid artery disease occurs on the luminal surface of the atherosclerotic lesion (20) The initial step in thrombus development is via the activation of platelets, usually through release of tissue factors from the damaged endothelium surrounding the atherosclerotic plaque (19,20). Platelets activate and adhere to the plaque surface and are known as “white thrombus” (21). The activated platelets attached to the plaque surface causes activation of the intrinsic clotting pathway via the generation of thrombin; this in turn leads to the accumulation of red blood cells forming a “red thrombus” (22). Subsequent growth of the thrombus then follows a distinct pattern which results in alternating bands of platelets with fibrin and bands of red blood cells which can be visualised histologically and are known as “Lines of Zahn” (22) *(Fig 6).*

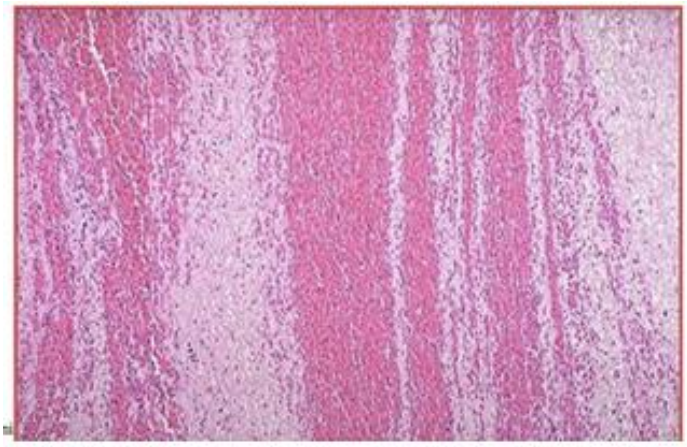


*Fig 6. Image to show lines of Zahn which develop as a thrombus forms in vivo. Pale pink bands are platelets and thrombin; red bands are red blood cells (22).*

Once formed, this thrombus is now precariously attached to the damaged endothelium surrounding the carotid plaque and is vulnerable to dislodging due to continued pressure from the passing flow of blood or following the development of plaque instability and rupture (22).

### 1.3 Clinical management of carotid artery disease

The clinical management of CAD patients is currently based on two parameters, the presence of cerebrovascular symptoms and the degree of stenosis within the internal carotid artery as measured by ultrasound (23,24).

Patients are firstly categorised as either symptomatic or symptomatic based on their recent clinical history (24). Patients who have suffered from a cerebrovascular event such as CVA or transient ischaemic attack (TIA) within 12 weeks prior to assessment are classed as symptomatic (25). Patients who have no history of cerebrovascular symptoms, who have suffered from amaurosis fugax (AF) alone of who have experienced a TIA or CVA more than 12 weeks prior to assessment are classed as asymptomatic (26). Symptomatic patients with a stenosis greater than 50% and asymptomatic patients with a stenosis greater than 70% are classed as candidates for surgical intervention via CEA (24,26).

All patients are also managed with lifestyle interventions and pharmacological regimes aimed to reduce disease progression and subsequent risk of stroke. Those patients not suitable for surgical

interventions are classed as medical patients and are solely treated via these lifestyle and pharmacological interventions (27).

### *1.3.1 Carotid endarterectomy*

The surgical management of CAD is based on the severity of carotid stenosis, which has been the main indication for treating carotid disease over the last four decades (24,28). Patients with a carotid stenosis >50% and recent symptoms of cerebral ischemia, such as AF, TIA or stroke, or those with a stenosis >70% are deemed to have a risk of stroke high enough to warrant surgical treatment and are therefore considered as candidates for surgical intervention via carotid endarterectomy (CEA) (24).

Around 10% of the general population have severe carotid stenosis requiring surgical intervention via CEA in which carotid surgery reduces the risk of ipsilateral stroke six-fold (26). During CEA surgeons remove atherosclerotic plaque from within the carotid artery to reduce stroke risk by removing the primary source of thromboembolism to the cerebral circulation *(fig 7)* (24).

The CEA procedure itself, like any surgical intervention, is associated with some risk and carries a 1-3% risk of CVA in asymptomatic patients and a 3-6% risk of CVA in symptomatic patients (24,29). There is also a 1% risk of death associated with CEA including the risks associated with undergoing a general anaesthetic (29).

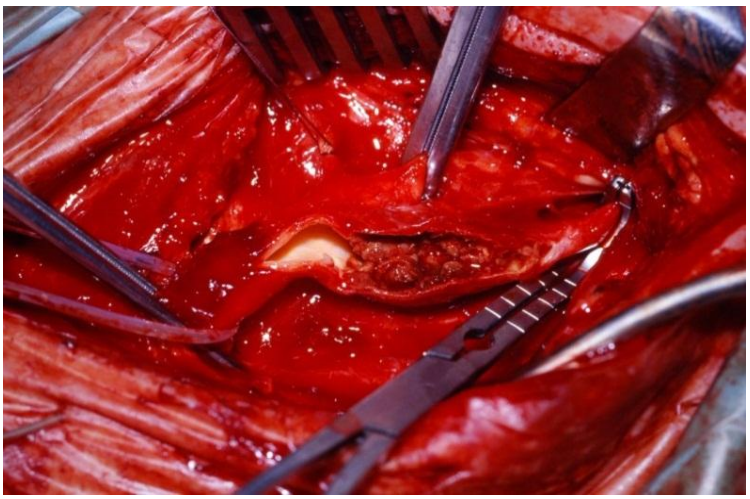


*Fig 7: A carotid artery opened during CEA to show a large disintegrating atherosclerotic plaque in patient only hours following an acute stroke (24).*

### *1.3.2 Medical Management*

All CAD patients, including those not considered suitable for surgical intervention via CEA (ie Symptomatic patients with a stenosis of < 50% and asymptomatic patients with a stenosis of <70%) and those who are not well enough to undergo CEA, as well as patients who are considered suitable for CEA are treated by medical therapies including lifestyle interventions and pharmacological therapies. (24).

#### *i) Lifestyle interventions*

The initial medical management of CAD involves lifestyle interventions aimed to slow the progression of the atherosclerotic disease by reducing exposure to known risk factors of plaque development and progression

(25,30). Key lifestyle related risk factors for the development of atherosclerosis include obesity, poor nutrition, sedentary lifestyle, tobacco smoking, excess alcohol intake and stress (30). Therefore lifestyle interventions aimed at CAD patients include reducing obesity and maintaining a healthy weight, cessation of smoking, reduction of alcohol intake, increased intake of fruit and vegetables in the diet and partaking in exercise (25). By adhering to diet and lifestyle advice CAD patients may also importantly experience a reduction in levels of hypertension and high blood cholesterol which are both key mediators of the progression and development of atherosclerotic disease (11,12). Therefore, the importance of lifestyle and diet intervention in CAD should not be underestimated and is a key strategy in the medical management of the disease, especially during early stages of progression. (12,27).

*ii) Cholesterol lowering and Antihypertensive Therapies*

In those patients in which lifestyle interventions are unsuccessful or whose disease is severely progressive, pharmacological interventions can be used in order to reduce exposure to risk factors which can impact the progression of the disease (31). The two commonly prescribed agents are drugs aimed to lower cholesterol levels and drugs aimed to reduce hypertension (31).

Statins are commonly used as Cholesterol lowering agents in CAD patients (31,32). The mechanism of action for Statin drugs is via

inhibition of the HMG Coa reductase enzyme; this enzyme converts ACoA, a product of lipid metabolism, to cholesterol in the liver (32). Therefore, by inhibition of HMG CoA reductase the circulating levels of cholesterol can be greatly reduced, as cholesterol plays such a key role in the development and growth of atherosclerotic plaques it is clear that reduction of serum cholesterol to normal levels would have a protective role in the further progression of the disease and the potential development of subsequent atherosclerotic lesions (11,12,30) In addition to the action of statins on cholesterol reduction there are wider pleiotropic effects of statin drugs which are also known to have a protective effect via the reduction of cardiovascular events. Importantly statin drugs have been shown to inhibit Matrix Metalloproteinases which are known to play a key role in the development of atherosclerosis by degrading the extracellular matrix causing vascular wall weakness and damage (31,32) .

There are several classes of anti hypertensive drugs which can be used in order to lower pathologically high blood pressure in CAD patients; these include Angiotensin converting enzyme inhibitors, thiazide diuretics, calcium channel antagonists and sympatholytic drugs (32). The particular category of anti hypertensive agent prescribed varies depending on the individual needs of the specific patient; the core mechanism behind the intervention is reduction of hypertension which is key risk factor in the development and progression of atherosclerosis seen in CAD due to its association with endothelial damage (11,12,32).

*iii) Platelet Inhibitory Therapy*

Antiplatelet therapy is a crucial therapeutic strategy employed to reduce stroke risk in CAD (33). This is exemplified by the fact that all CAD patients, regardless of symptom status, degree of stenosis, or their allocation as a surgical or medical candidate, are treated with antiplatelet drugs throughout the duration of their clinical management (31,33). These agents suppress the activation of platelets in order to reduce thrombus formation around the lesions and can therefore lower the risk of ischaemic stroke in CAD (12,33) (53). Additionally, following surgical intervention via CEA, CAD patients are treated via platelet inhibition in order to reduce their risk of post operative thrombus formation and lower the long term risk of further recurrent atherosclerotic development (32,33). Platelet inhibition can therefore be seen as the cornerstone of CAD management, a stance which is warranted by the ability of platelet inhibitory therapy to reduce annual stroke risk by up to 9% and its use has also been shown to prevent a fifth of recurrent strokes (30).

Platelet inhibitory therapy in CAD typically occurs via the administration of Aspirin or Clopidogrel (34). As these agents suppress platelet activation by two distinct pharmacological mechanisms, and as such are associated with differing adverse effects, patients are allocated to treatment via either Aspirin or Clopidogrel based on their individual medical history (24,34). Currently, the most widely prescribed antiplatelet agent for the prevention of secondary cardiovascular events is aspirin, but due to its suppression of prostaglandins its use is

associated with the development of gastrointestinal ulcers and as such it is rendered unsafe for use in patients with a history or gastric problems. (26,34). Therefore, currently the majority of CAD patients receive platelet inhibition via Aspirin and patients in whom Aspirin use is contraindicated are treated via Clopidogrel (34). From a financial perspective the use of Aspirin as the primary agent, unless contraindicated, is also warranted due to the average cost of a prescribed dose of Aspirin being significantly lower than that of Clopidogrel (35).

a) Physiological Action of Aspirin

Aspirin, pharmacologically known as 2-acetoxybenzoic acid, is an antiplatelet agent which is subcategorised into a group of drugs known as "Thromboxane inhibitors" (36). Physiologically, the stimulation and release of the chemical mediator Thromboxane A2 within the platelet leads to conformational changes associated with platelet activation and subsequent platelet adherence and aggregation. The pharmacological action of Aspirin is to manipulate this pathway by inhibiting the action of Cyclooxygenase-1, which is a key mediator in the enzymatic pathway which results in stimulation of Thromboxane A2 (36).

Aspirin acts to irreversibly inhibit the synthesis of Thromboxane A2 within the platelet and as such, inhibits platelet activation and adherence *(fig 8)* (30,36). As aspirin inhibits the Thromboxane A2 pathway irreversibly, the platelet is not able to restore the missing enzymatic activity required for its activation and platelet inhibition via Aspirin is

therefore maintained for the full life span of the circulating platelet, which is around 7-10 days (36).

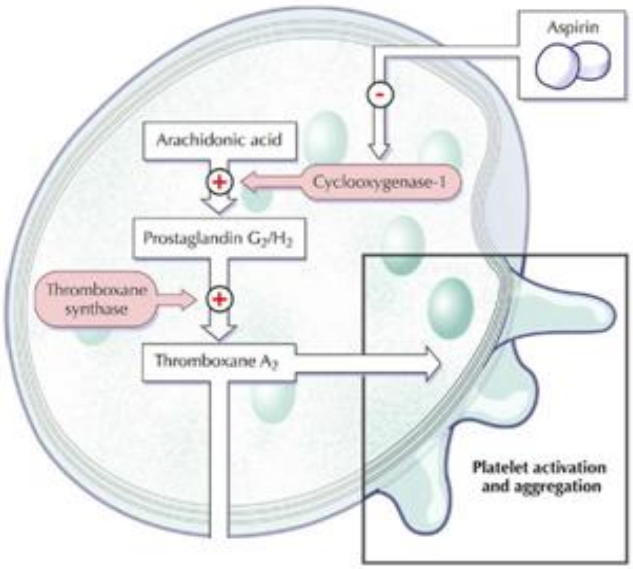



*Figure 8: Aspirin inhibits platelet activation via inhibition of Cyclooxygenase -1 resulting in platelet inactivation and subsequent inhibition of platelet aggregation (36).*

b) Physiological Action of Clopidogrel

Clopidogrel is an antiplatelet agent which is grouped in the subcategory of drugs known as Adenosine Diphosphate (ADP) receptor antagonists or Thienopyridines (36,37). Physiologically the stimulation of ADP receptors, which are located on the cytosolic surface of platelets, occurs through the specific binding of ADP following endothelial damage (12,37). This binding causes an intracellular cascade which results in the downstream activation of platelet P2Y12 receptors via the release of

calcium (37). P2Y12 receptor activation leads to the activation and subsequent aggregation of platelets around the area of endothelial injury (37,12). (61). The pharmacological action of Clopidogrel is to act as a specific antagonist of the ADP receptor, inhibiting the activation of P2Y12 receptors and subsequently impeding the activation and aggregation of platelets *(fig 9)* (32,37).

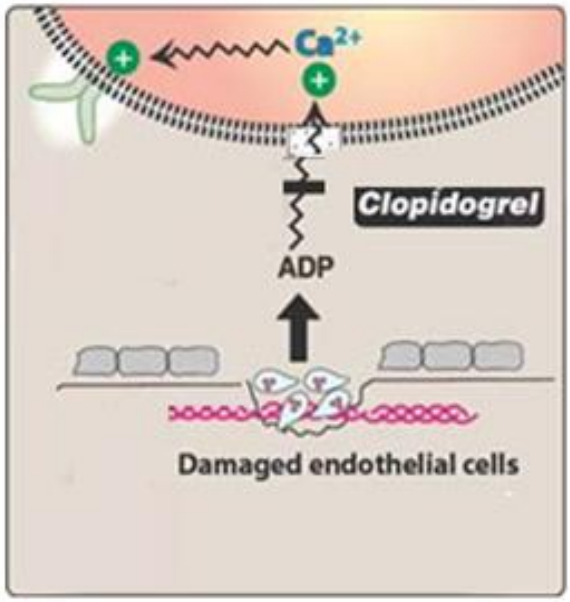



*Figure 9. Clopidogrel suppresses platelet activation via inhibition of ADP which is required to activate P2Y12 receptors within the platelet. Suppression of this platelet activation pathway results in subsequent inhibition of platelet aggregation (32).*

Once platelets are rendered inactive through the administration of an antiplatelet agent, this therefore results in the reduction of thrombus formation associated with platelet activation (33). As plaque development and subsequent thrombus formation is responsible for the

risk of ischaemic stroke seen in CAD, platelet inhibition through Aspirin or Clopidogrel can therefore reduce the risk of stroke and other cerebrovascular events associated with the disease (15,34). Platelet inhibition is therefore a highly significant therapy employed in the management of CAD (35).

## 1.4 Determination of stroke risk in carotid artery disease

CAD patients are classed as surgical candidates if their risk of stroke when left untreated is larger than that associated with CEA itself (2-3%) (24,29). Symptomatic patients with significant of stenosis (50-99%) are classed as highest clinical priority with an annual stroke risk of around 16.8% (24). Asymptomatic patients with a high degree of stenosis (70-99%) have an annual stroke risk of around 2% (24). Therefore symptomatic and asymptomatic patients with a high degree of stenosis are considered as candidates for CEA whereas asymptomatic and symptomatic patients with a stenosis of less than 60%, deemed to have an annual stroke risk of only 1.6%, are not deemed as candidates for surgical intervention (24,29).

### *1.4.1 Severity of stenosis*

Although carotid stenosis and symptom status are the primary factors used to determine stroke risk and clinical priority in CAD, stenosis is in fact a poor predictor of stroke risk (24,31). Specifically, in asymptomatic patients with >70% carotid stenosis, the risk of ipsilateral stroke is under

2% a year (31). Therefore in these patients the benefit of intervention with carotid endarterectomy is minimal, in simple terms around 19 CEA's would need to be undertaken to prevent just one stroke in a CAD patients over a five year follow up period (32). This clearly indicates that carotid stenosis itself is a poor indictor of stroke risk.

### *1.4.2 Carotid plaque volume*

Carotid plaque volume (CPV) is the volume of atherosclerotic plaque within the carotid artery. CPV may impact on stroke risk in CAD as an increased atherosclerotic plaque burden may disrupt carotid blood flow, leading to thrombus formation and therefore subsequently increasing risk of thromboembolisation and ischaemic stroke (38).

A recent pilot study by our research group recruiting over 200 patients demonstrated that CPV in patients undergoing CEA within four weeks of symptoms was almost double of that seen in asymptomatic patients, with mean CPV in the symptomatic group being 1.1cm3 compared with 0.68cm3 in the asymptomatic group ($p<0.001$) (39) *(fig 10).*
Interestingly, carotid plaque volume was also shown to fall rapidly following symptoms of cerebral ischemia from 1.1cm3 within four weeks of cerebral symptoms to 0.91cm3 at six weeks and a mean of only 0.62 cm3 more than eight weeks following symptoms (39). This data demonstrated that there may be a crucial link between CPV and cerebrovascular symptoms in CAD and therefore between CPV and stroke risk in the disease (39).

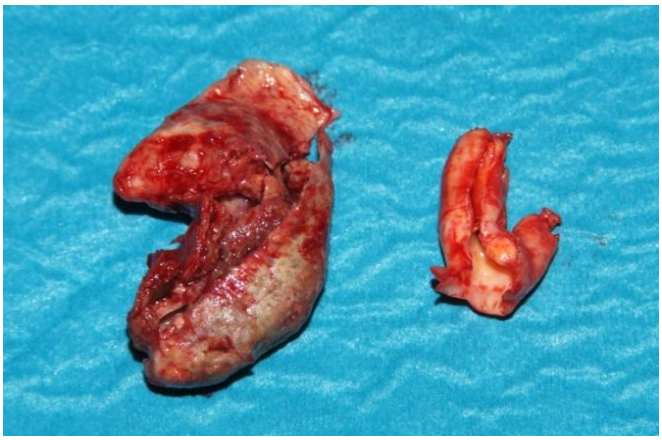


*Fig 10: Photograph of post CEA carotid plaque specimens. The large plaque on the left is from a symptomatic patient; the small plaque on the right is from an asymptomatic patient (39).*

### *1.4.3 Microembolic signals*

Microembolic signals (MES) are asymptomatic events occurring when small emboli pass into the brain (40). Although these fragments are not big enough to cause any cerebrovascular symptoms they can be detected via Transcranial Doppler ultrasound (TCD) inosonation of the middle cerebral artery (40). It is thought that there may be a link between MES and stroke risk in CAD as these silent embolic events may be precursors to the larger embolisations associated with CVA (37,40). MES are seen in 40% of recently symptomatic CAD patients with research suggesting that the presence of MES within 48 hours post stroke is associated with an increased likelihood of the recurrence of cerebral ischemic events (38). The Asymptomatic Carotid Emboli Study demonstrated that the presence of MES independently predicts 2-year stroke risk (39).

Additionally, the presence of MES is associated with an echogenic and hypoechogenic plaque morphology on ultrasound which we believe are associated with increased risk of plaque rupture and therefore increased stoke risk (40). Due to the association between stroke risk and the presence of MES it is argued that TCD scanning in order to identify MES should be incorporated into stroke risk prediction in CAD patients but currently, preoperative TCD monitoring is not a routine part of clinical management (41).

### *1.4.4 Antiplatelet resistance*

All CAD patients are treated with antiplatelet agents, commonly aspirin or clopidogrel, to reduce the incidence of thrombus formation around carotid plaques (29). This intervention is thought to reduce stroke risk by around 9% annually and is therefore a key therapy in the management of CAD (30). Research has suggested that up to 37% of the population may be resistant to the actions of antiplatelet agents; if this is true in CAD patients this would represent a severe shortfall in current treatment and the prevalence and impact of this resistance may therefore seriously affect the risk of stroke in CAD patients (42).

A recent pilot study involving 35 patients demonstrated a significant prevalence of antiplatelet resistance in CAD patients and showed an association between cerebrovascular symptoms and residual platelet aggregation (43). Interestingly a trend was also identified between residual platelet aggregation and the number of MES entering the middle

cerebral artery as measured via TCD, further strengthening they hypothesis that antiplatelet resistance may be associated with stroke risk in CAD (43). Data from this project demonstrates that antiplatelet resistance may be prevalent in the CAD patient population and that the impact of this resistance, although potentially significant, is as yet not fully understood (43).

### *1.4.5 Circulating Biomarkers*

Whether circulating biomarkers could predict increased risk in cardiovascular disease is not a novel idea but has yet to be thoroughly investigated in CAD (44,45). There are some studies which have explored the relationship between circulating biomarkers of inflammation and the risk of atherosclerosis, stroke or carotid stenosis, but no study as yet has specifically investigated circulating biomarkers in relation to stroke risk in CAD (46,47,48).

C reactive protein (CRP) was shown to be associated with increased cardiovascular risk in an observational study of 5888 patients (46). Patients with both carotid stenosis and significantly elevated CRP levels showed a 72% increase in risk of cardiovascular death compared to those with normal CRP levels indicating that CRP and risk and cardiovascular death may be linked (46). P selectin levels, determined through flow cytometry assessments of circulating platelets, were also correlated with carotid intimal media thickness (47).

Although these studies do indicate that plasma biomarkers may be involved in the development and progression of CAD, no study as yet is to specifically search for a biomarker which can be used to distinguish stroke risk in carotid patients following the development of an atherosclerotic plaque more accurately than carotid stenosis alone. There is therefore a clear need for investigation of an easily measured plasma marker which may be associated with stroke risk in the disease.

### 1.4 Research Question

Although the determination of stroke risk in CAD and subsequent clinical management of CAD patients is determined via the degree of carotid stenosis, carotid stenosis is in fact a poor predictor of stroke risk (31,48). It has been shown that in asymptomatic patients with >70% carotid stenosis, the risk of ipsilateral stroke is under 2% a year, indicating the carotid stenosis itself is a poor indictor of stroke risk (31). To allow the best clinical management of CAD patient's factors which more accurately predict stroke risk in CAD must be identified.

Although some studies have investigated parameters potentially relevant to stroke risk separately, no study has yet fully assessed the potential relationship between these factors or reached definitive conclusions on the importance of each factor in stroke risk determination. Additionally, no research has yet been undertaken to evaluate whether circulating biomarkers in the blood may be able help to predict stroke risk.

Therefore, an accurate risk prediction model to determine which patients are at substantially increased risk of stroke is yet to be developed.

Until further research has been undertaken to definitively determine the importance of these proposed risk factors the most accurate model of stroke risk determination in CAD will remain unresolved.

### 1.5 Hypothesis

This research was to explore whether the presence of MES correlated more closely with CPV and antiplatelet resistance than with the severity of carotid stenosis, indicating that these factors may be important in predicting stroke risk in CAD. It was expected that CPV and antiplatelet resistance may be associated with MES in recently symptomatic patients. We wondered whether a significant proportion of CAD patients would show “antiplatelet resistance” and if resistant patients would also have an increase in the factors known to be associated with stroke risk. The investigation of plasma biomarkers in CAD patients may lead to the identification of biological signals which help to determine stroke risk in CAD.

### 1.6 Aims

1. To investigate the relationship between cerebral emboli and resistance to platelet inhibitory therapy in CAD patients

2. To investigate the relationship between cerebral emboli and CPV in CAD patients.
3. To determine whether cerebral emboli are associated with the degree of carotid stenosis.
4. Identification of circulating biomarkers that are associated with cerebral emboli, symptom status or CPV.
5. To contribute to the development a risk prediction model based entirely on minimally-invasive data.

### 1.7 Impact of research

Ischaemic stroke is a leading cause of premature mortality and the single largest cause of long-term disability affecting nearly 400,000 stroke survivors in the UK each year (7). The frequency and long term clinical implications of stroke therefore have a massive impact on both patients and the UK economy (7). The direct costs of stroke to our NHS are £2.4 billion/year with the informal costs of caring for stroke victims adding a further £2.4 billion (44). Including the costs associated with lost employment and productivity, the total cost to the UK economy is thought to be over £7 billion/year (45). Approximately 30% of all ischaemic strokes are thought to be due to carotid artery disease, raising the possibility that identifying and treating carotid artery disease before stroke occurs may avoid up to 45,000 strokes/year in the UK alone (8,9).

# 2. MATERIALS AND METHODS

## 2.1 Patient recruitment

90 CAD patients from the vascular department of the University Hospital of South Manchester (UHSM) who were listed for carotid endarterectomy were recruited for this study, with both asymptomatic (65 patients) and symptomatic (25 patients) patients being included. Patients were classed as symptomatic if they had suffered from cerebrovascular symptoms, such as TIA or CVA, within 12 weeks prior to assessment. Those who had either no history of cerebrovascular symptoms or who had suffered from TIA or CVA more than 12 weeks prior to assessment were classed as asymptomatic.

Patients that were excluded from this study were those who had an occluded carotid artery, a mechanical heart valve, were under the age of 40 or who had a disability which may have caused discomfort during the TCD monitoring. Additionally, patients with underlying heart disease at high risk of emboli, i.e. those with atrial fibrillation, prosthetic valves, recent myocardial infarction or dilated cardiomyopathy, were excluded due to the uncertainty of the origin of the emboli (41,43). All patients were required to give informed written consent before recruitment to the study and had their full clinical history taken using a purposely designed data entry form (appendix 1).

## 2.2 Measurement of carotid stenosis

The severity of carotid stenosis was measured in 90 patients using a Siemens Acuson P300 Doppler ultrasound machine one day prior to surgery. Measurements were recorded by a trained ultrasound technician who was blinded to specific details of patient identification to ensure fairness throughout the trial. Patients were scanned using the NASCET criteria lying supine or in a sitting position to allow easy access to the neck which was extended and the head rotated to the opposite direction of the side being assessed (46). The carotid arteries were viewed from a lateral or antero-lateral approach using the sternocleidomastoid muscle as an acoustic window (46). Measurements taken were; velocities in metres per second, diameter (transverse anterior-posterior, medial-lateral) in centimetres and the length of disease in centimetres (46). The stenosis measurements were recorded on the data collection sheet and subsequently entered into a spread sheet prior to analysis.

## 2.3 Measurement of carotid plaque volume

Plaque specimens from 90 patients were removed by the vascular surgeons during CEA (24). Surgeons aimed to remove plaque samples "en bloc" to allow an accurate volume measurement of single specimen.

Following removal the plaque was washed in sterile water to remove any adherent blood. The volume of the specimen was then measured in all 90 removed plaques using a novel hydrostatic suspension technique identified and developed by the author

This technique involves determination of the volume of an object through measurements taken whilst the object is immersed in fluid of a known density (47). The volume of the object was determined by measuring increase in the weight of a set amount of fluid with a known density when the object in question was submerged and then dividing the increase in weight by the density of the fluid (48). All weights were recorded using an Adam equipment fine balance scale, accurate to six decimal places.

The standard equation used was "V = ^w/D" where D is the measured density of the fluid, ^w is the change in weight recorded when the object is suspended and *V* is the unknown volume (48) *(Fig 11).* This technique is a more accurate method of assessing the volume of small irregularly shaped objects than a classical Archimedes water displacement method as the quantities of displaced water are too small to be accurately measured on a repeatable basis (48).

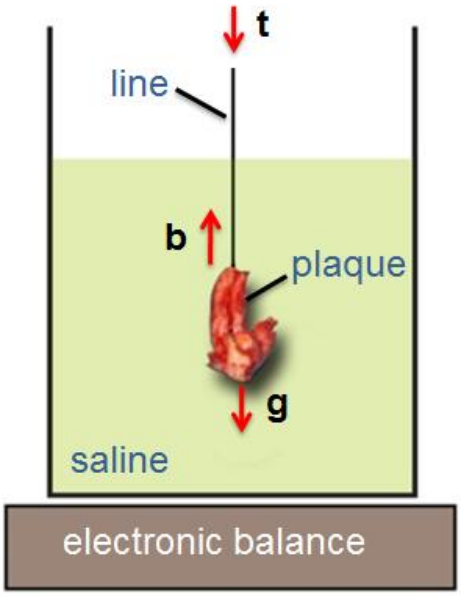



*Figure 11: Diagram to show the principles behind the hydrostatic suspension technique for measuring plaque volume. "Since the immersed object is stationary; the downward gravitational force (g) is balanced by the upward buoyancy (b) and line tension (t). The immersed object is equivalent to a 'virtual' volume of water of exactly the same size and shape." (48).*

### 2.4. Measurement of residual platelet aggregation

Residual platelet aggregation was measured in 90 CAD patients. Following consent the patient's full medical history, including details of the duration and dose of current antiplatelet therapy, was recorded in a data collection sheet. Medication charts were checked on the day of testing to confirm that patients had been compliant with their antiplatelet medication, compliance was also verbally confirmed directly with the patient. Patients underwent venepuncture prior to surgery with blood

samples being drawn between one and six hours post administration of medication to ensure adequate time for physiological activation of the antiplatelet agent (44). Two millilitres of peripheral blood was drawn and stored in a 2 millilitre double wall Hirudin blood tube (Roche Multiplate). Samples were then transported carefully by hand with excess shaking of the storage tube being avoided to ensure minimal platelet activation during transportation (45). Samples were stored in the designated area of the Multiplate machine until analysis at precisely 90 minutes post venipuncture, this was to ensure both uniformity and a high level of accuracy as some of the reagents used to activate platelet aggregation are time dependant (46).

Blood samples were analysed using a Multiplate model 5.0 impedance aggregometer, the Multiplate manufacturer guidelines were strictly adhered to at all times to ensure accuracy (49). One Millilitre of blood was pipetted into each Multiplate test cell, a separate platelet agonist was then added to each individual cell with care being taken to avoid cross contamination of agonists by using a new pipette tip for each reagent. All reagents used were supplied by Roche Multiplate for use with the Multiplate protocol. Arachadonic Acid (Roche AA test) was added to test cell one which stimulated platelets via the aspirin inhibited pathway, ADP (Roche AA test) was added to test cell two which stimulated platelets via the Clopidogrel inhibited pathway and thrombin receptor activating peptide (Roche TRAP test) was added to test cell three which acted as positive control for platelet activation by

stimulating aggregation through a common pathway not suppressed by antiplatelet drugs, therefore ensuring platelets are responsive and capable of aggregation (46).

Samples were then incubated for seven minutes and the resulting aggregation data was presented as an "aggregation curve" which plots aggregation, recorded in Aggregation units (Au) against time (46) *(Fig 12).* Upon analysis, patients were classed as resistant if their aggregation curve was above 40 AU for Aspirin and 47 AU for Clopidogrel patients as per Multiplate recommendations (45,49). (Appendix 2)

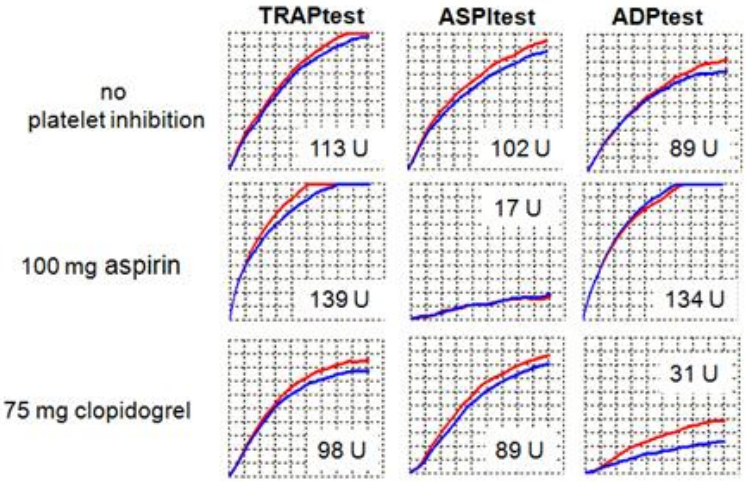



*Fig 12: Example of a Multiplate aggregation curve used to assess platelet function. The top line represents typical normal values without platelet inhibitory therapy classified by the steep curves representative of high platelet activation. These curves are flattened in patients where antiplatelet medication has inhibited platelet aggregation to the relevant aggregant, as seen in the middle and bottom lines (49).*

## 2.5 Plasma Metabolomics

### *2.5.1 Sample collection for plasma metabolomics*

20 CAD patients underwent venipuncture one day prior to surgery to ensure uniformity in results. Samples were collected using the centre for advanced discovery and (CADET) standard operating protocol in combination with the carotid plaque volume study protocol (Appendix III). 5mls of peripheral blood was drawn into a lithium heparin plasma collection tube (Greiner cat no 455084) which was then gently inverted three times to thoroughly mix the anticoagulant. The plasma fraction was prepared immediately by centrifugation at 3000 x g for 20 minutes at $4^{o}$c. Samples were then divided into 0.5 ml aliquots in cryovials (Greiner cat no 122261) and stored in a -80 freezer until analysis.

### *2.5.2 Plasma analysis*

The plasma metabolome was interrogated using state-of-the-art mass spectrometric techniques to identify molecular markers which reflect patient outcome. Plasma was compared from patients from the varying patient population, i.e. asymptomatic and symptomatic patients, patients with MES and without MES and antiplatelet resistant and antiplatelet responding patients.

Thawed plasma samples were mixed using a vortex mixer for 10 seconds before 200 µL of each sample was transferred to its new eppendorf tube

(labelled with the sample's identification number). 100µl of internal standard and 600 µL of methanol were added to the eppendorf and the sample was mixed for a further 10 seconds to enable protein precipitation. The use of isotope-labelled internal standards in GC enables the relative quantification of metabolites present within the samples (50). Deproteinisation aids to preserve the chromatography column/mass spectrometer as it eliminates involatile material from the samples (50). If not removed, this material would cause a fast impairment in the performance of these instruments and considerably decrease reproducibility (50). In order to pellet the precipitated protein, samples were then centrifuged at 13000rpm for 15 minutes.

Following centrifugation, 200 µl of the supernatant was transferred to the two equivalently labelled eppendorf tubes. With two copies of each original sample, both liquid chromatography (LC)- and gas chromatography (GC)-mass spectrometry (MS) analysis could be performed. This was important, as using both techniques enables a greater breadth of metabolite coverage (51). All supernatant samples were placed within an Eppendorf Concentrator and dried under vacuum at 35˚C for approximately 18 hours. This transformed the liquid samples into a dried, concentrated sample residue in preparation for analysis. Samples were then randomised using Microsoft Excel in order to prevent any potential bias being introduced as a result of sample analysis order.

For the purpose of quality assurance, quality control (QC) samples were used. Twenty replicates of a pooled QC sample were produced by combining any unused plasma from the participant samples and

preparing them in the same manner as for the study samples. This provided sufficient 'bracketing' QC samples, to be analysed at regular intervals during the analytical run. However, for the lead-in samples which are required to equilibrate/condition the analytical instruments prior to sample analysis to ensure reproducibility, commercial human plasma (adult male) was used. These samples were prepared using the same methodology as for the previous samples

*2.5.3 Polar metabolite analysis - Gas chromatography-mass spectrometry (GC-MS)*

Metabolites in the polar fraction were derivatised to methoxime/trimethylsilyl derivatives. 60 µL of 20 mg/mL methoxylamine hydrochloride (Acros Organics) in dry pyridine (Acros Organics) was added to each sample and samples heated to 80°C for 20 min. Samples were allowed to cool, 60 µL of N-methyltrimethylsilyltrifluoroacetamide (MSTFA) was added and sample heated for a further 20 minutes at 80°C. The samples were allowed to cool and 20 µL of retention index markers (selected n-alkanes covering the range C12 to C32) in pyridine were added. Samples were centrifuged at 16,000 x g for 5 min, and 95 µL transferred to autosampler vials.

GC-MS analysis was carried out using a Gerstel MPS2 autosampler, an Agilent 7890A Gas Chromatograph with Split/Splitless inlet, and a LECO Pegasus HT time-of-flight mass spectrometer. Gas chromatography was conducted using an Agilent/J&W DB-17MS

column (30 m x 0.25 mm x 0.25 µm) with a 3m deactivated Fused Silica retention gap (0.25mm), and helium carrier gas at 1.4 mL/min in constant flow mode. 1 µL sample injections were made in Pulse Splitless mode at an inlet temperature of 270°C. Initial column temperature was 50°C, held for 6 min then ramped to 300°C at 10°C/min and held for a further 4 min. This resulted in a total cycle time of 42 min between injections. After an initial 450s solvent delay to allow solvent and reagents to elute without damaging the detector, nominal resolution mass spectral data was acquired at 10 spectra per second, for the *m/z* range 45-800 Da. Standard 70 eV electron energy was employed, at a source temperature of 220° C.

For polar metabolites, data were prepared by peak deconvolution of representative samples with nominated metabolites listed (ChromaTOF 4.5; LECO Instruments). Putative identities were provided by matching mass spectra and retention time to data available in the NIST mass spectral library, the Golm metabolite library and an in-house library developed at the University of Manchester (52). A reference table consisting of mass spectra and their expected retention times was compiled and was applied as a target list of features. The initial list of nominations was also edited to remove ambiguous and low quality spectra prior to use.

In order to use the edited reference table as a reporting tool, appropriate parameters were specified: mass spectral match threshold (600) and retention time tolerance (± 3 sec), and the table was initialised using a

pooled QC sample to provide reference *m/z* peak areas. By determining which internal standard yielded the lowest variance for a given metabolite across all the QC injections, the most suitable internal standard was assigned to each metabolite. The resulting data for each sample was compiled into of a matrix of metabolite intensity data for the experiment, which was merged with experimental metadata for visualisation and statistical analysis.

*2.5.4 Non-polar metabolite analysis - Ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS)*

Metabolites in the non-polar fraction were resuspended in 120 µL MeOH and centrifuged for 15 min at 16,000g before being transferred to autosampler vials. Samples were analysed on an Accela UHPLC system coupled to an Orbitrap Velos mass spectrometer equipped with a heated electrospray ionisation source (HESI) (ThermoFisher Scientific). Samples were analysed separately in positive- and negative-ion modes. Chromatographic separations were performed on a Hypersil GOLD column (100 x 2.1 mm, 1.9 µm; ThermoFisher Scientific) operating at a column temperature of 50°C. Two solvents were applied (solvent A - 0.1% formic acid in $H_2O$ (v/v) and solvent B - 0.1% formic acid in MeOH (v/v)) at a flow rate of 400 µL/min. Solvent A was held at 100% for 0.5 min followed by an increase to 100% solvent B over 4.5 min, which was then held at 100% solvent B for a further 15.5 min. A step change to 100% solvent A was performed at 20.5 min and then held at 100% solvent A to equilibrate for 1.5 min. All column eluent was

transferred to the mass spectrometer and full-scan profiling data were acquired in the Orbitrap mass analyser (mass resolution 30,000 at *m/z* = 400). The source and ion transfer parameters applied were as follows; source heater 200°C, sheath gas 50 (arbitrary units), aux gas 15 (arbitrary units), capillary temperature 300°C, ISpray voltage 4.5kV (positive-ion mode) and 3kV (negative-ion mode), slens 60% (positive-ion mode) 65% (negative-ion mode) and AGC 5 x $10^5$.

XCMS was employed to convert each LC-MS dataset into a matrix of detected peaks versus sample identification with peak response for detected metabolites reported, where a peak response is defined as the sum of intensities over a window of specified mass and time ranges. Default settings were employed in XCMS with the exception of S/N threshold (3), step (0.02), *m/z* diff (0.05) and for grouping bandwidth (10), mzwidth (0.05) and minfrac (0.15). Putative metabolite identification was performed applying the PUTMEDID-LCMS workflows as previously described with an RT window of 3 seconds and a *m/z* error of 5 ppm (51).

Both GC-MS and UHPLC-MS data sets were processed similarly. A principal components analysis (PCA) visualisation was used to confirm overall integrity of the experiment and univariate statistical tests (mean ratio with confidence intervals, Mann-Whitney p-value, FDR-corrected q-value) applied to compare metabolite intensities between classes.

### 2.6 Identification of cerebral emboli by Transcranial Doppler

20 CAD Patients underwent one hour TCD monitoring in the week prior to endarterectomy using an Acuson Multiprobe JH-6007 TCD machine. TCD readings were recorded bilaterally using the transtemporal statoacoustic windows (both ipsilateral and contralateral to the diseased carotid artery). The bilateral recording allowed more accurate differentiation to be made between artefacts and MES; any signal appearing simultaneously on both readings is highly unlikely to be a genuine MES and could therefore be considered an artefact (52).

At the beginning of each TCD reading the patient was asked to undertake movements which may create artefacts on the recording (such as taking a deep breath, coughing, talking) and the technician tapped and moved the probe wire to identify any further source of potential artefacts. If any signal with all the features of a supposed MES appeared when created by an artefact the MES was discounted as it was considered to unsure in origin (52). During the recording the patient was required to remain still and silent where possible to reduce the level of artefacts recorded. The researcher was present during the full recording to identify any issues and to record the timings of any artefacts and suspected MES. The presence of an MES was be defined by the criteria outlined by the Consensus Committee of the Ninth International Cerebral Hemodynamic Symposium (53). An MES was therefore confirmed only when "the signal duration is of <300 ms at a high intensity, during random periods in the cardiac cycle, present on one side only of the baseline and accompanied by a typical chirping sound" (53) *(Fig 13).*

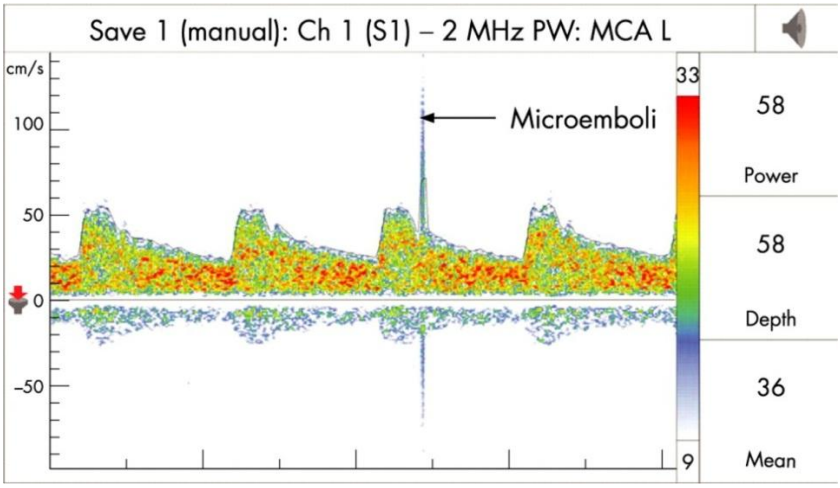



*Fig 13: Screenshot of a TCD recording showing the presence of an MES, denoted by the arrow, as recorded during TCD analysis (47).*

Following recording the TCD reading was reviewed by the researcher to validate the number of MES for each patient. The TCD reading was also reviewed by a separate researcher to validate the presence of an MES and to reduce any source of potential error.

### 2.6 Statistical Analysis

Results were collated in a SPSS version 20 spreadsheet to allow the selection of appropriate statistical analysis. Data was categorised as either parametric/normally distributed or non parametric/not normally distributed via analysis of a histogram created with SPSS. Following categorisation an appropriate statistical test was identified; statistical calculations and graph development was undertaken using SPSS version 20 and Microsoft Office Excel 2012.

# 3. RESULTS

## 3.1 Patient populations

90 patients were enrolled into this study between March 2013 and April 2014. All patients had CPV and residual platelet aggregation recorded and 20 also had TCD measurements and additional blood taken for metabolomic analysis. 63 patients were taking aspirin and 27 patients were taking Clopidogrel, with the majority of patients taking a daily dose of 75mg or 150mg. The mean age of patients was 68 years. The majority of patients (65/ 72%) were symptomatic with a high degree of carotid stenosis (60-99) with the remainder (25/ 28%) being asymptomatic with a high degree of stenosis. All patients were in patients of the vascular ward at UHSM and had been recorded as compliant with therapy on the day of testing. Full demographic details are shown in table one overleaf.

| Variable | Resistant<br>(n=37) | Responder<br>(n=53) |
|---|---|---|
| Mean age | 67.9 | 69.3 |
| sex | 19 male (51%)<br>18 female (49%) | 41 male (77%)<br>12 female (23%) |
| Antiplatelet | 24 Aspirin (65%)<br>13 Clopidogrel (35%) | 39 Aspirin (74%)<br>14 Clopidogrel (26%) |
| Anticoagulant | 3 (8%) | 3 (6%) |
| Ethnicity | 37 Caucasian | 53 Caucasian |
| Diabetes | 6 (16%) | 9 (17%) |
| Hypercholesterolemia | 18 (49%) | 37 (70%) |
| Hypertension | 19 (51%) | 32 (60%) |
| Smoking<br>Current<br>Ex<br>Never | <br>15 (40%)<br>19 (50%)<br>4 (10%) | <br>20 (38%)<br>26 (50%)<br>7 (12% ) |

*Table 1: Demographic details of 90 recruited patients*

## 3.2 Antiplatelet resistance

### *3.2.1 Relation to patient characteristics and symptom status*

Antiplatelet resistance was measured in 90 CAD patients. 65 patients were symptomatic and 25 were symptomatic. 63 patients were taking aspirin and 27 were taking Clopidogrel. Mean (± SD) platelet aggregation was 28.5 ± 2.8 AU in asymptomatic patients as compared to 55.5 ± 3 AU in symptomatic patients (Mann-Whitney U test U=411.5, Z=-2.98, p=0.003).

Mean aggregation in symptomatic patients exceeded the resistance thresholds for aspirin (>40 AU) and clopidogrel (>47 AU) *(Fig 14).* A significant association was found between symptomatic status and antiplatelet resistance (Chi Squared test X2 5.23/ p=0.003)

Resistance was seen more frequently in patients taking Clopidogrel with 48 % of Clopidogrel users showing resistance compared to 30% of aspirin users *(Fig 15).*

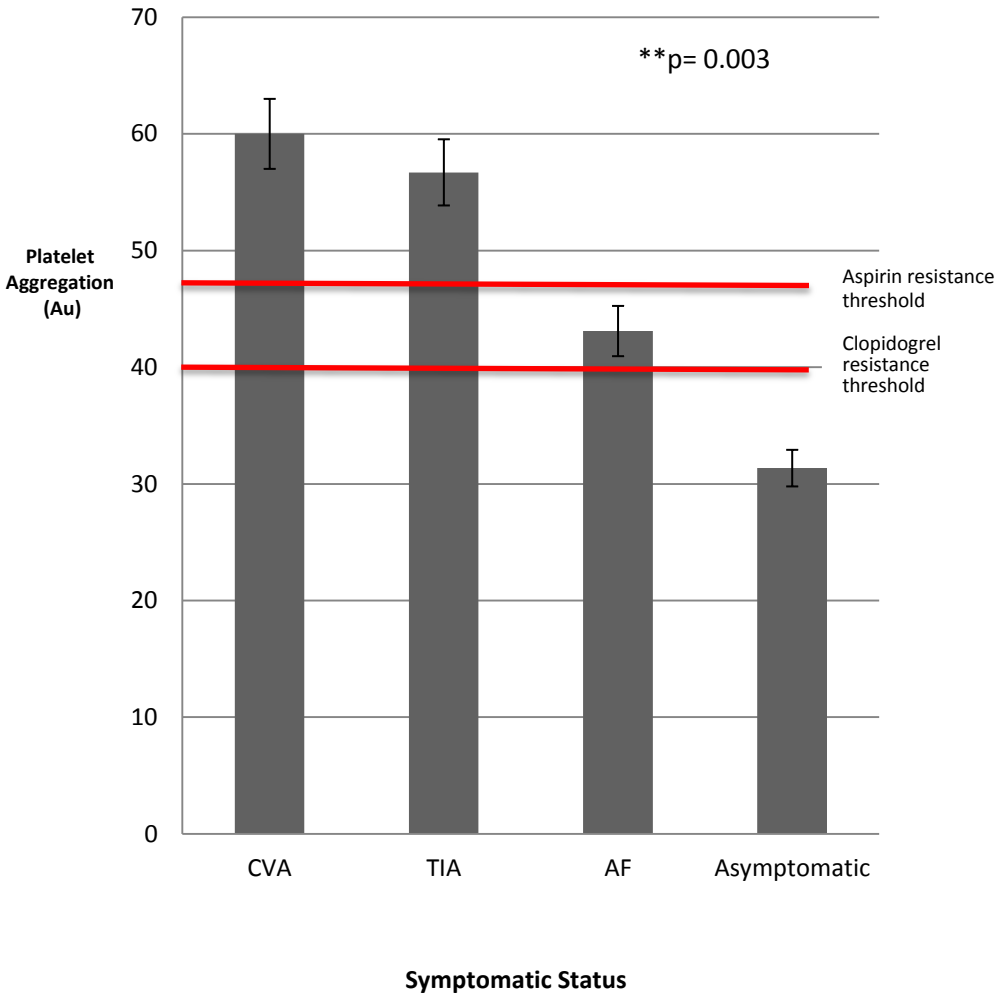
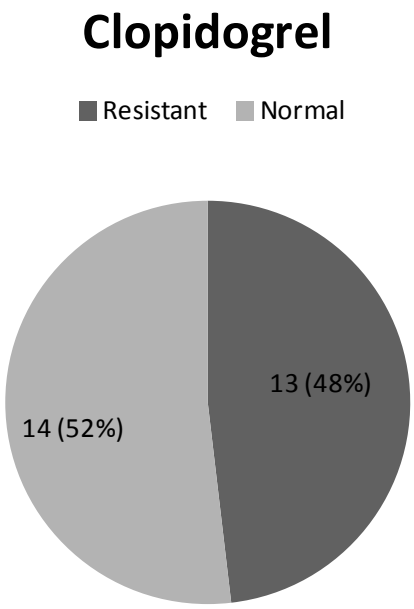



*Fig 14: Graph to show the association between cerebrovascular events and antiplatelet resistance (n=90). A significant association was seen between antiplatelet resistance and symptomatic status with average residual platelet aggregation in symptomatic patients (CVA/TIA/AF) being significantly higher than in asymptomatic patients (Chi Squared test X2 5.23/ p=0.003)*



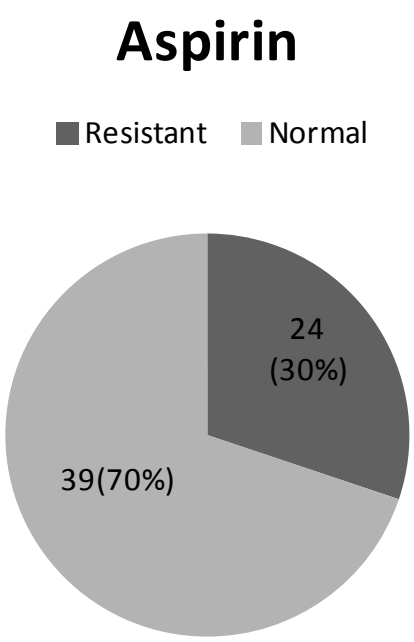




*Fig 15: Charts to show the proportion of resistance seen in patients taking aspirin (n=63) and in those taking Clopidogrel (n=27). Antiplatelet resistance was prevalent in both groups .There is an increase in resistance seen in the patients being treated with Clopidogrel (48%) as compared to those taking aspirin (30%).*

*3.2.2 Relation to Cerebral emboli by TCD*

TCD ultrasound was undertaken in 20 patients, 10 symptomatic and 10 asymptomatic. Resistant patients had more frequent MES at a mean rate (± SD) of 6.6 ± 2.8 /hour compared with 1.8 ± 1.7 /hour in non-resistant patients (p = 0.008 unpaired t test). MES were therefore seen an average of 3.5 times more often per hour in resistant patients. Importantly, an association between the frequency of MES and the level of residual platelet aggregation was demonstrated (Spearman rank correlation $r_S$ (18) = 0.816 p=0.002) *(fig 16).*

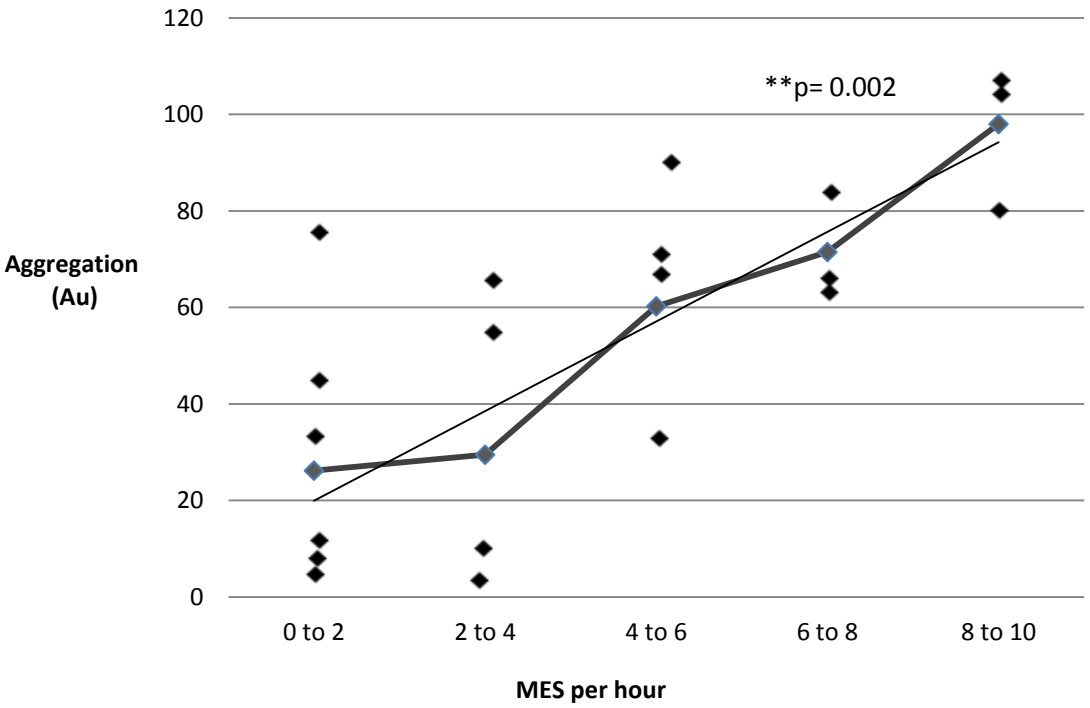



*Fig 16: Graph to show the relationship between MES and residual platelet aggregation (n=20). A significant association was found between increasing levels of residual platelet aggregation and the numbers of MES (Spearman rank correlation $r_S$ (18)= 0.816 p=0.002).*

*3.2.3 Relation to Carotid plaque volume*

CPV was measured in 90 CAD patients, 25 asymptomatic and 65 symptomatic. There was no significant association noted between average carotid plaque volume and antiplatelet resistance (Chi squared test p=0.8) *(Fig 17).*

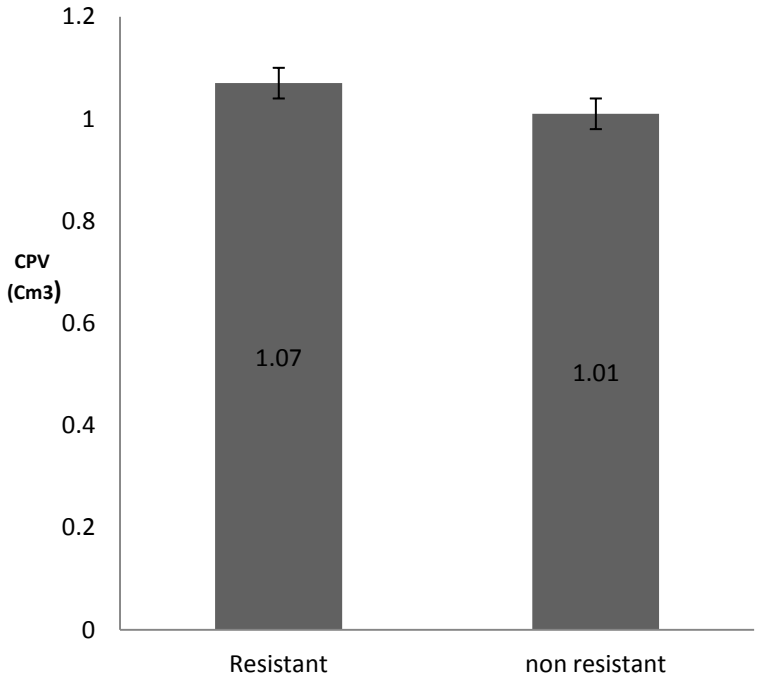



*Fig 17: Graph to show the relationship between carotid plaque volume and resistant status (n=90). No significant association as found between antiplatelet resistance and the volume of plaque measured (Chi squared test p=0.8).*

### 3.3 Carotid plaque volume

*3.3.1 Relation to patient characteristics and symptom status*

CPV was measured in 90 CAD patients, 25 asymptomatic and 65 symptomatic. Mean CPV (± SD) was significantly higher in symptomatic patients at 1.18 ± 0.413 $Cm^3$ than in asymptomatic patients at 0.673 ± 0.528 $Cm^3$ (Chi Squared test X2 5.23 p=0.02) *(fig 18).*

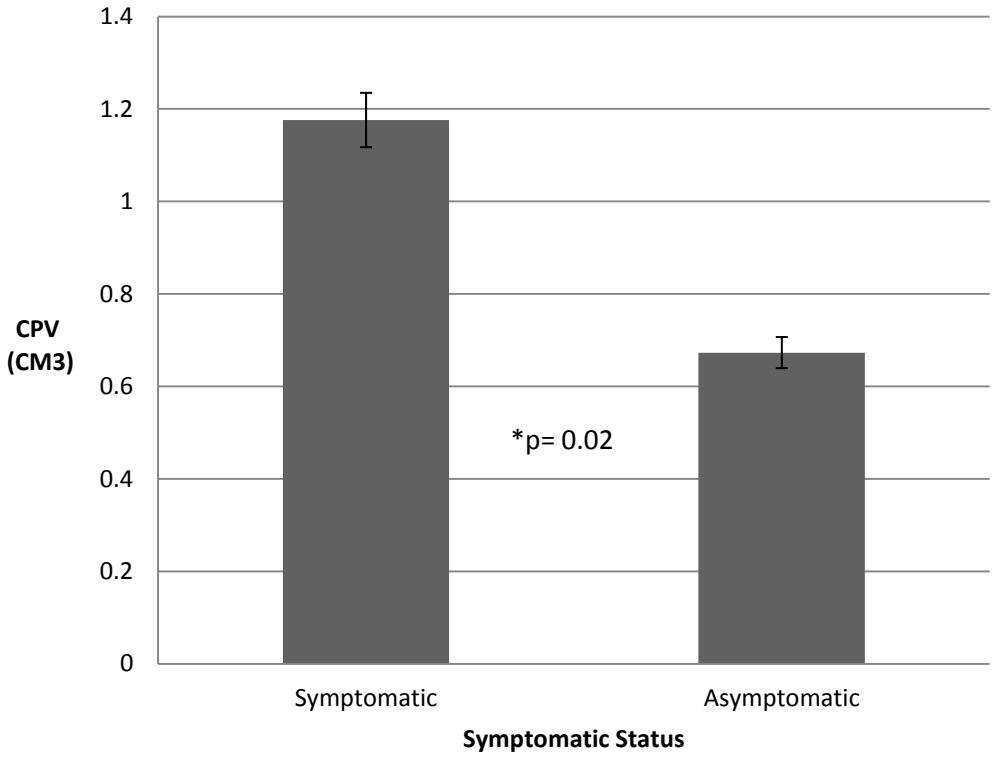



*Fig 18: Mean carotid plaque volume (CPV) was significantly higher in symptomatic patients (n=65) as compared to that seen in asymptomatic patients (n=25) (Chi Squared test X2 5.23 p=0.02).*

*3.3.2 Relation to Cerebral emboli by TCD*

TCD ultrasound was undertaken in 20 patients, 10 symptomatic and 10 asymptomatic. CPV postoperatively and carotid stenosis preoperatively was measured in all 20 patients. No significant association was found between the degree of carotid stenosis and the number of MES seen on TCD (Chi Squared test p=0.7) (fig 19). A significant association was seen between CPV and the number of MES seen on TCD (Chi Squared test p=0.03) *(fig 20).*

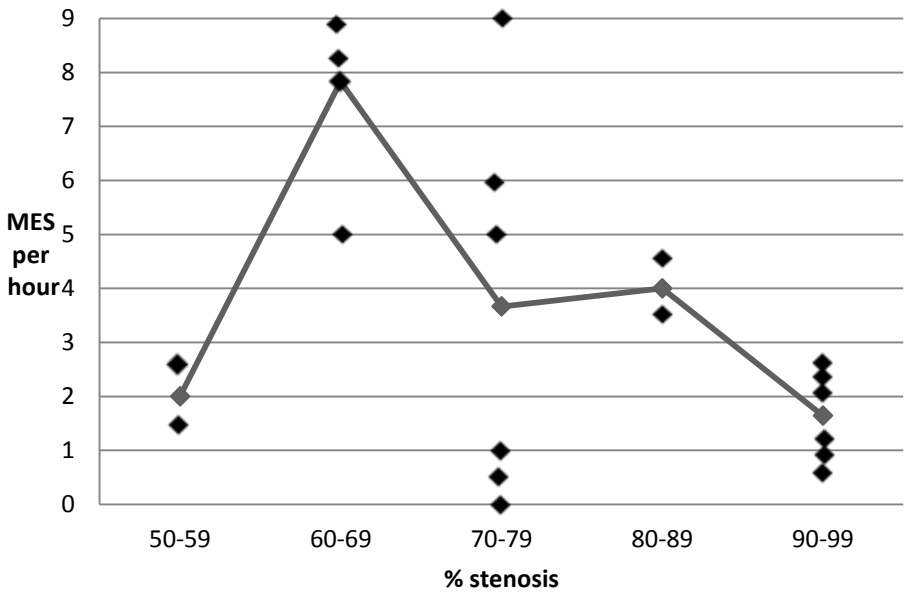



*Fig 19: Graph showing the relationship between MES and percentage of carotid stenosis (n=20). The black diamond's show individual data points and the grey diamonds show the mean value for each data group. No significant association (Chi squared test p=0.7) was seen between increasing levels of carotid stenosis and the number of MES seen.*

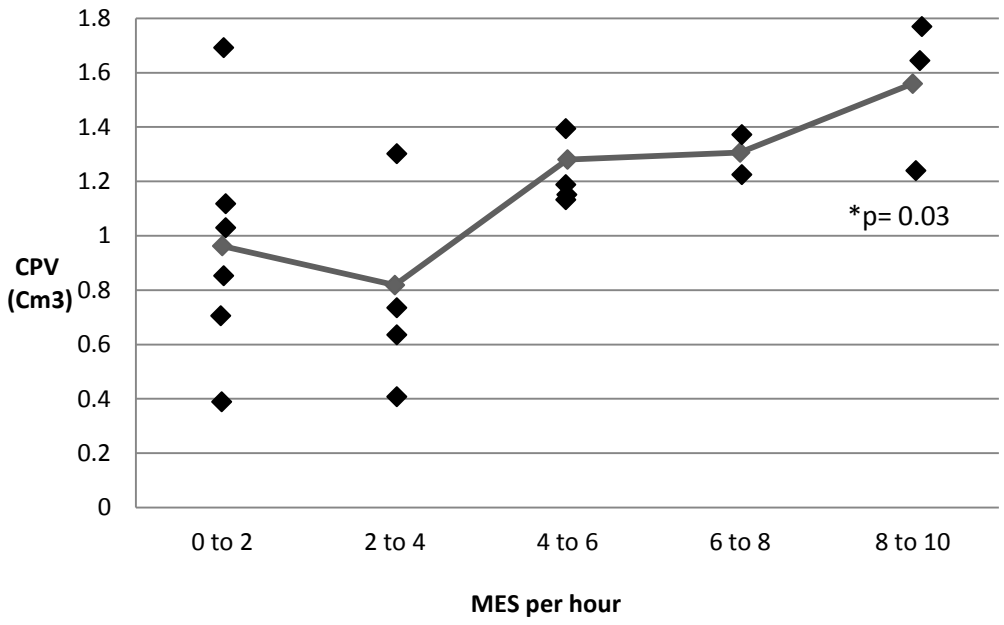



*Fig 20: Graph showing the relationship between CPV and MES (n=20). The black diamond's show individual data points and the grey diamonds show the mean value for each data group. A significant association was seen between increasing numbers of MES and the volume of the carotid plaque (Chi squared test p=0.03).*

### 3.4 Plasma metabolomics

Plasma samples from 20 CAD patients, 10 symptomatic and 10 asymptomatic underwent metabolomic analysis. In addition plasma from 10 healthy controls, free from any history of vascular disease, were also analysed in order to highlight any global differences between CAD patients and the general population. In control vs symptomatic comparison, 620 features were initially detected as significantly changed between the groups ($p < 0.05$) *(fig 21).* This reduced to 141 features after further analysis to remove known common biological variables based on

age, sex and medication history. Despite there being no separation of experimental groups based on PCA analysis, univariate statistical analysis (using Kruskal-Wallace Test) on individual metabolites did show some differences in levels of phospholipids, specifically phosphatidylcholines and phosphatidylserines, which were decreased in symptomatic patients and in some bile acids, which were increased in symptomatic patients.

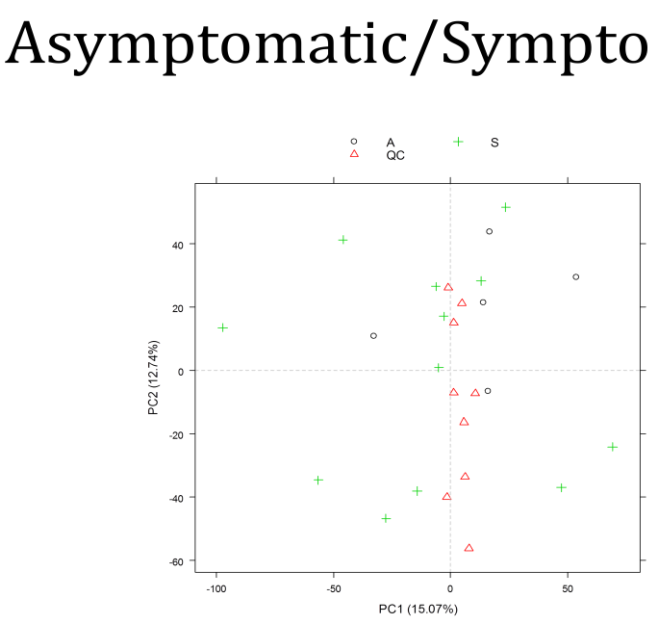



*Fig 21: Example of a PCA plot generated during metabolomic analysis of plasma samples. Despite there being no separation of experimental groups (plus signs symptomatic/ triangles asymptomatic/zeros quality control) based on PCA analysis, univariate statistical analysis (using Kruskal-Wallace Test) on individual metabolites did show some differences in levels of phospholipids, specifically phosphatidylcholines and phosphatidylserine.*

# 4. DISCUSSION

## 4.1 Summary of key findings

Resistance to antiplatelet therapy was frequent amongst CAD patients. Resistant patients had an increase in factors known to be associated with increased stroke risk in CAD, with significantly increased MES and higher frequencies of symptoms for cerebrovascular disease. CPV was associated with both symptom status and cerebral emboli, with the presence of cerebrovascular symptoms (CVA or TIA) and rates of MES significantly higher in patients with large plaque volumes. This strengthens the hypothesis that CPV may a useful factor in the determination of stroke risk in CAD. As resistant patients have increases in the factors currently known and thought to be associated with stroke risk in CAD it is possible that resistant patients have an increased stroke risk when compared to non resistant patients. The association of MES with CPV but critically not with the severity of carotid stenosis indicated that carotid stenosis alone may be poorly related to stroke risk in CAD. Plasma metabolomics may be a useful tool in the identification of a circulating biomarker for stroke risk in CAD, further research needs to be undertaken in order to identify markers seen at significantly different levels between symptomatic and asymptomatic patients.

## 4.2 **Novel data arising from research**

The association between antiplatelet resistance and an increase in the occurrence of both MES and cerebrovascular symptoms strengthens our hypothesis that antiplatelet resistance is associated with an increase in stroke risk as measured against known risk factors for stroke. The increase in stroke risk associated with both MES and cerebrovascular symptoms in CAD has been effectively demonstrated through a multitude of research studies (51, 52, 54, 55, and 56). By demonstrating a significant increase in risk factors known to be associated with stroke risk in resistant CAD patients it may indicate both an association between stroke risk and antiplatelet resistance and highlights the need for antiplatelet resistance testing to be incorporated routinely into the clinical management of CAD to help determine accurate stroke risk in individual CAD patients.

Our data also serves to support and expand upon findings in the surrounding literature which have noted a correlation between antiplatelet resistance and the presence of MES. Dawson et al (57) recruited 62 patients with significant CAD, the majority of which were symptomatic, who were taking aspirin. MES signals were recorded and results were compared between aspirin resistant and non-aspirin resistant patients. Results indicated that MES were seen more frequently in patients who were aspirin resistant, with 50% of resistant patients having MES signals compared to only 17% of non-resistant patients (57). These results support our findings that MES are more likely to occur in aspirin resistant patients and our research has expanded these findings by

additionally incorporating data from Clopidogrel resistant patients, an important factor considering the wide use of Clopidogrel as an antiplatelet agent in CAD.

The lack of association between carotid stenosis and the number of MES is also supported in the surrounding literature. Censori et al studied the correlation between MES and ischemic symptoms in patients with symptomatic carotid artery stenosis, their results indicated that patients with high numbers of MES who subsequently suffered cerebrovascular symptoms had varying degrees of carotid stenosis (from 60-99%) and stenosis did therefore not correlate with stroke risk (58). These results were supported in a study by Sztajzel et al who assessed MES in patients with stenosis of 30-99% and failed to show any association between stenosis and the occurrence of MES (59). A study of 71 patients with carotid stenosis of 30-99% by mayor et al also failed to show any association between stenosis and increased incidence of MES (60). By incorporating measurements of antiplatelet resistance, cerebrovascular symptoms, carotid stenosis and plaque volume together in our patient population our data serves to expand on current research by not only showing the inaccuracy of carotid stenosis in assessing risk in CAD but providing evidence that alternative risk factors may provide a more accurate solution.

The significant increase in carotid plaque volume seen in symptomatic patients is in agreement with our current research which has documented large volumes of plaque in symptomatic patients and small volumes of plaque in asymptomatic patients (47). Therefore, this study has provided

further evidence to support the hypothesis that plaque volume is associated with increased stroke risk in CAD and may be a useful tool for determination of clinical priority in the disease. The association of MES with CPV but critically not with carotid stenosis further indicates that carotid stenosis alone is an inaccurate factor for the determination of stroke risk in CAD.

## 4.3 Strengths and Limitations of Research

Previous studies which have aimed to identify antiplatelet resistance have commonly received criticism relating to ensuring compliance of patients with their antiplatelet medication (37,38,39). Typically, these studies have recruited patients from outpatient clinics or general practice and have therefore relied solely on the patient's own recollection of medication compliance (37,38,39).. It could therefore not be definitively proven that patients had in fact taken their medication within a suitable time frame prior to testing to allow a suitable physiological reaction of the drug to be elicited or in fact if they had actually taken their medication at all. This lead to doubt about the accuracy of the prevalence of antiplatelet resistance and its subsequent importance in clinical practice. A great strength of this study therefore lies in the recruitment of in patients on the vascular ward prior to carotid endarterectomy. Pre surgical inpatients have stringent records taken to ensure compliance with medication which include records of the exact date and time all medications are taken which is supervised, witnessed and signed by a

medical professional. Therefore, the design of this study this meant that it could be definitively and accurately proven that patients had been compliant with their medication prop to resistance testing, eliminating the primary cause for criticism seen in previous studies. This study design therefore translates to an increase in the accuracy of our research data as compared to previous studies.

The measurement of CPV on post operative endarterectomy specimens posed a significant challenge before the identification and implementation of the hydrostatic suspension technique. This technique was identified and subsequently applied and validated for the measurement of endarterectomy specimens by the author herself and has allowed accurate measurement of CPV in post operative specimens. The use of the hydrostatic suspension technique in this research has allowed an accurate measurement of CPV to be made which further strengthens the specificity of the data as a whole (47).

Of the 90 CAD patients tested for aspirin resistance only 20 were able to undergo TCD monitoring to measure MES. This was due both to the exclusion criteria set for the investigation and the lengthy nature of the readings, leading to the identification of suitable patients being restricted and subsequently patients who were classed as suitable for TCD monitoring being reluctant to consent to undergoing the scan. Additionally, it is difficult to obtain TCD readings from those undergoing CEA as an acute emergency as the necessity for their rapid admission to theatre supersedes the TCD requirement of the research.

Although investigation of plasma biomarkers was undertaken in 20 patients only 10 age and sex matched controls were recruited to the study. Originally, it was not planned to include plasma controls in the study and to simply compare markers between symptomatic and asymptomatic groups. During plasma analysis it became apparent that control data, from age and sex matched patients with no history of vascular disease, would in fact be a useful tool for accurate comparison of results. This accounts for the relatively small number of suitable controls which could be recruited due to time constraints following ethics amendments to incorporate the addition of control recruitment. Although some trends were noted in the proteomic and metabolomic data no significant biomarker could be identified. The addition of more controls and the recruitment of additional patients would be beneficial to future research. During data collection several aliquots of plasma were frozen for each patient and only one was subsequently used during analysis, this will allow experiments to be repeated when additional data is collected which will be beneficial to the research.

Additionally, carotid plaque samples removed during endarterectomy were sectioned and frozen alongside plasma samples for future proteomic, metabolomic and histological analysis. It was decided that it would be beneficial to hold analysis of these samples until more data was collected to ensure the most accurate and useful use of all specimens. There is therefore much scope for future research into this are and the continuation of this research project will no doubt be highly beneficial to moving the accuracy of the treatment and management if CAD.

## 4.4 Future Research

In order to solidify this research and further strengthen the association between cerebral emboli and both CVP and antiplatelet resistance it would be beneficial to recruit further patients to this study, and specifically to increase the numbers of patients undergoing measurement of MES via TCD. The recruitment of patients to TCD posed a significant challenge in the study and in order to overcome this in future research it would be beneficial to identify and recruit subsequent patients during outpatient clinics. This would allow more suitable patients to be identified and additionally, by informing patients of the study prior to their admission to hospital this will allow them more time to consider their participation during a less stressful time as the immediate preoperative period, potentially leading to higher recruitment rates.

In order to translate CPV measurements to a useful clinical measure of stroke risk which can be used in order to make decisions relating to clinical priority and the need for surgical or medical intervention in CAD it will be necessary to develop of method of taking these measurements preoperatively. A three dimensional ultrasound measurement of carotid plaque volume in vivo is currently being developed and validated by our research group and poses an exciting avenue for more accurate stroke risk determination in carotid disease.

It is clear from our data that antiplatelet resistance is significantly prevalent in CAD patients and may pose a significant impact on stroke

risk in the disease. In order to continue this research the realities of incorporating this measure into routine clinical practice should now be investigated. This could be through pilot schemes which introduce the antiplatelet testing method in several vascular departments throughout the country; this would provide greater data on the impact of resistance and give an indication of the ease of its incorporation into routine clinical practice.

This research showed that metabolomic analysis of frozen plasma samples is a useful technique for the potential identification of plasma biomarkers of strike risk in CAD. Future research should therefore focus on the recruitment of further patients and controls in order to generate more data to allow a significant biomarker to be identified. During data collection several aliquots of plasma were frozen for each patient and only one was subsequently used during analysis, this will allow samples taken from our initial patients to be re analysed and incorporated into future data sets. Carotid plaque samples removed during endarterectomy were also sectioned and frozen alongside plasma samples for future analysis. These samples represent a further avenue for research and the metabolomic and histological analysis of these specimens would no doubt yield further useful data.

There is clearly great scope for future research and the continuation of this research project will no doubt be highly beneficial to improving the accuracy of stroke risk determination in CAD and the subsequent treatment and management of the disease.

### 4.5 Clinical impact of this research

If an accurate model of stroke risk prediction in CAD can be formulated it will significantly impact the clinical management of CAD patients. Currently, patients may not be receiving the best treatment as stenosis and cerebrovascular symptoms are the only parameters which are taken in to account when calculating risk and subsequent treatment (24, 25). If stroke risk can be more accurately predicted it will allow personalised treatment of CAD patients resulting in safer and more efficient clinical management. Currently, patients who could benefit from CEA may be denied surgical treatment as their level of stenosis is not deemed severe enough to warrant intervention and may experience strokes whilst receiving medical treatment (26). Additionally, patients considered as candidates for surgical intervention may be incurring unnecessary risk by undergoing CEA when in reality it is not in their best interests (26).

## 5. CONCLUSIONS

Antiplatelet resistance is associated with an increase in factors known to be associated with increased stroke risk in CAD, with significantly increased MES and higher frequencies of symptoms for cerebrovascular disease and higher levels of CPV. CPV is associated with symptomatic patients suffering from CVA or TIA and the number of cerebral emboli, indicating that CPV may a useful factor in the determination of stroke

risk in CAD. Investigating patients with CAD for antiplatelet resistance would allow resistant patients to be prescribed an alternative antiplatelet regime and may be a realistic option to improve the current clinical management of CAD. It is clear from this study that measurement of carotid stenosis alone is an inaccurate method of determining stroke risk in CAD. Plasma metabolomics may be a useful tool in the identification of a circulating biomarker for stroke risk in CAD, further research needs to be undertaken in order to identify significant markers.

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## APPENDIX I: DATA COLLECTION SHEET

**Research Number:**

**Data Collection Sheet version 3: Does carotid plaque volume and morphology predict stroke?**

**Patient Demographics: Date:**
Patient Name:…… Patient Hospital ID number:……..…….
NHS number:…….. Hospital:……
Consultant:…….
DOB:…… Age:….. Sex: M/F Weight:……..
Height:……. BMI:…….
Patient address:……..……... GP name and address:…….
…….....
…….....
……..
……..……...
Contact number:……....
Contact number:……..

**Patients Ethnicity (please tick one)**

**A : White**

British

Irish

Any other White background (please write in) **……..**

**B : Mixed**

White and Black Caribbean

White and Black African

White and Asian

Any other mixed background (please write in)**…….**

**C : Asian or Asian British**

Indian

Pakistani

Bangladeshi

Any other Asian background (please write in) **…….**

**D : Black or Black British**

Caribbean

African

Any other Black background (please write in)**……...**

**E : Chinese or other ethnic group**

Chinese

Any other (please write in) **……..**

**Not stated**
Not stated

**Past Medical History (please tick where appropriate):**

1). Do you have Diabetes?
2). Have you ever been told you have Kidney Failure?
3). Have you ever had a Kidney Transplant?
4). Do you have High Blood pressure?
5). Do you have High Cholesterol?
6) Do you suffer from rheumatoid arthritis?
Have you ever been told you have Heart Failure?
7). Have you ever been told you have and irregular heart beat? (Atrial Fibrillation)
If 'Yes' has this been corrected? Yes No
8). Have you ever been told you have Ischaemic heart disease?(angina/chestpain)
9). Have you ever had a heart attack? (Myocardial Infarction)
Date of MI:……..
10). Have you ever had a Previous Stroke/Mini Stroke (TIA)/Amaurosis fugax (ask patient symptoms)
11). Have you previously had any cardiac intervention such as a stent or heart bypass?
If 'Yes': date:……..
Type of Intervention:….....
12). Have you previously had any surgery to your neck or carotid? (stent / surgery)
If 'Yes': date:……..
Type of Intervention:….....

13). Do you have a family history of TIA/ Stroke /Heart disease? (circle appropriately and note AGE)
If yes, please explain……...
14) do you have a first degree relative who has suffered from a MI before the age of 60?
15) do you have a first degree relative who has suffered from a stroke/TIA before the age of 60?

**Smoking history**:

Do you smoke or have you ever smoked?
YES: ……. Cigarettes a day FOR ……years
NO: Never smoked
Ex smoker: Stopped …… years ago

**Alcohol Intake:**

How much alcohol do you drink a week?
Units per week:……..

**Baseline Rankin score:**
How were you before the episode? Were you able to do everything for yourself?

| | |
|---|---|
| 0 | No symptoms at all. |
| 1 | No significant disability despite symptoms; able to carry out all usual duties and activities. |
| 2 | Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance. |
| 3 | Moderate disability; requiring some help, but able to walk without assistance |
| 4 | Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance. |
| 5 | Severe disability; |

| | |
|---|---|
| | bedridden, incontinent and requiring constant nursing care and attention. |
| 6 | Dead. |

**Pre-operative Morbidity:**

1. Referring speciality:…….

2. Referring hospital:……

3. Date of Referral:……..

4. Date of vascular review……..

5. Date of Operation:……..

6. Was it more than **14 days** from onset of symptoms to surgery: YES NO

If yes, what is the reason for delay?:…….....

**Symptoms Status**:

How did you feel before you episode? Did you have any symptoms? (Symptomatic if symptoms described <12 weeks)
Asymptomatic

**IF SYMPTOMATIC:**

Date of most recent symptoms:……..

Can you describe your symptoms to me? Did these symptoms resol hours? If not, when?

TIA
(If symptoms resolved in 24 hours)

Stroke
(If symptoms resolved after 24 hours or have yet to resolve)

Amaurosis Fugax
( Loss of vision in one eye, 'like curtains closing')

Cranial nerve

description……
…….....
Other
symptoms:……
…….

CT scan to confirm stroke: YES NO

Date of CT Scan:…….

If 'YES' results:
……
……

**What type of symptoms did you have?**:

Motor
Sensory
Cranial nerve eg:
facial drooping/numbness
Speech problems

**What is the patients preoperative blood pressure?**

Systolic BP
Diastolic BP

**Medication (please circle if appropriate):**

1. Are you currently taking any medication for high cholesterol / Statin therapy?

Yes No

type: ……..

2. Are you currently taking any medication for high blood pressure / hypertension?

Yes No

type: ……..

Any Other
medications:……
……

| | |
|---|---|
| **Preoperative bloods:** | |
| | Creatinine |
| Cholesterol | |
| Triglycerides | Hb |
| HDL | WBC |
| LDL | Platelets |

**Antiplatelet and anticoagulant therapy:**

1. Are you currently taking any of these medications to thin your blood? / Antiplatelet or Anticoguloant?

Aspirin 75mg
Aspirin 150mg
Aspirin 300mg
Clopidogrel 75 mg
Warfarin

What dose of medication are you taking?
How long have you been on it?.....

1. Have you been compliant with your antiplatelet therapy? YES NO
2. Was antiplatelet therapy stopped before surgery? YES NO

If 'YES' for how many days: …….. days

Have you had any concurrent use of NSAID's for > 1 week over the last 3 months?

**Ultrasound Duplex: (PLEASE INCLUDE A COPY OF THE DUPLEX)**

1. Duplex on presentation (indication for vascular referral) Date: ……

% stenosis Left: …….. % Right: …… %

2. Preoperative duplex (if repeated) Date: …….

% stenosis Left: …….. % Right: ……. %

3. Peak systolic velocity:

Left: …….. Right: …….

4. Ration peak ICA/peak CCA:

Left: …….. Right: …….

5. Disease length (on most recent duplex): ……

6. Carotid internal diameter (cm): ……..

7. Carotid outer diameter (cm): ……...

*PVI= ½ (Outer diameter- internal diameter) x disease length*

8. Plaque volume index (mm3): ……

**Intraoperative findings:**

Operation side: RIGHT LEFT

Date of Operation: ……

Diameter of the Internal carotid artery (mm): …….…….
Carotid closure: Graft? YES NO

If 'YES' Tube graft Patch graft
Graft material:……..

Drain inserted: YES NO

Shunt YES NO

Blood loss: …….. mls

**Carotid Plaque Specimen:**

**What is the quality of the carotid plaque specimen (as specified by the sticker on the specimen bottle)?**

Intact
Not intact but complete removal
Not intact and incomplete removal

**Disease length (cm):…….**

Specimen measurements
Plaque weight(g)
Suspended weight(g)
Carotid plaque volume(mm3)

**CPV = Suspended weight / 0.997 569**

**Post operative complications:**

| Complications | Yes | No |
|---|---|---|

Post operative stroke
If 'YES': how long after operation? ……

Post operative TIA
If 'YES': how long after operation? ……

Post-operative MI
If 'YES': how long after operation? ……

Post-operative haematoma
If 'YES': how long after operation? ……

Cranial Nerve injury
If 'YES': how long after operation? ……

Death
If 'YES': how long after operation? ……

## APPENDIX II : MULTIPLATE RESISTANCE

## RECOMMENDATIONS

### *Performing a measurement*

The Multiplate® analyzer is an easy-to-use, compact system. It deploys a Windows® based interface programmed with a comprehensive menu of whole blood platelet function assays. Its flexible design allows for a fast turn around time of 10 minutes per test a period. Combined with its 5 channels, this permits the processing of up to 30 tests per hour that require only 300 µL blood per analysis.The performance of a Multiplate measurement is an easy four-step process. The results are available within 10 minutes.

*1) Place the test cell into the measurement position*

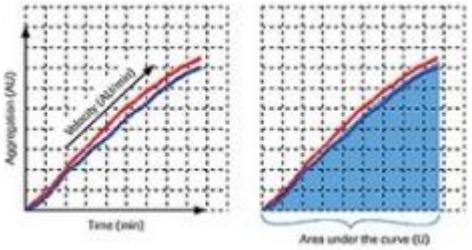
*2) Attach the sensor cable*

*3) Pipette 300 µl of saline + 300 µL of hirudin blood*

*4) Warming and equilibration*


*5) Pipette reagent*

*6) Results after 10 minutes*



## APPENDIX III: CPV STUDY PROTOCOL

1. **Recruitment – Consent form signed and patient given information sheet**

2. **Medical history – Complete data collection sheet**

3. **Bloods – Aspirin resistance test and plasma for metabolomics**

- Ensure blood for AR test is taken first
- Both tests are time sensitive, it is vital that analysis is completed quickly - as soon as the blood is taken prepare the plasma in the centrifuge, this takes 20 mins, while plasma is being prepared undertake the AR test then return and complete plasma protocol

Aspirin resistance test

1. Ensure patient has taken antiplatelet medication before initiating blood test
2. Record time/date / medication and dose in data collection sheet
3. Draw 3ml of blood into a multiplate hirudin blood tube
4. Invert gently 3 times to ensure mixing of anticoagulant
5. Transport carefully to avoid excess shaking and analyse within 30 minutes of venepuncture
6. Analyse bloods following multiplate manufacturer recommendations
7. Patients are classes as resistant if area under the curve is >40 for asp or >47 for ADP

Plasma collection for metablolomics

1. Draw blood into lithium heparin tube (green lid) and invert 3 times
2. Prepare plasma immediately by centrifugation at 3000xg for 20 min at $4^0$c
3. Aliquot plasma into cryovials (minimum 4)
4. Label cryovials with date, patient ID and DOB
5. Freeze at -$80^0$c

4. **3D and 2D ultrasound scan – Notify vascular studies of patients involvement in the study**

## 5. TCD Scanning

- Ensure patient is free from all exclusion criteria for TCD scanning
- Scan for 1 hour with probes placed bilaterally
- Watch TCD monitor during scan and record the time of any suspected MES

## 6. Plaque collection

1. Data label must be attached to sample pot and completed with the following data :

- Removal (intact, not in tact but complete removal, not intact)
- Hospital
- Surgeon
- Patient ID
- DOB
- Date

2. Ensure plaque is collected DRY
3. Place plaque on ice immediately after removal
4. Undertake plaque volume measurement

- Measure the length, weight and state of the plaque and record
- Fill a glass beaker with 9% saline and place on a fine balance scale then "zero" scale
- Secure the plaque sample using a suture and lower the plaque beneath the surface of the saline in the beaker and hold in place until weight stabilises
- Record the weight.
- If the plaque is fragmented repeat this step was repeated for each individual fragment and record the sum of the volumes
- CPV is calculated as the suspended weight divided by the density of the fluid (0.997569)

5. Divide plaque into 3 sections

6. Divide each section into three samples

7. From each section (red, amber, green) remove one sample store In a cryovial (labelled with date, patient ID, DOB and test) in -80$^0$c freezer for H+E analysis

8. From each section (red, amber, green) remove one sample and prepare for metabolomic analysis

- Wash samples in ice cold saline
- Weigh samples and record weight then store in a cryovial (labelled with weight, date, patient ID, DOB and test) in -80$^0$c freezer

9. From each section (red, amber, green) remove one sample and prepare for proteomic analysis

- Wash tissue twice in ice cold PBS then in ice cold 0.25M sucrose and dry on tissue
- Weigh samples and record weight then store in a cryovial (labelled with weight, date, patient ID, DOB and test) in -80$^0$c freezer