Is carotid plaque volume more important than severity of stenosis when predicting stroke risk in Carotid Artery Disease?

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<td>ACST</td>
<td>Asymptomatic Carotid Surgery Trial</td>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>ASP</td>
<td>Arachidonic Acid</td>
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<td>CAD</td>
<td>Carotid Artery Disease</td>
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<tr>
<td>CADET</td>
<td>Centre for Advanced Discovery and Experimental Therapeutics</td>
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<td>CEA</td>
<td>Carotid Endarterectomy</td>
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<td>CPV</td>
<td>Carotid Plaque Volume</td>
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<td>ECST</td>
<td>European Carotid Surgery Trial</td>
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<td>GC-MS</td>
<td>Gas Chromatography – Mass Spectroscopy</td>
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<td>High Density Lipoprotein</td>
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<td>Induced Coupled Plasma</td>
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<td>Interleukin</td>
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<td>Lipoprotein Associated Phospholipase A2</td>
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<td>MES</td>
<td>Micro-embolic Signal</td>
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<td>Matrix Metalloproteinase</td>
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<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
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<td>OPG</td>
<td>Osteoprotogerin</td>
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<td>oxLDL</td>
<td>Oxidised Low Density Lipoprotein</td>
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<td>PCA</td>
<td>Principal Component Analysis</td>
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<td>RANK</td>
<td>Receptor Activator Nuclear Factor Kb</td>
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<td>TIA</td>
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<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>TPA</td>
<td>Total Plaque Area</td>
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<td>TRAP</td>
<td>Thrombin Receptor Activating Protein</td>
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<td>UHSM</td>
<td>University Hospital of South Manchester</td>
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Abstract

Each year 152,000 people suffer from Stroke in the United Kingdom. 25.5% of these Strokes are caused by Carotid Artery Disease. This project set out to develop the foundations of a stroke risk prediction profile in carotid artery disease patients. The main focus of the project was investigating Carotid Plaque Volume as an alternative indicator for Carotid Endarterectomy. The secondary aim was to establish the various parameters that differ between symptomatic and asymptomatic patient groups. 2D and 3D ultrasound, Transcranial Doppler Monitoring for micro-emboli, antiplatelet resistance testing and patient medical history were all performed as part of the study. Blood samples and carotid plaques removed during carotid endarterectomy surgery were processed for metabolomic, proteomic and histological analysis. 121 Carotid Artery Disease patients were recruited to the study (Symptomatic n = 93, asymptomatic n = 28). Symptomatic patients displayed a significantly larger plaque volume than asymptomatic patients (0.836 (SD ± 0.035cm$^3$) vs 0.629 (SD ± 0.056cm$^3$), P <0.05), whilst no relationship could be made between carotid plaque volume and degree of stenosis (F(4,87) = 0.3380, P 0.852). There was a general trend of increasing plaque volume correlating with increasing symptom severity, with plaque volume gradually increased from Amaurosis Fugax (0.721 (SD ± 0.056cm$^3$), n = 12), to TIA (0.844 (SD ± 0.056cm$^3$), n = 43) and again to Stroke (1.041 (SD ± 0.118cm$^3$), n = 25). Patients that were resistant to antiplatelet medication displayed generally raised carotid plaque volumes (1.33 SD ±0.242) vs 0.817 (SD ±0.052), P 0.059). Trace metal analysis showed trends of raised Iron (P <0.05) and copper (P 0.065) levels in symptomatic plaques. Using Linear Regression of correlation, all plaques across both patient groups displayed a close positive correlation between Calcium and Lead ($R^2$ 0.7113; p <0.0001), Calcium and Zinc ($R^2$ 0.6867; P <0.0001), Calcium and Sodium ($R^2$ 0.9499; P <0.0001) and Calcium and Magnesium ($R^2$ 0.9736; P <0.0001). Due to time constraints and equipment failure, full ’omic analysis of plaque and blood samples could not be completed. We have provided further evidence for the use of plaque volume as an alternative to stenosis when deciding upon best treatment for carotid artery disease patients. This project has also helped to lay the foundations of a stroke risk prediction profile by determining several key differences between symptomatic and asymptomatic patients.
**Declaration**

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The Author

I am a Biomedical Sciences graduate, a Research Assistant and Postgraduate Student at the University of Manchester. I strive to help implement innovative academic research and clinical trials undertaken at the Institute of Cardiovascular Sciences in clinical, surgical and laboratory settings. My experience extends through Vascular, Cardiac, Stroke and Respiratory specialities. I have seen multiple studies through from conception to completion, including research presented at meetings of the American Thoracic Society, The British Medical Ultrasound Society and the Charing Cross Vascular Symposium. My main research is focused on a multi-factorial approach to stroke prediction in Carotid Artery Disease patients, based mainly on carotid plaque volume as a feasible alternative to degree of stenosis when deciding on medical management of patients.
1. Introduction to Stroke

Approximately 152,000 people in the U.K. suffer from stroke every year, causing 7% of male deaths and 10% of female deaths annually \(^1\). It is the single largest cause of adult disability, with 1.1 million stroke survivors living in the United Kingdom 50% of which require social care \(^1\). Not only is stroke a major cause of morbidity and disability, it also incurs huge healthcare costs. Each year, including healthcare costs, lost working hours and care costs, stroke costs the UK approximately £7 billion \(^2\).

Stroke can be separated in to two distinct subgroups (Fig.1); haemorrhagic stroke and ischemic stroke. Haemorrhagic stroke occurs due to a rupture of the cerebral blood vessels \(^3\). This form of stroke can be further categorised by the location of the bleed. Intracerebral rupture will lead to accumulation of blood in the brain tissue, whereas subdural rupture will cause application of pressure to the brain as blood accumulates in the cranial cavity \(^4, 5\). Ischemic stroke is frequently a consequence of carotid disease.

1.1 Ischemic Stroke

Ischemic stroke is far more frequent than haemorrhagic, accounting for 85% of all strokes \(^6\). 30% of ischemic strokes are caused by carotid artery disease (CAD), development of an atherosclerotic plaque in the carotid arteries. In CAD, thrombus develops at the site of the plaque and travels to the cerebral arteries where it may cause blockage of a vessel and a subsequent cerebrovascular attack. Ischemic stroke may also be caused by atrial fibrillation. Irregular rhythm of the heart can cause a blood clot to develop. The clot travels to the brain causing an ischemic event in the same manner as seen in CAD. Symptomology of the cerebrovascular event is largely dependent on the location of the clot and the amount of time over which the event takes place \(^7\). Vessel blockages that resolve quickly will give less severe symptoms such as amaurosis fugax or a transient ischemic attack, whereas prolonged non-spontaneously resolving clots can lead to severe strokes and death. The quicker a person undergoing a cerebrovascular event is treated, the more likely that they will make a full or partial recovery \(^8\). Most common symptoms include slurring of speech, difficulty in expressing oneself, loss or alteration of vision, numbing or drooping of the face and numbing or weakening of one or more limbs.
FIG. 1 An ischemic stroke occurs when a thrombus derived from elsewhere in the vasculature becomes lodged in a vessel supplying blood to the brain. The tissue supplied by the blocked vessel becomes starved of oxygen, dying if left untreated. Haemorrhagic stroke is caused by the bursting of a weak cerebrovascular vessel. The vessel bursts and blood leaks directly into the brain tissue, the blood accumulates and compresses the brain tissue \(^{(9, 10)}\), Adapted from \((9)\) and \((10)\).

1.2 Carotid Artery Disease Development, Progression and Thrombus Formation

Risk Factors for CAD (Table 1.) are similar to the risk factors seen in almost all atherosclerotic disease patients. Age is positively correlated with development of atherosclerosis and pathophysiology involved with the disease. Smoking contributes hugely to cardiovascular morbidity and impacts upon all stages of atherosclerosis from initial endothelial disruption through to clinically acute thrombus derived embolic events\(^{(11)}\). Diabetes, in particular diabetic retinopathy, is independently associated with CAD. Type 2 diabetes patients with diabetic retinopathy present with higher plaque burden and more frequent early carotid plaque development than those without retinopathy \(^{(12, 13)}\). Metabolic syndrome, similar in symptomology to pre-diabetes, has also been independently associated with at risk CAD patients\(^{(14)}\). Hypertension presents frequently in CAD patients. High blood pressure can cause stress and damage to the artery walls, which in turn causes an inflammatory cascade within the walls of the artery leading to plaque formation \(^{(15)}\). Recent research also indicates the change of blood flow and subsequent turbulence of blood passing through arterial bifurcations could potentially be a major cause of arterial wall stress that results in activation of localised inflammatory mechanisms \(^{(16)}\).
TABLE 1. Main risk factors for Carotid Artery Disease (7).

The primary factors that contribute to development of atherosclerosis are endothelial injury, turbulent blood flow and hypercoagulability (17). As mentioned, high blood pressure can result in endothelial injury. This initial injury is a primary instigator of plaque development. The subsequent inflammatory cascade and progression of the atheroma results in localised turbulent blood flow. As the plaque progresses it narrows the lumen and affects the motility of the artery wall, further increasing localised blood pressure (15, 17).

FIG.2 A) In the first stage of plaque development immune cells and lipid droplets accumulate in the intima of the carotid artery following activation of the arterial endothelium via shear stress. B) White blood cells are recruited from the blood, passing through the endothelium into the intima, here they differentiate into foam cells C) Smooth muscle cells migrate to the intima of the lumen.
Smooth muscle cell proliferation, causes the artery wall to expand into the lumen. There is also a significant increase in synthesis of inflammation inducing extracellular matrix derived macromolecules. As the lesion progresses towards an advanced stage the necrotic core of the plaque develops and expands. D) At this advanced stage of disease, intraplaque pathophysiology occurs. These processes are all capable of disrupting and destabilising the plaque, leading to rupture, thrombosis and a subsequent catastrophic ischemic event. Taken from (18).

As the carotid atheroma develops it may become unstable, exhibiting a large lipid rich necrotic core and a thin fibrous cap (Fig.2). Rupture of this cap exposes the contents of the plaque to the arterial blood flow. But what causes this rupture? A likely explanation is the crystallisation of free cholesterol within the core of the plaque(19). The process of crystallisation within a plaque is deemed to be sudden and irreversible, occurring upon development of a saturated solution of free cholesterol within the necrotic core (20). Conversion from a soluble to a crystallised state results in a volumetric increase. These increases will stretch the fibrous cap, which may already be thin and fragile. Cholesterol crystals have the ability to perforate this thin fibrous cap, exposing them to arterial blood flow and causing rupture of the weakened fibrous cap (Fig.3) (21). The initial thinning of the fibrous cap is possibly initiated by macrophage derived matrix metalloproteinases, as macrophages accumulate in the necrotic core levels of these proteolytic enzymes increase, gradually degrading the fibrotic cap (22). One reason that statins may be seen to alter plaque morphology is due to their ability to dissolve cholesterol crystals. This process will reduce plaque volume, relieve pressure on the fibrous cap and effectively stabilise the plaque (23).

![Fig.3](image)

*Fig.3. This scanning electron micrograph shows cholesterol crystals penetrating the fibrous cap of a patient who has suffered TIA and is not on statin therapy. Taken from (23)*

If the plaque does rupture, exposure of plaque contents to the arterial blood flow can incur thrombosis. A monolayer of circulating platelets aggregates to the area of damage. The adhered platelets become activated via an increase of intracellular calcium. Some platelets may be activated by the presence of localised thrombin, produced as a result of tissue factor release; others are
directly activated by interaction with plaque contents such as collagen. As a result of activation, platelets change their morphology and release various potent pro-aggregates and pro-activators such as thromboxane-A2. Platelet glycoprotein receptors are also activated allowing for platelet-platelet aggregation. Increased recruitment and aggregation of platelets to the area of injury results in thrombus formation (24).

At the site of plaque rupture an initial ‘white thrombus’ consisting of active platelets develops, covering the ruptured area. Presence of localised thrombin and thrombin production by activated platelets causes the adherence of red blood cells to the ‘white thrombus’ via pro-coagulation mechanisms. A mixture of red blood cells and fibrin coats the pre-existing thrombus forming a ‘red thrombus’. This layering process of white and red thrombus creates a distinct histological profile named ‘Lines of Zahn’ (Fig.4) (25, 26).

**FIG.4** This histological image depicts the Lines of Zahn which characterise thrombus formation at sites of rapid arterial blood flow. Platelets form the pale layers, whilst the dark layers consist of fibrin and red blood cells (25). Taken from (25)

The developing thrombus is a contributing factor in increased localised blood flow disruption. Increased turbulent flow can result in particles of thrombus breaking free and embolising from the carotid arteries to the cerebral vasculature. Smaller emboli may pass through the cerebral vasculature undetected, however a percentage of emboli are large enough to become lodged within a cerebral or ocular vessel, causing a Stroke, TIA or other cerebrovascular symptoms.

Following rupture, ulceration and cap degradation, the fibrous cap may exhibit a healing process over time. This process can be particularly slow, with many ulcerations persisting for long periods of time(27). A successful healing process is associated with positive patient outcomes, showing reduced reoccurrence of cerebrovascular symptoms related to disease (28). Frequency of this healing process is unclear and in depth, large cohort research is lacking. Teng et al (28) used cardiovascular magnetic resonance imaging to follow up symptomatic CAD patients at 3 and 12
months. Only 5 of 54 (9%) patients showed plaque healing at 12 months, though the authors noted limitations in their imaging technique as well as their use of lumen curvature and roughness as their descriptor of plaque morphology. Research by Gils et al \(^{(27)}\), using serial multidetector computed tomography angiography on 83 patients followed up at 21 ± 13 months, indicated similarly persistent ulceration and infrequent plaque healing. Of 15 patients showing ulceration, 4 showed regression of plaque morphology, 10 remained unchanged and 1 progressed. Contrary to the argument that ulceration repair is an elongated and infrequent process, Qiao et al \(^{(29)}\) presented a case study of an asymptomatic 72 year old male with bilateral stenosis of >80%, the patient was scheduled for left carotid endarterectomy, with right endarterectomy scheduled two months later. The patient presented with an ulcerated right sided carotid atheroma that showed significant repair over just 2 months between MRI scans. At the time of the second scan, a new collagen rich fibrous cap could be seen covering the previously ulcerated area. It would appear repair of ruptured and ulcerated carotid plaques is highly variable.

### 1.3 Treatment of Carotid Artery Disease

Severity of disease is currently measured by carotid stenosis, the percentage of the carotid artery lumen blocked by the atherosclerotic plaque. Those with a high level of stenosis will be referred for urgent surgery, usually in the form of carotid endarterectomy, in order to remove the plaque. Degree of stenosis requiring surgery is dependent on whether the person is symptomatic (Stroke/TIA/Amaurosis fugax in the past 12 weeks), or asymptomatic. In asymptomatic patients, European Carotid Surgery Trial (ECST) investigators suggested that surgery is only beneficial to those with stenosis of over 70% \(^{(30)}\). Symptomatic patients are generally operated on when it is deemed that they have high level stenosis along with some form of cerebrovascular event, 50-99% \(^{(31)}\). Those with an occluded artery (100% stenosis) do not undergo surgery as thrombus cannot embolise to the brain.

150,000 carotid disease related surgeries are undertaken in the U.S. every year\(^{(32)}\). The number of carotid endarterectomies performed has gradually decreased since the introduction of techniques such as carotid stenting which require less frequent short term re-intervention \(^{(33)}\). Success of such surgical techniques as well as better public understanding of how to prevent stroke through lifestyle and dietary habits have resulted in a fall in the number of Carotid Endarterectomies performed each year (Fig.5) \(^{(33)}\).
Patients not indicating surgical intervention will be treated with medical therapy and lifestyle interventions. It is recommended that patients strive to maintain a healthy weight, stop smoking, reduce alcohol intake and alter their diet. Medical Therapy consists of prescription of several medications. Antiplatelet medications such as aspirin and clopidogrel are prescribed in an effort to reduce occurrence of thrombosis at the site of disease. Patients are also prescribed statins to reduce cholesterol levels and anti-hypertensives to lower blood pressure. These medications are prescribed in order to slow plaque progression by restoring arterial physiological conditions to normal levels.

1.4 Symptomatic and Asymptomatic Carotid Artery Disease

The process described in section 1.2 is effectively a step by step progression of symptomatic CAD, in which the patient develops symptoms of cerebral ischemia due to an embolic event. Symptomatic patients are at high risk for stroke over a short period of time, decreasing as the time from event increases, whereas asymptomatic patients have a low risk of stroke over many years\(^{34}\). But what pathological mechanism causes a previously asymptomatic patient to become symptomatic? Severe stenosis may present without symptoms, whereas symptomatic patients may experience cerebrovascular events with much lower levels of stenosis.
Research by Freilinger et al (35) indicated that approximately a third of cryptogenic stroke patients (patients with no clear cause of stroke) presented with non-stenosing plaques (<50% stenosis) that were actually highly progressed, complicated atheromas. These potentially symptomatic, complex atheromas would not be operated on under current guidelines due to this low level of stenosis. Intraplaque haemorrhage was present in 75% of these atheromas and fibrous plaque rupture present in 50%. Similar results were presented by Saam et al (36), who concluded that complicated atheromas are frequently found in non-stenosed or mildly stenosed arteries (<50% stenosis). Research such as this presents evidence that it is not stenosis that is the main cause of embolism and seriously questions stenosis as a primary risk factor in CAD, as well as highlighting the lack of reliable parameters upon which plaque stability can be analysed.

In the recent paper ‘Why is the Management of Asymptomatic Carotid Disease So Controversial’ (37) Naylor presents a comprehensive overview of why asymptomatic carotid disease patients represent a modern day medical minefield, with contrasting evidence and advice on how these patients should be treated. The key points presented by Naylor revolve around the idea of a percentage of ‘highly selected’ asymptomatic patients in whom surgery will be of benefit in preventing cerebrovascular attack. The issue is that no true guidelines have been set as to how these patients should be selected, other than a series of vague outlines. There is no definitive profile for these patients to be compared to in order to decide who should undergo CEA. The crux of the issue is summed up succinctly when indicating that >90% of revascularisations in the U.S. are carried out on asymptomatic patients, as opposed to zero asymptomatic revascularisations in Denmark (38, 39). Opinions on asymptomatic CEA exist at extreme ends of the scale.

Naylor concludes that realistically only a small percentage of asymptomatic patients will actually benefit from prophylactic surgical intervention. In a breakdown of asymptomatic surgical data, largely from the two biggest asymptomatic surgery trials to date ACST and ACAS (Asymptomatic Carotid Surgery Trial and Asymptomatic Carotid Atherosclerosis Study), figures suggest that only 46 strokes would be prevented at 10 year follow up for every 1000 CEA performed on asymptomatic patients (32). Although this may seem like a very small benefit when weighed up against cost/risk, those patients in whom stroke is averted represent the 16,500 of 150,000 annual strokes in the U.K. which occur in CAD that was previously asymptomatic (37). Naylor highlights that although prophylactic surgery on asymptomatic patients is limited in its effectiveness, there is a definite group of these ‘highly selected’ patients, perhaps 10-20%, who will benefit from the procedure, stating that it is vastly important that the medical community has the capacity to identify these specific patients.

Ultimately the key issue facing ischemic stroke research is; why do some CAD patients go on to suffer plaque rupture and subsequent embolism, whilst others do not? Patients may have exactly the same level of arterial narrowing (stenosis) caused by the disease, but one may suffer a stroke whilst the other will continue to be asymptomatic. Clearly the level of stenosis is not the sole factor
in occurrence of stroke in these patients. The answer may lie in the characteristics of symptomatic plaques, with ‘complex’ plaque characteristics shown to be significantly increased in recently symptomatic patients when compared with asymptomatic controls \(^{(40)}\). This research will tie together the various parameters associated with stroke risk, alongside plaque volume in order to provide a more comprehensive overview of the difference between symptomatic and asymptomatic patients, providing potential parameters than can be used to identify the most at risk asymptomatic patients, as well as reiterating the advantages of plaque volume over stenosis as a primary risk factor.

1.5 Research Problem

Current treatment for CAD has been based on degree of stenosis and patient symptom status for 40 years. Recent studies have suggested that stenosis may not be as reliable a treatment indicator as previously thought and that only a highly selected group of asymptomatic patients will benefit from surgical intervention. Biller et al \(^{(41)}\) discovered the existence of developed carotid plaques that present with no luminal narrowing and would therefore not be considered for surgical intervention. Asymptomatic patients with >70% stenosis are found to be at a meagre 2% risk of stroke per annum, bringing into the question the true necessity of the intervention that would potentially be carried out on discovery of such disease. It could be argued that stenosis is too basic a measurement, not paying relevant attention to the true morphology and structure of the plaque \(^{(42)}\). The contribution of specific plaque characteristics to occurrence of cerebrovascular symptoms has been given increasing attention in recent research. Intraplaque haemorrhage, cholesterol levels within the disease and subject co-morbidities such as diabetes have all been associated with stroke risk \(^{(43-45)}\). Research in this investigation hopes to investigate morphological aspects of carotid disease with known risk factors in an attempt to provide aspects of a stroke risk profile based on carotid plaque volume as opposed to degree of stenosis. Plaque metabolomics, proteomics and histological content will be investigated alongside circulatory biomarkers, plaque characteristics, micro-embolic signals and patient demographics.

2. Research Background

2.1 Carotid Plaque Volume

2.1.1 Stenosis, Total Plaque Area and Carotid plaque Volume

Degree of stenosis is the current primary risk factor when predicting Stroke and deciding upon patient treatment \(^{(41)}\). Stenosis is effectively the narrowing of the artery lumen caused by atherosclerosis. This narrowing is presented as a percentage of the artery that has been occluded by the disease. For example a patient with 99% stenosis, has only 1% of their expected artery lumen unaffected by atherosclerosis. ECST guidelines indicate that symptomatic patients (those who have experienced stroke, transient ischemic attack or amaurosis fugax in the preceding
weeks) with stenosis less than 50% should be treated with best medical therapy, whilst patients with stenosis between 50% and 99% should be considered for carotid endarterectomy (CEA) [30]. Asymptomatic patients will only be considered for CEA when indicating stenosis of 70-99% [30].

Over the past few years, carotid plaque volume (CPV) has become an emerging contender as an important stroke risk factor in CAD patients. Carotid plaque volume is effectively the total mass of the plaque that resides in the affected carotid artery. Carotid plaques can greatly vary in their surface area, composition and morphology (Fig.6) [46]. These factors are simply not taken into account when measuring degree of stenosis, whereas plaque volume is a potentially more thorough indicator of these pathological plaque characteristics. In simple terms, CPV has the capability to represent a more accurate overall plaque burden on CAD patients. Such is the potential of CPV that many researchers are already advocating it as a better descriptor of disease severity than stenosis in CAD patients [47].

**FIG 6.** Plaques vary in length, size and morphology, often dependant on whether the patient is symptomatic or asymptomatic. The plaque on the left is from a symptomatic patient, whilst the plaque on the right has been removed from an asymptomatic patient [48]. Taken from [48].

In order to obtain accurate CPV measurements, the plaque must be analysed 3-dimensionally. This is unlike measurements for degree of stenosis and total plaque area which are measured using a standard 2D duplex ultrasound. 3D ultrasound analysis of CPV has been deemed, less expensive and more sensitive than alternative methods of imaging CPV 3-dimensionally [49]. Multiple studies have deemed 3D ultrasound evaluation of carotid plaque progression to be an accurate, reliable and reproducible monitoring technique [50-52]. Through implementation of strict quality control and standardised protocols, 3D ultrasound can achieve highly reliable re-readings of plaque images, further supporting the clinical capabilities of the technique [52].

Analysis of CPV progression and regression allows investigation of multiple important plaque parameters such as volume changes occurring in various dimensions, plaque surface morphology and distribution of the plaque within the carotid artery [51]. Evaluation of stenosis and total surface
area (Fig.7) does not allow for such thorough evaluation of plaque characteristics and therefore these measurements are limited in their analytical contributions.

Alongside CPV, Total plaque area (TPA) is a measurement that has become a frequent topic of stroke research in CAD patients over the past several years. Research has indicated that perhaps a larger plaque surface area would provide a bigger source for thrombosis and subsequent embolism to occur, thus becoming more likely to predict stroke than stenosis \(^{50}\,^{53}\). Although this is along the right mode of thinking, in a similar situation to degree of stenosis, merely looking at TPA is simplifying the problem. Researchers have found that TPA may well be useful for identifying high risk patients, but does not account for the more intricate details that can be evaluated by measuring CPV \(^{53}\). For example, research by Wannarong et al presented CPV as a strong predictor of cardiovascular event or death in 5 year follow up of cardiovascular disease patients, opposed to TPA which was described by the authors as a weak predictor \(^{54}\). TPA may have a role in secondary stroke prevention and treatment monitoring, but research has indicated that in a stroke free population TPA is not a useful screening tool \(^{55}\,^{56}\).

FIG.7 Shown in this figure are 3 examples of identical atheroma within the arteries. Each artery represents a different method of measuring the disease present. 'A' shows measurement of stenosis. This is the percentage of the artery impeded by the atheroma, indicated by the red arrow. 'B' indicates total plaque volume. Plaque volume takes into account the complete 3D size of the disease within the artery. 'C' shows total plaque area. This is the amount of plaque surface that is in contact with arterial blood flow.

2.1.2 Carotid Plaque Volume – An alternative to Stenosis?

As CPV has emerged as a potential focus area for CAD stroke prevention and prediction, the reliability of stenosis has been questioned. Current research indicates that CPV may indeed be a better indicator of atherosclerotic severity than stenosis \(^{47}\). Ouhlous et al \(^{47}\) studied patients with
cerebrovascular symptoms that had an atherosclerotic plaque with no luminal narrowing according to NASCET criteria. Despite a lack of stenosis, these patients were experiencing symptomatic events, indicating that other parameters must be considered as the causative factor. Not only can extensive atherosclerotic disease be present without measurable stenosis of the lumen, but a relatively small plaque volume may be focally accumulated causing a high degree of stenosis with a minimal amount of disease \(^{(57)}\). It is clear that stenosis does not address all aspects of plaque morphology and pathologies that must be considered when analysing stroke risk in CAD patients.

Plaque distribution is distinctly variable; some plaques will be very focal and densely packed over a small area whilst other plaques may be more spread out, less dense and covering a larger area of the artery \(^{(58)}\). Smaller more focal plaques may present as a high degree of stenosis with a low overall volume, whereas more evenly distributed plaques may present with little to no stenosis but a large overall volume. This is a key issue with treatment of CAD patients. The patient presenting a smaller focal, highly stenosing plaque may be forwarded for surgery, however the patient with the larger plaque volume causing no stenosis may not be correctly treated when following guidelines such as those provided by NASCET/ECST. CPV not only provides an alternative indicator for surgery in CAD patients, but may also be used to monitor the effects of medical therapy upon the carotid atheroma. In one study by Braybrook, Patients with known CAD were prescribed 80mg Atorvastatin or placebo daily and underwent 3D scanning of the carotid arteries at baseline and 3 month follow up. Carotid plaque volume was significantly lower in those receiving Atorvastatin, indicating that carotid plaque volume is a reliable indicator of response to medications in the treatment of atherosclerotic disease \(^{(59)}\). Similar results were presented by Du et al, in this study patients were also followed up at 3 months and showed a significant, rapid decrease in plaque volume in response to Rosuvastatin \(^{(60)}\).

2.1.3 Carotid Plaque Volume and Plaque Morphology

There are many factors that influence the volume of the plaque in atherosclerotic disease. Plaques of varying degrees of development have vastly different components that form the abundance of the disease. For example, developed and ulcerated plaques are more likely to have a larger plaque volume as well as a larger, more lipid rich core whilst presenting reduced levels of fibrous tissue \(^{(61)}\). Fibrous tissue is mostly located at the ‘cap’ of the plaque; this cap separates the plaque contents from the artery lumen. Large plaque volumes can therefore be associated with several pathological features of unstable atherosclerotic disease. Plaque size has been associated with cardiovascular events, even in those with low levels of stenosis \(^{(62)}\). This is a hugely important factor for the argument of CPV as an alternative risk predictor than stenosis. If patients presenting with low stenosis have high plaque volume, they may require intervention that would not necessarily be offered by current measures.
2D B-mode ultrasound can be used to measure plaque echogenicity. This is one way of investigating how advanced a plaque is via measurement of the necrotic core. This method of ultrasound relies on the core of the plaque reflecting the emitted ultrasound signal. Necrotic cores that are more lipid rich will appear more echolucent, reflecting back fewer waves due to the high content of acellular and lipid components. Smaller and less necrotic cores, usually found in more stable plaques, will reflect back a higher level of ultrasound waves. Echolucent plaques have been associated with higher risk of plaque rupture and cerebrovascular events \(^{(63)}\). Frequency and degree of plaque echolucency is also associated with cardiovascular risk factors such as diabetes, as well as intraplaque pathologies such as haemorrhage. Echogenic plaques are associated with more stable pathophysologies such as fibrosis and calcification \(^{(61, 63, 64)}\). Plaques may not be completely echolucent or echogenic and thus can be described as homogenous or heterogeneous. Homogenous plaques present a uniform echo-intensity, indicating that the majority of the plaque is comprised of similar components. Homogenous plaques are more likely to be echogenic and stable. Heterogenous plaques showing varying levels of echo-intensity throughout the plaque, indicating varied components contributing to plaque structure. These plaques are more likely to be unstable and echolucent \(^{(62)}\). Meta-analysis of several carotid plaque echogenicity studies revealed that there is significant positive association between plaque echogenicity and ipsilateral stroke across all severities of stenosis. Gupta et al also concluded that plaque echogenicity provides better insight than luminal stenosis when predicting future CAD derived thrombotic events, but is not sufficient as an independent indicator for intervention\(^{(65)}\).

Plaque progression can be slowed by prescription medications such as anti-hypertensives, cholesterol lowering drugs and antiplatelets e.g. aspirin. Changes to lifestyle habits including dietary changes and smoking cessation. Making these lifestyle changes results in a reduction of inflammatory triggers that perpetuate the pathophysiological processes involved in atherosclerosis. Antihypertensives will reduce systemic blood pressure, subsequently lowering localised blood pressure at the site of carotid disease. Thinning of the blood with antiplatelet medications will reduce turbulence of the blood flow that is passing over the plaque, as well as reducing the number of platelets binding to the endothelium covering the atheroma. Dietary changes and smoking cessation reduce further intra-plaque build-up of lipid and inflammatory molecules such as free radicals that drive the atherosclerotic development process.

**2.1.4 Summary**

CPV is capable of giving a more accurate representation of parameters such as surface morphology and plaque distribution. The pathological effect of these plaque variables is summed up by Tatar et al \(^{(42)}\) who suggest that the ‘structure of atherosclerotic plaques causing carotid stenosis is more important than their diagnosis alone’. Ultimately, knowing that a patient has atherosclerotic disease in the carotid artery is not sufficient information alone when attempting to provide the best possible medical treatment.
The key to truly analysing stroke risk in CAD patients could be through a universal risk score type system, similar in practice to that constructed by Prati et al. Though not completely redundant, stenosis should be included in a system that involves multiple factors, primarily CPV and the various parameters addressed in the research carried out as part of this study. This research hopes to make a considerable contribution to the confirmation of CPV, and the components that influence CPV, as the most important stroke risk factors in CAD.

### 2.2 Emboli Detection by Transcranial Doppler Monitoring

Micro-embolic signals (MES) are asymptomatic embolic events associated with increased risk of stroke. These micro-emboli are thrombus particles breaking away from the carotid plaque and moving towards the cerebral vasculature. Though these emboli move to an area in which devastating ischemia can occur, unlike a full sized thrombus they are too small to cause symptoms and the patient goes unaffected. MES are detected using a technique called Transcranial Doppler Ultrasound (TCD) (Fig 8/9/10). This technique utilises a small ultrasound Doppler probe attached to a band placed around the patient’s head (Fig.8). The probe is then used to detect the pulsatile flow of the Middle Cerebral Artery (MCA), which branches from the Internal Carotid Artery (ICA) (Fig.9). A plaque located at the bifurcation of the Carotid Artery may produce MES that can be detected by the Doppler monitoring the MCA.

**FIG.8** Figure 8 shows a patient undergoing a routine TCD examination with the probe placed on the temporal window, insonating the middle cerebral artery. Taken from (68).
**FIG.9** Figure 9 visualises the cerebral vasculature, highlighting the carotid arteries leading to the cerebral arteries that are examined for emboli during TCD (68, 69). The area of the vasculature monitored during TCD is highlighted by a green circle. Taken from (69).

Although TCD is a useful technique for measuring MES, not every patient can undergo monitoring. Many patients have an inadequate temporal acoustic window through which ultrasound waves cannot pass, up to 30% of patients present with this issue (70). A temporal bone window which is simply too thick is the most common cause preventing TCD monitoring (67). As a result of this, MES must be used in conjunction with other risk factors when analysing CAD patients.

**FIG.10** Depicted above is the classic waveform observed upon insonation of the middle cerebral artery during T.C.D. monitoring. Emboli present as a clear visible disturbance in the waveform, accompanied by a characteristic ‘chirp’ sound caused by rapid reflection of the ultrasound waves.

Research associating MES with risk of stroke in CAD patients is abundant. Meta-analysis of various studies indicated that MES have the capacity to predict stroke risk in acute stroke, symptomatic carotid stenosis and post-operatively following CEA (CEA) (71). MES may only be detected in ~43%
of symptomatic CAD patients and ~10% of asymptomatic patients, but the importance of detecting MES in these patients should not be underestimated (72). Studies have associated MES occurrence with plaque volume and morphological pathologies such as irregular plaque surface, plaque inflammation and unstable plaque. (73-75). Such evidence is further presented by Zhang et al (71); MES were found more frequently in patients with irregular or heterogenous plaques when compared with those presenting regular and homogenous plaques. These plaque based pathologies are associated with an increased stroke risk. Other research has focused predominantly on MES association with antiplatelet resistance. Aspirin resistance occurs more commonly in CAD patients who are MES positive than those who are MES negative. Dawson et al (76) reported that out of a cohort of 62 symptomatic CAD patients; 50% of MES positive patients presented aspirin resistance, opposed to 17.4% of MES negative patients.

The importance of MES monitoring in risk of stroke is further supported in CEA post-operative studies. Siebler (77) et al present research indicating that after CEA, MES frequency is significantly lower or disappears altogether, suggesting that removal of plaque burden has a dramatic effect on thrombosis derived embolic events. Abbott et al (78) report that patients who continue to show significant levels of MES following CEA are approximately 15 times more likely to suffer TIA or ipsilateral stroke.

MES occurrence is an important factor to include in the research being carried out as part of this study. With presence of MES already widely associated with increased stroke risk in CAD patients, it will be important to correlate MES alongside the other risk factors being investigated in this research. Previous research has shown a positive correlation between MES and antiplatelet resistance, being able to further link these factors with a variety of other stroke risk factors in CAD patients could be a huge step forward in stroke prediction.

2.3 Antiplatelet Resistance

The mainstay of medical therapy for those with CAD is the prescribing of antiplatelet medications. The function of these drugs is to stop or reduce the occurrence of a thrombotic event within the patient’s vascular system. In CAD these drugs are prescribed in order to prevent a thrombus forming at the site of the plaque in the carotid arteries, reducing the chance of a cerebral ischemic event taking place. Aspirin and Clopidogrel are two of the most frequently prescribed antiplatelet medications (79). Clopidogrel is mainly prescribed for secondary stroke prevention, though patients may also be placed on dual antiplatelet therapy (Aspirin + Clopidogrel)(80).

2.3.1 What is Antiplatelet Resistance?

Prescription of antiplatelet drugs is a frequent, widespread and generally effective method of reducing occurrence of ischemic events, however during recent years the phenomenon of ‘Antiplatelet Resistance’ has been established (81). Resistance to pharmacological agents can be
defined as an “insufficient expected biological reaction to a given dose of drug” (82). Specifically, in laboratory terms, antiplatelet resistance is an insufficient increase of platelet inhibition following the administration of an antiplatelet medication (82). In clinical terms, resistance can be defined as treatment failure (83). Thrombosis derived ischemic events occurring in patients receiving antiplatelet therapy indicates resistance or hypo-responsiveness.

Resistance to antiplatelet therapy is associated with occurrence of major adverse outcomes (84). Ineffective antiplatelet treatment will not have the desired effect of preventing thrombus formation, potentially leading to major ischemic events and subsequent death or disability.

FIG.11 A patient that is responsive to aspirin will display a clear reduction in aggregation units for the ASP test of the multiplate assay, as seen in the top row of results. A patient that is either not taking aspirin, or is compliant but resistant, will display normal aggregation levels for the ASP test, as shown in the bottom row of results. In those who are resistant, this test indicates that their circulating platelets are aggregating in spite of antiplatelet therapy. Patients are deemed to be resistant if their aggregation curve for aspirin is above 40Au or above 47Au for Clopidogrel (85, 86).

Adapted from (85) and (86).

Topçuoglu et al (87) indicate that up to a third of strokes occur in patients already receiving antiplatelet medication, whilst Helgason et al (88) concluded that 25% of previous ischemic stroke sufferers showed antiplatelet resistance. Research carried out by members of the team at University Hospital of South Manchester found an antiplatelet resistance prevalence of 37% amongst patients undergoing CEA (48). Such resistance levels were further supported in a meta-analysis of 3000 patients involved in multiple small studies. It was concluded that antiplatelet resistance in cardiovascular disease patients occurs at a prevalence of 39% (89). In this meta-analysis, 49% of resistant patients had experienced an adverse cardiovascular event following initiation of antiplatelet therapy, compared to adverse events in just 16% of non-resistant patients. A further meta-analysis of 6,450 resistant and non-resistant patients indicated a significant
increase in resistance in those who had suffered from an adverse cardiovascular event, with resistance more significant in those presenting with acute phase of the disease (90).

Resistance to these drugs is not a case of 'all or nothing'; within previous Stroke and TIA sufferers antiplatelet resistance can vary wildly from patient to patient. Collet et al showed individual patient aspirin resistance levels varying from 3% up to a huge 85%, with Clopidogrel presenting a smaller range of 28-44% (91, 92). Resistance figures are astonishingly high, given the global dependence on drugs such as aspirin and clopidogrel. Lack of a uniform patient response to antiplatelet medication is a clear indicator that this phenomenon must be carefully considered and monitored in those at high risk of thrombus derived ischemic events.

2.3.2 Aspirin and Clopidogrel Mechanisms of Action

Although Aspirin and Clopidogrel achieve the same overall effect, they reach their pharmacological outcomes via differing pathways and mechanisms (Fig.12). Aspirin blocks the binding of platelets by inactivating COX enzyme and subsequently inhibiting production of thromboxane, which is a key mediator of platelet-platelet interaction (87). Clopidogrel is a pro-drug requiring CYP2C19 for activation. Clopidogrel blocks specific ADP receptors required for platelet-platelet interaction and fibrin cross linking (93). The effect of these drugs is hugely important in preventing ischemic events in at risk patients. Aspirin alone can reduce the chances of an arterial thrombotic event by 25% in patients at high risk for heart attack and stroke (82).

**FIG.12** Diagram showing the pathways involved in platelet activation that are blocked by aspirin and clopidogrel respectively.
2.3.3 Mechanisms of Resistance

Antiplatelet resistance can be split into two sub-groups, pseudo-resistance and true drug resistance (94). Pseudo-resistance is patient non-responsiveness caused by a modifiable source, not the inability of aspirin to act upon its pharmacological target (94). Mechanisms of resistance between aspirin and clopidogrel are largely the same, with pharmacological differences varying between the two.

Factors included in pseudo-resistance are; poor patient compliance, inadequate dosage, co-medication interference or accelerated Thrombopoeisis (82, 95, 96). Lack of consistent patient compliance is a frequent and widespread issue. Non-compliance is possibly the primary factor involved in the occurrence of pseudo-resistance; up to 40% of cardiovascular disease patients are not fully compliant with their aspirin therapy (82). In absence of patient compliance aspirin levels in the blood will shift outside of the therapeutic window and the risk of a cardiovascular event occurring will increase.

Interfering co-medications are another cause of pseudo-resistance. Non-steroidal anti-inflammatory agents (NSAIDs) such as ibuprofen and naproxen both effect aspirin’s inhibitory mechanism. NSAIDs carry out reversible binding of COX-1 at the site of aspirin interaction, providing direct competition for aspirin itself. NSAIDs binding capacity outlasts the pharmacological activity of aspirin. When NSAID-COX-1 binding reverses, aspirin is no longer active and platelet thromboxane-A2 production resumes (94, 97). Reoccurrence of thromboxane production gives the appearance of aspirin resistance, when aspirin has simply been blocked from its pharmacological target. Unfractionated heparin is also known to diminish antiplatelet response to aspirin (98).

Pseudo resistance may also present via accelerated Thrombopoeisis. Patients will usually undergo a ~10% turnover in total platelets over a 24 hour period, however this can increase sharply in situations of physiological stress such as surgery or systemic inflammation (97). Studies carried out on platelet turnover in patients undergoing antiplatelet therapy indicated that platelets with intact cyclo-oxygenase, platelets which are no longer inhibited, can be detected 4 to 6 hours following aspirin dosing. Those with diabetic angiopathy presented with uninhibited platelets at an even earlier stage (99). Association between antiplatelet resistance and diabetes is well established and diabetic related enhanced platelet production has been well documented in literature (99-101). Accelerated occurrence of uninhibited platelets restores levels of activation and aggregation promoters, giving rise to the appearance of resistance to antiplatelet therapy.

True antiplatelet resistance is present at a genetic and biochemical level. COX-1/2 genetic polymorphisms as well as that of thromboxane synthase have been detected in those with antiplatelet resistance. Polymorphism of these cyclooxygenase enzymes incurs thromboxane-A2 production in spite of antiplatelet therapy, giving evidence to genetically induced structural
changes to aspirin binding sites on the enzymes (82, 94, 99). In Clopidogrel, polymorphisms that induce resistance occur in the cytochrome P450 family (96).

Biochemically, alternate platelet activation and aggregation pathways have the capability of inducing aspirin resistance. Switching of primary platelet activation pathways from COX-1 to COX-2 is one such route that may present as aspirin resistance. COX-2, similar to COX-1 is a cyclooxygenase capable of inducing thromboxane-A2 production. COX-2 is less sensitive to aspirin than COX-1 and not as easily inhibited (102). Platelets may also develop increased sensitivity to extracellular catalysts of thromboxane production such as cholesterol and ADP (82). Cholesterol and ADP induce thromboxane production via pathways that are not blocked by aspirin, therefore platelet sensitivity to these inducers of activation and aggregation will result in thromboxane production and resistance to antiplatelet therapy.

Duration of therapy is a more controversial area of true antiplatelet resistance. In a study of 281 patients, 51 exhibiting antiplatelet resistance, it was concluded that duration of antiplatelet therapy in association with resistance was not significant (103). These results are in contrast to those presented by Pulcinelli et al (104). The authors monitored platelet resistance over 2 years in 150 patients taking aspirin. Patients were separated into two cohorts, one receiving 100mg/day of aspirin and the other 300mg/day. Long term aspirin therapy showed association with a progressive reduction in platelet responsiveness to medication in both cohorts (104). This area of true resistance needs wide scale, long term investigation. Patients with cardiovascular disease are placed on antiplatelet medication indefinitely, a percentage of these patients showing lowered response over time means that adjustments to their treatment must be addressed as a matter of urgency.

TABLE 2. A summary of the key mechanisms involved in antiplatelet resistance.

<table>
<thead>
<tr>
<th>Pseudo Resistance</th>
<th>True Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Non-Compliance</td>
<td>• Genetic polymorphisms e.g. COX-1/2, thromboxane synthase</td>
</tr>
<tr>
<td>• Co-medication interactions e.g. NSAIDS</td>
<td>• Alternate activation and aggregation pathways</td>
</tr>
<tr>
<td>• Inadequate dosing</td>
<td>• Duration of therapy</td>
</tr>
<tr>
<td>• Hyper-thrombopoiesis</td>
<td></td>
</tr>
</tbody>
</table>

2.3.4 Summary

Pseudo-resistance is a phenomenon that is as important to investigate as true resistance, especially considering that it is incurred by a modifiable or treatable cause. A clear process must be established in antiplatelet medicated patients. Any level of resistance should firstly be identified and subsequently re-tested, to allow for the dynamic nature of such resistance. The root cause of
resistance must then be investigated whilst the patient’s antiplatelet response is regularly monitored. Investigating the primary cause of resistance, in those presenting pseudo-resistance, will allow effective and potentially lifesaving therapeutic intervention. Solutions may be as straightforward as ensuring patient’s compliance, or altering co-medications. Treatments for true drug resistance are less clear cut and more difficult to thoroughly analyse, it is this aspect of resistance that must be prioritised in future work.

Investigation into antiplatelet resistance has increased in recent years; however there is a lack of thorough research incorporating antiplatelet resistance alongside other risk factors in atherosclerotic disease. Initial groundwork has been successfully made in small scale studies such as that conducted by Dawson et al; the authors presented a positive correlation between antiplatelet resistance and micro-embolic signals per hour in CAD patients (71). Larger scale studies involving consistently accurate point of care platelet aggregation testing have been attempted but have encountered cost and feasibility issues (76, 105). An area of further exploration is to therefore look at antiplatelet resistance alongside parameters with which it may correlate positively, rather than just resistance itself.

By measuring antiplatelet resistance alongside CAD patient demographics and various parameters such as carotid plaque volume, micro-emboli, plaque histology and plaque ‘omics, this research aims to broaden the understanding of how antiplatelet resistance contributes to occurrence of cerebral ischemic events and the relationship this phenomenon holds with other atherosclerotic disease risk factors.

2.4 Serum Biomarkers

Biomarkers, particularly those that are disease related, have been used in a variety of medical specialties for many years. When relating to disease, biomarkers can be predictive, indicative of risk, diagnostic and prognostic. A biomarker can be a wide range of biological substances and products such as antibodies, proteins and biochemical molecules. Diagnostically, biomarkers are used as a measurable endpoint to indicate abnormalities in levels of detectable substance which may indicate disease associated pathological processes. Changes in biomarkers levels can indicate progression or regression of disease and response to treatment. In autoimmune diseases biomarker tests will indicate irregular autoantibody presence, for example autoantibodies can be detected before onset of Rheumatoid Arthritis allowing for a pre-emptive treatment response (106). In cardiovascular disease the damaged heart muscle cells will release troponin following myocardial infarction; troponin is therefore used as a biomarker for indication of heart attack (107). Not all biomarker measurements can be this straightforward and some diseases may need a variety of specific biomarker changes in order to be characterised, whereas other disease have no detectable biomarkers. CAD is one such disease that currently has no biomarker or biomarker profile deemed
to be robust and reliable enough to help predict occurrence of cerebrovascular events or disease progression.

2.4.1 Biomarkers in Carotid Artery Disease

In a disease like CAD, the structure of the main pathology is important when researching potential biomarkers. The primary pathology of CAD is the atherosclerotic plaque in the wall of the carotid artery. Research over the past several years has uncovered the complex structure of the carotid plaque, as well as many of the pathological mechanisms leading to plaque formation. It is these processes and the structure of the plaque itself that must be closely considered when investigating potential biomarkers produced in CAD. The first stages of plaque development mostly involve accumulation of immune cells and deposition of small amounts of lipid within the intima of the carotid artery \(^{(21)}\). The developed, pathological plaque is a far more complex structure (FIG.13).

**FIG.13** a) The initiation of plaque development consists of lipid deposition and inflammatory cell recruitment to the artery intima. B) Early stage plaques will begin to impede upon the artery lumen. At this point plaque composition is more complex. C) More advanced plaques will show an acellular necrotic core as well as advanced intraplaque pathologies D) This is the very advanced stage of atherosclerosis. The necrotic core is large and the fibrin cap may be thin leading to plaque instability. The plaque impedes on a large volume of the lumen causing significant narrowing and severe local turbulence of blood flow \(^{(108)}\). Taken from \(^{(108)}\).

There are many components that make up a typical advanced carotid atheroma. Activated immune and inflammatory mediating cells, smooth muscle cells, vascular endothelial cells, lipids, extracellular matrix and acellular debris \(^{(109)}\). The acellular debris is known as the necrotic core and consists of a high density of lipids and an acellular soup \(^{(21)}\). This ‘soup’ consists of cells necrotized by the localised infiltrating immune and inflammatory cells. The necrotic core is surrounded by collagen fibres and smooth muscle cells which form a fibrous cap. Immune cells are present throughout the plaque, both in tissue and the necrotic core \(^{(104)}\). The final component of the plaque is the barrier between the lumen of the artery and the plaque itself; this is a layer of vascular endothelium. Typical opinion is that the carotid plaque presents as a localised thickening of the carotid intima, however recent research has questioned this, indicating that a percentage of
symptomatic CAD patients may present with advanced plaques that do not cause luminal narrowing (47). Biomarkers that can be associated with CAD are generally the result of atherosclerotic progression and plaque vulnerability. This includes processes such as inflammation, lipid accumulation, apoptosis, proteolysis, thrombosis and angiogenesis.

Pathologies not seen in the common advanced atheroma do exist. This can include varying degrees of ectopic calcification within the plaque, which in some cases may even result in bone formation within the vessel wall (110). Biomarkers associated with calcification and bone formation are therefore another subset of measurable and quantifiable indicators of plaque pathologies. Research has already indicated that such biomarkers may be useful in non-invasively determining calcification of the carotid artery, one such biomarker being undercarboxylated osteocalcin (111). Other indicators include bone matrix proteins, which have been shown to be elevated in symptomatic CAD patients (112). Biomarkers of calcification will be discussed in more depth in this section.

2.4.2 Inflammatory Biomarkers

Many pathological processes leading to the instability and progression of a plaque can potentially produce measurable systemic biomarkers that could aid in analysis of degree of CAD. Inflammation is arguably one of the best processes for producing detectable disease associated biomarkers. In atherosclerosis, inflammation is closely related to plaque instability and probability of plaque rupture (113). Measurement of clear plaque associated inflammatory biomarkers has the potential to greatly contribute to analysis of plaque stability and CAD progression. Two studies into High Sensitivity C-Reactive protein have shown it to positively correlate with highly progressive carotid plaque development and also with a significantly higher risk of first time stroke in asymptomatic patients (114, 115). Due to the high influx of immune cells into advanced lesions there are a number of interleukins and associated inflammatory mediators present in carotid atheromas. IL-6 is a pro-coagulant and pro-inflammatory cytokine shown to be increased in plaque lesions (116, 117). Tumour Growth Factor–B1 levels also vary between stable and unstable plaques, this biomarker is present at increased levels in stable plaques when compared to unstable plaques (118). Another pro-inflammatory interleukin is IL-18, this biomarker is elevated within the plaque and is a promotor of matrix metalloprotease (MMP) production (119). MMP’s have a profound effect in atherosclerosis. MMP-9 for example is found to be present throughout atherogenesis, is significantly high in unstable plaques and is higher in plaques of TIA and stroke patients when compared with asymptomatic and amaurosis fugax patients (120-122). MMP-9 is also found to positively correlate with age (123). MMP-12, elastin and collagen, mediators that are associated with plaque stability are lower in diabetics. The stabilising properties of elastin were highlighted in the study conducted by Asciutto et al in which low plaque elastin levels were associated with higher ipsilateral stroke risk (123). MMP-12 is also found to represent adverse outcomes following CEA. Such is the
inflammatory potential and role in plaque instability of MMPs, some researchers are suggesting that imaging MMP activity may be a potential method for identifying unstable plaques \(^{(124,125)}\).

### 2.4.3 Vascular Calcification Biomarkers

One potential source of circulatory biomarkers that can be linked to plaque stability is vascular calcification. This process is the formation of mineralized tissues within the artery walls \(^{(126)}\). Development of calcification in the arteries is not dissimilar to bone formation and is a fairly common occurrence \(^{(127)}\). With aging, many people will develop increasing calcium deposits within the walls of the major arteries \(^{(110)}\). In arterial specimens removed during vascular surgery, an average of 5-15% will show some form of calcification. The effects of vascular calcification can vary in their pathological outcomes. In CAD, calcification of the atheroma can actually be a stabilising process, reducing occurrence of symptoms when compared with less calcified plaques \(^{(128,129)}\). Extensive calcification, resulting in bone formation, occurs in plaques that are heavily calcified with no ulceration or intra-plaque haemorrhage, two processes that are directly linked to increased likelihood of plaque rupture. It would appear that in diseased tissue such as carotid atheroma, vascular calcification can potentially provide a protective effect, reducing the occurrence of plaque rupture and subsequent thrombosis seen in more necrotic, less fibrotic plaques \(^{(128)}\).

**FIG. 14** shows a highly calcified area of carotid plaque surrounded by fibrotic tissue with a high level of infiltrating macrophages.

The effects of arterial calcification are mixed. Whilst in CAD calcification can be seen as a preventative measure as it slows progress of the necrotic core, the process can also be wholly negative. Calcification of healthy arterial tissue results in reduction of the elasticity of tissue. Over time this disrupts local haemodynamics causing localised hypertension and stenosis of the artery. Examples of such are aortic calcification and valvular calcification \(^{(110)}\). Formation of calcified
tissues such as that visualised in FIG.14, result in normal tissues structures becoming disrupted and unable to carry out their appropriate function.

Atherosclerotic calcification occurs through differentiation of vascular cell subpopulations into an osteogenic phenotype. This is a process that appears to be driven by inflammatory mediators that are derived from the atheromatous components of the plaque\(^{(110)}\). High levels of circulating glucose are also linked to this osteogenic differentiation process, this correlates with research indicating that calcification can occur in response to a high fat, diabetogenic diet as well as being affected by smoking, age and hypertension\(^{(110)}\). The process that leads to calcification is well described by Dellegrottaglie et al\(^{(130)}\). The author breaks this down into 4 factors: Initiation of osteogenesis in the vessel wall, inhibitory imbalance, enhanced bone production and mineral metabolism abnormalities. Infiltrating macrophages are key players in the initiation of the process. Upon arriving at the site of inflammation, these cells release a cascade of inflammatory cytokines including Interleukin-1 (IL-1) and Tumour Necrosis Factor-α (TNF-α). IL-1 and TNF-α are potent inducers of osteogenic stimulating factors such as osteoprotegerin (OPG) and receptor activator nuclear factor kB (RANK). This increase of osteogenic mediators overwhelms osteoclastic, calcification preventing, mechanisms and results in gradual increase of calcification, aided by overwhelming of normal mineral metabolism processes\(^{(130)}\).

Calcification with the carotid artery atheroma results in a reduction of viable volume in which necrosis and pathological processes can take place. Calcification has been linked with asymptomatic plaques, lower intensities of fibrous cap inflammation and more stable echogenic plaques on B-mode ultrasound\(^{(110, 128, 130)}\). Although calcification is distinctly pathological, the results of this process in CAD provide a degree of stabilisation to the plaque, when compared with plaques with higher levels of inflammation and necrosis. Vasuri et al found that patients with diffusely calcified carotid artery tissue have a similar level of histological complications but significantly lower occurrence of symptoms when compared with non-calcified plaques\(^{(131)}\). Inflammatory mediators produced by vascular osteogenesis are certainly potential measurable biomarkers that can be linked with the calcification process, one such variety of biomarkers are sphingomyelinases which have only recently been closely associated with the vascular calcification process\(^{(126)}\). When combined with clinical techniques such as B-mode ultrasound, used to measure plaque echogenicity, measurable biomarkers associated with calcification could certainly play a role in clinical decision making when considering individual plaque stabilities.

### 2.4.4 Lipid Accumulation

Lipid accumulation is another pathological process which produces detectable biomarkers. In advanced plaques heightened localised lipid accumulation results in cell death, production of a necrotic lipid rich core and subsequent plaque instability\(^{(132)}\). A biomarker associated with such a process is oxidized low density lipoprotein (oxLDL). This biomarker has been shown to relate to
plaque instability \(^{(133)}\). There is also a detectable link between oxLDL and the lipoprotein-associated phospholipase A2 (Lp-PLA2) enzyme. OxLDL is known to upregulate Lp-PLA2 production. Lp-PLA2 is pro-inflammatory in its actions and is highly prevalent in diseased vessels \(^{(134)}\). Lp-PLA2 is also known to increase macrophage lipid uptake, resulting in increased foam cell differentiation and subsequently perpetuating localised inflammation and plaque instability. In CAD this enzyme is expressed at higher levels in symptomatic patients than in asymptomatic patients \(^{(133, 134)}\). High Density Lipoprotein (HDL) and lipoprotein(a) levels are further examples of detectable lipid accumulation biomarkers, low HDL and high lipoprotein(a) were associated with ultrasound findings that indicated plaque instability \(^{(135)}\).

2.4.5 P-Selectin

One biomarker that has already been fairly substantially investigated throughout atherosclerotic diseases is P-Selectin. P-Selectin is a cell adhesion molecule located on the surface of activated endothelial cells that line the endothelium of the inner surface of blood vessels. P-Selectin is also present on activated platelets \(^{(136)}\). These activation processes usually occur as a result of thrombin or histamine interactions \(^{(137)}\).

P-selectin is a major component of response to vascular injury. When damage to the endothelium occurs, P-selectin facilitates recruitment of leukocytes to the area of injury \(^{(136)}\). Along with recruitment of leukocytes, P-Selectin is also largely responsible for recruitment and aggregation of platelets to the area of injury. In platelets that are inactive, P-Selectin is located on the inside of α-granules. When the platelet is activated membrane flipping occurs \(^{(138)}\). This is a process by which the in the inner walls of the α-granules are exposed to the outside of the platelet. Exposed P-selectin is able to bind circulatory leukocytes, as shown in fig.15, as well as initiating platelet aggregation. Platelet aggregation is then facilitated by the newly exposed P-Selectin via platelet-fibrin and platelet-platelet binding \(^{(139)}\).
**FIG. 15** In the event of damage or disturbance, activated P-selectin on arterial endothelial cells will bind circulatory white blood cells and draw them into the site of injury. This is a key initiator of the cascade inflammatory processes that resulted in atheroma development (140). Adapted from (140).

P-Selectin is an essential component of atherosclerotic plaque progression and is an independent factor associated with carotid plaque development (141). Stable and less active plaques show a reduced level of P-selectin expression (142). This indicates a possible link between plaque instability and increased levels of this cell adhesion protein. Preliminary data collected by Blann et al (142), shows promising results that indicate a link between raised P-selectin levels and adverse outcomes in patients with atherosclerotic disease.

Another interesting aspect of P-selectin is the protein’s response to antiplatelet drugs. Some research has shown that clopidogrel, regardless of concurrent aspirin usage, can suppress P-selectin expression (143). Other studies have shown similar results only using aspirin (144). Research into this area of P-selectin antiplatelet response is anything but clear. As opposed to the aforementioned research, many studies have found contrasting results. Some studies indicating that anticoagulants have no effect on P-selectin levels, with others even suggesting that anticoagulants increased circulatory p-selectin levels (145-147).

With research indicating that P-selectin inhibitors can reduce thrombus formation, there is a potentially important link between such inhibitors and prevention of ischemic stroke (148, 149). With particular regards to the research carried out as part of this investigation, it will be of interest to see if there is any association between circulatory levels of P-selectin and aspirin resistance measurements taken as part of this study.
2.4.5 **Summary**

Being able to detect serum biomarkers that are characteristic of plaque pathophysiologies could be a great contributing factor when deciding upon treatment of CAD patients. Knowing the morphology of a patient’s plaque is changing or that a plaque is becoming less stable would be beneficial in the specific care of each patient. Investigation of such occurrences through a simple blood test would be a great leap forward in CAD treatment and stroke prevention. As summed up by Hermus et al. (113), ‘Non-invasive risk assessment in CAD by determination of serum biomarkers may play a role in clinical practice’.

Like many of the various facets of this research, serum biomarkers may not be beneficial as solitary indicator of stroke risk, but may correlate with other risk factors. Histological, proteomic and metabolomic analysis of the carotid plaques may tie in closely with biomarkers that can be detected in the blood. For example, a biomarker in the blood may be directly linked with a specific metabolomic process occurring in unstable plaques, therefore increased biomarker levels represent an increase of the metabolomic process and a decrease in plaque stability. The potential of such findings is vast.

3. **Relevance of Research**

Current methods of stroke risk analysis and subsequent treatment decisions are based on limited and outdated parameters. Degree of stenosis as an indicator for surgery has been questioned in recent years, with potential alternatives becoming apparent. Small scale studies have already confirmed that carotid plaque volume has the ability to predict stroke and could be a long term viable alternative (47, 52). Carotid plaque volume addresses the more in depth aspects of the disease, allowing for morphology and distribution to be considered when deciding upon treatment. Combining this parameter with others, such as circulatory biomarkers, antiplatelet resistance, micro-embolic signals and plaque composition, could lead to more personalised and specialised treatment recommendations for CAD patients. One potential outcome could be the introduction of a stroke screening programme for CAD patients who fulfil a number of disease related risk factors. Ultimately this research hopes to address some of the shortcomings in the decision making process of treatment for CAD patients, particularly those who are asymptomatic.

4. **Impact of Research**

The medical, economic and social burden of stroke is huge. 152,000 strokes occur each year in the U.K. alone (1). Approximately 85% of these strokes are ischemic in origin, with a substantial number occurring directly as a result of CAD (2). Half of all stroke survivors require some level of social care, creating significant financial burden and a vast social burden upon family and carers (1). Including direct NHS costs, informal care costs and lost working hours, stroke incurs an annual cost of £7 billion to the U.K economy (2). This research hopes to reveal more about the intricate
pathology of CAD, contributing to a change in the way that treatment is decided upon from patient to patient. Research conducted as part of this study will reaffirm CPV as a viable alternative to stenosis in stroke risk prediction, as well as displaying any and all links between this primary factor with various secondary factors such as antiplatelet resistance and MES. It is hoped that this research will contribute to relieving the global burden imposed by stroke.

5. Project Aims

The research in this thesis is designed to answer the following research questions:

- Is carotid plaque volume associated with symptoms in patients undergoing surgery for CAD?
- Is carotid plaque volume associated with cerebral emboli detected by TCD in CAD?
- Is antiplatelet resistance associated with symptoms or cerebral emboli in CAD?
- What circulatory biomarkers can be associated with CAD?

6. Methods

6.1 Overview

Patients were recruited from University Hospital of South Manchester. Patients listed for CEA, both electively and urgently, underwent a variety of investigational procedures before and after surgery. Before surgery patients underwent ultrasound scanning of the diseased artery in order to determine severity of stenosis, Transcranial Doppler Scanning to detect any micro-embolic signals as well as blood sampling for circulatory biomarkers and platelet aggregometry. Obtaining full data sets was of huge importance, however not all patients chose to undergo all aspects of the study. In such cases, as much data was collected as possible.

Patient informed consent allowed the research team to obtain the carotid plaque from theatre upon removal. The plaque was be measured and weighed upon removal. Suspended weight of the plaque was also measured in order to provide a total plaque volume for each sample. The plaque was then be trisected, with each section subsequently trisected again. Three sections of plaque were selected for each of proteomic, metabolomic and histological investigation.

Post operatively patients were followed up at 4-6 weeks to check for occurrence of cerebrovascular symptoms and to obtain a blood sample. The blood sample was used for biomarker and aggregometry analysis in order to quantify systemic plaque burden.

6.2 Patient Recruitment

Patients presenting with CAD at UHSM who were listed for CEA were approached to take part in the study. Both symptomatic and asymptomatic patients were be included. Patients were deemed to be symptomatic if they had suffered cerebrovascular symptoms such as Amaurosis Fugax, TIA
or Stroke within 12 weeks prior to assessment. Asymptomatic patients are those who have experienced no cerebrovascular symptoms or are symptomatic beyond the 12 weeks preceding the assessment.

Exclusion criteria were as follows; patients with an occluded carotid artery, a mechanical heart valve, those who are under 40 and those who are incapable of undergoing a T.C.D reading. Origin of emboli recorded during T.C.D must also be considered; therefore those with underlying heart disease at high risk of emboli were excluded. Excluded patients included those with atrial fibrillation, prosthetic valves, recent myocardial infarction or dilated cardiomyopathy. These co-morbidities are capable of producing emboli that would interfere with the true CAD derived emboli count. Patients who are not completely cognitively able were also excluded.

Every patient had to provide informed written consent before they were involved in the study. Patients were given time to consider their participation and consult family members and friends if they wished. Patients were free to withdraw from the study at any time and did not have to take part in every aspect of the study. The G.P. of the consenting patient was be informed of their involvement in the study and contacted in the event of an incident or discovery relevant to the patient’s health. Patient demographics, including but not limited to, smoking history, medical history and current medications were noted on study CRFs. Patients were followed up in Vascular outpatients clinics approximately 4-6 weeks post-surgery.

**6.3 Carotid Stenosis**

2D ultrasound duplex scan was used to analyse each patient’s degree of stenosis in the carotid arteries (Fig.16), stenosis was measured using ECST guidelines. This ultrasound measurement is conducted as routine clinical analysis in all CEA patients before surgery, duplex scanning allows for visualisation of the disease in order to guide the operating surgeon. Measurements were conducted by a trained sonographer blinded to specific patient details in order to reduce bias between study groups.
Image A is a colour Doppler 2D ultrasound image of a patient with a stenotic, ulcerated plaque within the carotid artery. Blood flow is disrupted, highlighted by the encircled area, this disturbance is not homogenous and the ulceration can be seen by the pitting of the blood flow into the middle of the plaque where the ulceration is present. This patient would be high risk for a CAD derived ischemic event. Image B shows a patent, undisturbed carotid artery in a patient unaffected by atherosclerosis. Images taken from (150) and (151)

6.4 Carotid Plaque Volume

Carotid Plaque Volume was measured by two different approaches. Before surgery, patients underwent a 3D ultrasound scan as part of the study. Unlike the 2D scan, this is not yet a routine method of pre-surgery analysis. 3D ultrasound scanning was used to measure the carotid plaque volume in the affected artery in vivo (Curefab 3D Ultrasound, Munich)(Fig.17). We could then compare this with a manual volume measurement post-surgery, using the hydrostatic suspension technique.
FIG. 17 Images A-D show the process of measuring a carotid plaque using the 3D ultrasound technique. a) Visualisation of the plaque in a similar manner to 2D ultrasound. b) Successive longitudinal slices of the plaque are taken until the entire plaque has been transversed. c) This successive ‘slicing’ allows 3D visualisation of the plaque. d) A virtual ‘mesh’ is fitted to the 3D plaque highlighting plaque morphology in its entirety and providing thorough visualisation of the disease. Image taken from: (50)

During CEA, the operating Vascular Surgeon removed the plaque ‘En Bloc’. This ensures that the plaque can be measured in its entirety, reducing error in plaque volume and dimensional measurements, as well as allowing accurate trisection. The plaque was then placed into a sterile sample pot and immediately submerged in ice in order to preserve the sample. Plaques were measured, processed and frozen within 20-30 minutes of surgical removal to maintain sample viability. Following transportation to the laboratory, the plaque was washed in sterile water to remove blood, tissue and any other contaminants. Once cleaned, length and weight measurements were taken. The weight of the plaque was taken using a highly accurate set of scales (Adam, UK) Suspended weight of the plaque was then measured, as described in the method below. From these measurements a manual carotid plaque volume can be calculated.

Hydrostatic suspension technique was used to measure true plaque volume of the sample. This technique determines volume of the sample through measurements recorded whilst the sample is submerged in fluid of a known density. In order to maintain a high degree of accuracy, the plaque
must be suspended whilst it is submerged. It must not be resting on the side or base of the container in which the measurement is taking place. Plaque volume is determined by noting the weight increase of a set amount of fluid once the plaque sample has been submerged. This figure is then divided by the known density of the fluid. The equation is as follows:

\[ V = \frac{\Delta w}{D} \]

V is the unknown plaque volume, \( \Delta w \) is the change in weight of the fluid following submersion of the plaque and D is the known density of the fluid.

FIG.18 Diagram to show the hydrostatic tension technique used to manually measure carotid 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." Adapted from (152)

6.5 Antiplatelet Resistance

Patient antiplatelet dose and length of time on the given dose was recorded when patient demographics are taken. Patient compliance on day of data collection was monitored using drug charts as all patients will be inpatients on the vascular wards. Antiplatelet sample was not collected if patient had not taken antiplatelet medications within the last 24 hours.

Patients underwent venepuncture prior to surgery and 2ml of blood was collected into a double wall hirudin blood tube. Samples were transported carefully in order to avoid excess shaking and ensure sample preservation. According to guidelines, Multiplate analysis must be carried out 90 minutes after the sample is taken.
**FIG.19** A standard Roche Multiplate analyser (Basel, Switzerland). Adapted from (153).

A Roche Multiplate Analyzer, identical to that shown in Fig.19, was used for analysis of antiplatelet resistance. The Multiplate Analyser works using impedance aggregometry, a process in which platelet agonists activate platelets within the blood sample through various biochemical pathways. These pathways can be inhibited by antiplatelet medications. Activated platelets aggregate to electrodes in the cuvettes, this increases electrical resistance. Blood samples with a high abundance of activated platelets will give a high level of electrical resistance indicating that no inhibition is taking place.

The patient subject number was entered into the Multiplate system. 300µl of blood was then pipetted into each Multiplate test cuvette along with 300µl saline buffer. This mixture was incubated for 90 seconds. Following this incubation, 30µl of a different individual platelet agonist was added to each cell. In one cell arachidonic acid was added, this activates platelets via the aspirin inhibited pathway. In the next cell ADP was added, this activates platelets via the Clopidogrel inhibited pathway. A thrombin receptor activating peptide (TRAP) was added to the final cell. This peptide acts as a control, activating platelets via a pathway uninhibited by antiplatelet drugs. This control confirms that the platelets are functional and capable of aggregation (154, 155). Sample mixtures are further incubated for 6 minutes and data is automatically plotted onto individual platelet aggregation curves. These graphs present data as aggregation units (Au) against time, as per Multiplate instructions. Platelets are deemed to be resistant if their aggregation curve for aspirin is above 50Au and above 44Au for Clopidogrel (154-156). If the TRAP control does not successfully cause platelets to aggregate, then the test is deemed invalid and
must be repeated if possible. Figure 20 depicts the various responses given by patients of varying antiplatelet resistance.

**FIG. 20** The platelet aggregation curves displayed above show the platelet response in patients responding normally to antiplatelet medications and patients displaying a level of resistance, low responders. Patient ‘A’ shows the expected response to aspirin, a large reduction in aggregation units. Patient ‘B’ shows the expected response to dual antiplatelet therapy, a reduction in aggregation units for both aspirin and clopidogrel mediated pathways. Patient ‘C’ shows low response to aspirin, presenting platelet aggregation levels ten times higher than patient ‘A’. Aggregation levels in patient ‘A’ are high despite therapy. Patient ‘D’ is also on dual therapy, this patient is responding to aspirin but shows a low response to clopidogrel, aggregation units are over four times higher than in the responding patient. Adapted from (85) and (86).

6.6 Transcranial Doppler Monitoring

Along with measurement of carotid stenosis, plaque volume and aspirin resistance, patients underwent an hour TCD reading prior to CEA. The temporal window was used to detect micro-embolic signals in the middle cerebral artery ipsilateral to the diseased carotid artery, as shown in
Figures 8 and 9. Due to the sensitive and difficult nature of maintaining simultaneous ipsilateral and contralateral TCD signals, one 60 minute ipsilateral T.C.D recording was deemed to be sufficient. Two technicians analysed the recording in order to reduce potential sources of error.

In regards to measurement of MES, current and previous research dictates that an MES is present when a signal meets the following criteria:

1) A duration of >300ms.
2) An amplitude at least 3 decibels higher than the background blood flow signal
3) Is typically unidirectional
4) Occurs at random in the cardiac cycle
5) Is accompanied by an audible 'chirp' sound (157).

Before carrying out TCD monitoring, potential sources of artefacts were ruled out for each patient. Artefacts can be described as MES-like signals created by interference from the patient, technician or equipment (158). These artefacts appear as false MES (158). An example of a true emboli is given in figure 10. Artefacts were ruled out by asking the patient to carry out movements such as coughing and talking prior to TCD monitoring. The technician also ruled out equipment derived artefacts by tapping and moving the TCD probe.

Patients were required to remain still and silent throughout monitoring and a researcher was present at all times to record any occurrence of MES or artefacts. A specific data sheet (Appendix 1) was used to record TCD activity throughout the hour; the technician will note any artefacts that occur as well as the cause if possible, i.e. patient movement or speech. After monitoring, the TCD recording was reviewed by the same researcher to validate the MES number. A different researcher then carried out a further review in order to decrease any potential sources of error and confirm MES numbers. If both researchers give differing opinions on an MES, a third technician reviewed the MES before casting a deciding opinion.

6.7 Histology + Mass Spectroscopy Based Proteomics, Metabolomics and Trace Metals

Our intention was to analyse both the plasma proteome and metabolomics profile of each patient using cutting edge mass spectroscopy techniques at CADET, based at the Central Manchester Foundation Trust. Omic analysis was to be carried out in order to identify any molecular markers that can be used to predict patient outcome. Following data collection it was possible to analyse patient plasma in various subgroups comparisons. For example symptomatic and asymptomatic, patients with MES and without MES, those with antiplatelet resistance and those without.

Due to time constraints and other prioritised projects within the proteomics lab, metabolomics was the main focus of ‘omic analysis. Preparation and processing of metabolomics samples for mass spec analysis is very complex, these intricate process are detailed in appendices 3 and 4.
Not only were patient blood samples be analysed using this technique, but the plaques removed during surgery were also subjected to ‘omic and histological analysis. In order to facilitate different methods of analysis of the plaque, samples were cut up into several sections as shown in Fig. 24. Following collection and measurement of the plaque, it was tri-sected and subsequently tri-sected again to give 9 sections from one sample. Due to the varied morphology between different regions of plaque, the 9 samples were numbered from 1-9, with 1 being the most proximal, moving across to 9 as the most distal. This allowed correlation of analysis of each segment with its overall location within the plaque (Fig. 21).

**FIG. 21** Image A shows a symptomatic plaque in its entirety following endarterectomy and before dissection. Image B shows the same plaque dissected into 9 separate samples for proteomic, metabolomic and histological analysis. Image C shows the plaque sections in pre-labelled cryovials, this reduces bias by pre-determining which parts of the plaque undergo which form of analysis.

Before the plaque was removed during the CEA 9 cryovials were labelled 1 to 9, with the patient’s subject number and surgery date. Each cryovial was assigned to either histological, proteomic or metabolomic analysis. This pre-labelling helped to reduce any potential bias that may arise in selecting which plaque section will undergo which form of analysis upon visual examination of the
sample. Sections that were to be analysed for histology and metabolomics were placed straight into the corresponding cryovials. Plaque sections for proteomics were washed twice in Phosphate Buffered Saline and once in 0.25M sucrose solution. Plaques sections were then frozen at -80°C.

Once sufficient samples numbers were collected, histological samples were sent to the Histology Research Group at Manchester Royal Infirmary to be processed. Samples were analysed by a trained histology technician who noted the presence of any and all histopathological processes in each plaque segment. Histological stains included: Haematoxylin and Eosin, Elastika Van Giesen, Alcian Blue and Period Acid Schiff, ORCEIN, Masson’s Trichrome, Martius Scarlet and Blue and Congo Red. Specific histopathological processes that samples were examined for were: inflammation, fibrosis, necrosis, bone formation, bony metaplasia, calcification, neovascularisation, collagen deposition, infiltrating fibroblasts and macrophages, elastic tissue pathologies and lipid clefts. Visually distinguishable presence of ulceration, ruptured fibrous plaque or necrotic core was also be noted. Plaques were graded on their severity of disease using American Heart Association guidelines (159).

Metabolomic and proteomic plaque samples were taken to CADET to be processed and analysed using the same techniques as the blood samples. It was hoped that it would be possible to link metabolomic and proteomic anomalies within the plaque, as well as histopathological processes, to circulatory biomarkers in patient blood samples. Proteomics and metabolomics were be analysed using mass spec in combination with both liquid and gas chromatography.

Before samples are passed through the mass spectrometer they must be processed according to CADET protocols. Samples must undergo processes such as digestion and extraction in order to reach a stage in which they can be passed through the Mass Spec. As previously mentioned, the techniques required for these processes are extensive and full details can be found in Appendices 3 and 4.
This image visualises the GC/LC-MS process. Beginning at the right of the diagram, the sample is injected into the chromatograph. In the case of GC a carrier gas, the mobile phase, will carry the sample through the capillary tube, containing the stationary phase. The molecules elute into the mass spectrometer where they are further separated, identified and quantified. Taken from (160).

GC-MS (Gas Chromatography – Mass Spec) and LC-MS (Liquid Chromatography - Mass Spec) are techniques that merge the methods of chromatography and mass spec in order to identify and quantify the individual components of a biological or chemical sample. As an example, GC-MS is comprised of two major components; the gas chromatograph and the mass spectrometer (Fig.22). The gas chromatograph uses a capillary column containing a stationary phase through which the mobile phase containing the sample is passed. In GC-MS the mobile phase is gas, whereas in LC-MS it is liquid. The column separates molecules in the sample by their varying degrees of affinity to the stationary phase. Affinity to the stationary phase will determine the retention times of molecules in the sample. Higher affinity results in a longer retention time and therefore the molecule elutes from the column at a slower pace than molecules with lower affinity and shorter retention times.

This initial sample separation by chromatography allows the mass spectrometer to process each molecule separately, allowing for a more refined identification process. The mass spectrometer consists of 3 components, an ion source, mass analyser and detector. The molecules from the chromatograph are passed into the mass spec’s ionizer, here voltage is applied which ionizes the molecules in the sample. As a result of this process ions can fragment, these full ions and fragments are passed into the mass spec’s analyser. Passing through an electric field, the speed and degree of deflection of the ions increases or decreases dependant on their mass to charge ratio. The ions are deflected onto the mass spec detector which can identify and quantify the ions and subsequently the molecules present in the original sample. The results are presented on a mass spectrum, pictured in FIG.23.
FIG. 23 A mass spectrum showing the molecular components of dodecane. Taken from: http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/MassSpec/massspec1.htm

Each bar in the mass spectrum represents an ion with a specific mass/charge ratio. The height of the bar is the relative abundance of that ion. The heaviest ion, the one with the highest m/z ratio, is assumed to be the mass ion, which is effectively the complete molecule. In the example of Fig. 23, the complete molecule is Dodecane, which is to the far right of the spectra with an m/z of 170. Identification of the ions and subsequent molecules, in both blood and plaque samples, allows us to attempt to draw comparisons between metabolic processes within the plaque and any markers in the blood that reflect these processes.

Our preliminary mass spec analysis indicated that there would be excess plaque tissue available for alternative methods of analysis. It was decided that this tissue should be used for inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS specifically analyses trace metals within the tissue and is capable of detecting these elements at levels as low as 1 part in $10^{15}$. Plaque tissue left over from the extraction process for GC/LC-MS, was digested according to local digestion protocols (Appendix 4). Digestion acid comprises of a mixture of nitric acid and ICP-MS internal standard. The digestion acid was added and the samples were heated, during which digestion takes place. During the digestion process it is possible to lose a small amount of sample during boiling. In order to counteract the loss of sample when carrying out data analysis the sample is weighed both pre (wet weight) and post (dry weight) digestion. This allows data analysis to be corrected to either of these weights and give a more accurate set of results. Dry weight is deemed the most reliable as use of this weight accounts for any tissue lost during the digestion phase. Following digestion the samples were mixed with LC-MS water to provide the solution required for analysis by the ICP mass spectrometer. Digested samples were ionized with inductively coupled plasma and the ions separated and quantified by the mass spectrometer.

6.8 Post-Operative Follow up

Patients underwent follow up at 2-3 months post CEA, coinciding with the patient’s post-op clinical follow up. Occurrence of cerebrovascular symptoms was the primary outcome measure of this
follow up. Serum biomarkers were also repeated alongside further antiplatelet measurements. This would potentially present the effects of plaque removal upon circulatory biomarkers and patient antiplatelet resistance. Follow up biomarker measurements that vary from those measured pre-operatively could be identified as biological signals associated with plaque burden and therefore stroke risk. Due to work load and time constraints, this follow up work was converted into a full side-project including further ultrasound scans of the previously affected artery. This side project was being carried out by a colleague and has not yet been completed.

6.9 Data analysis

Sample size calculations were necessary in order to define required sample numbers for aims 1 and 2. Aim 1 was to regress against the primary outcome measures of plaque volume and carotid stenosis. Aim 2 was the identification and addition of antiplatelet resistance to this model. Using the multiplate system for impedance aggregometry, antiplatelet resistance can be defined as an insufficient increase of platelet inhibition following the administration of an antiplatelet medication.

Further analysis included known risk factors of stroke between platelet resistance and non-resistance groups. This included levels of MES and CPV measurements in order to determine any link between antiplatelet resistance and MES/CPV. Another aim was to create prediction models incorporating the multiple factors analysed as part of this project in order to provide more accurate prediction capacity. This aim was not reached in this project due to limitations of data available. It is hoped that increased patient numbers incorporated by colleagues furthering this project in my absence will result in data sufficient for full risk prediction modelling. Associations between CPV, carotid stenosis and MES in each patient were be analysed in particular detail.

Mass spectrometry trace metal and metabolomic data was analysed and levels of any potential serum biomarkers, as well as those present in plaque tissue, were compared between patients from varied sub-categories. For example asymptomatic and symptomatic patients, patients with and without MES. These sub-groups were analysed in order to identify any association between stroke risk and serum biomarkers.

In regards to our ‘omic analysis, it is usually the case that the number of features reported is much larger than the number of samples analysed. This makes ‘false discovery’ (i.e. a significant difference which is purely chance), likely. This topic has been addressed in some detail and a number of useful approaches described. The method used in our current work (5% as defined Benjamin-Hochberg) is generally accepted, allowing us to be confident in our analysis of the data collected. Trace metals were analysed using a series of linear regression models. Linear regression was selected over non-linear regression due to our preference for keeping the complex preliminary analysis as straight forward as possible. With the information available to us from previous work, it seemed highly unlikely that non-linear regression would present us with anything that linear modelling would not.
7. Results

121 CEA patients (Symptomatic $n = 95$, asymptomatic $n = 26$) at University Hospital South Manchester were recruited to the study. Patients underwent a medical history and lifestyle review, 2D ultrasound scanning of the carotid arteries, 3D Ultrasound scanning of the carotid arteries, transcranial Doppler monitoring for presence of micro-emboli, antiplatelet resistance testing and phlebotomy. Plaques were removed during surgery, collected by our research team, dissected and frozen. Plaques were later transferred to the Centre of Advanced Discovery and Experimental Therapeutics for ‘omic and histological analysis.

7.1 Demographics, Co-Morbidity and Laboratory Data

**TABLE.3** - Demographic, Co-morbidity and Laboratory data for Symptomatic and Asymptomatic patient groups. Parameters highlight in bold showed a significance level of $P = <0.05$.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Asymptomatic mean (SD)</th>
<th>Symptomatic Mean (SD)</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>70.54 (1.60)</td>
<td>70.52 (0.92)</td>
<td>0.99</td>
</tr>
<tr>
<td>BMI</td>
<td>28.21 (0.98)</td>
<td>27.54 (0.69)</td>
<td>0.61</td>
</tr>
<tr>
<td>Pack Years</td>
<td>27.78 (3.13)</td>
<td>34.43 (4.17)</td>
<td>0.32</td>
</tr>
<tr>
<td>Alcohol (Units)</td>
<td>7.08 (2.19)</td>
<td>6.96 (1.41)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Co-morbidity</th>
<th>$n$ Asymptomatic (%)</th>
<th>$n$ Symptomatic (%)</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>23 (79.3)</td>
<td>65 (73.0)</td>
<td>0.62</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>21 (75.0)</td>
<td>58 (65.2)</td>
<td>0.36</td>
</tr>
<tr>
<td>Ischemic Heart Disease</td>
<td>7 (25.0)</td>
<td>14 (15.7)</td>
<td>0.27</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7 (33.3)</td>
<td>19 (27.1)</td>
<td>0.80</td>
</tr>
<tr>
<td>Previous Cerebrovascular</td>
<td><strong>11 (39.3)</strong></td>
<td><strong>15 (16.9)</strong></td>
<td><strong>0.019</strong></td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood result</th>
<th>Asymptomatic mean (SD)</th>
<th>Symptomatic Mean (SD)</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.92 (0.28)</td>
<td>4.50 (0.16)</td>
<td>0.18</td>
</tr>
<tr>
<td>Albumin (mmol/L)</td>
<td>36.53 (1.05)</td>
<td>37.56 (0.46)</td>
<td>0.35</td>
</tr>
<tr>
<td>Alkaline Phosphatase (mmol/L)</td>
<td>79.07 (3.80)</td>
<td>83.08 (3.33)</td>
<td>0.57</td>
</tr>
<tr>
<td>ALT (mmol/L)</td>
<td>17.40 (1.67)</td>
<td>20.35 (1.42)</td>
<td>0.31</td>
</tr>
<tr>
<td>Bilirubin (mmol/L)</td>
<td>8.8 (0.91)</td>
<td>10.03 (0.68)</td>
<td>0.40</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>6.4 (0.37)</td>
<td>6.42 (0.28)</td>
<td>0.99</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>84.08 (4.44)</td>
<td>88.83 (3.51)</td>
<td>0.50</td>
</tr>
<tr>
<td>Haemoglobin (mmol/L)</td>
<td>134.6 (3.21)</td>
<td>133.5 (2.56)</td>
<td>0.82</td>
</tr>
<tr>
<td>White Blood Cells (mmol/L)</td>
<td><strong>7.44 (0.34)</strong></td>
<td><strong>8.55 (0.24)</strong></td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>Platelets (mmol/L)</td>
<td>236.2 (9.11)</td>
<td>246.2 (7.34)</td>
<td>0.48</td>
</tr>
<tr>
<td>C-Reactive Protein (mmol/L)</td>
<td>3.8 (1.23)</td>
<td>9.19 (1.93)</td>
<td>0.15</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>139.7 (0.51)</td>
<td>138.7 (0.37)</td>
<td>0.20</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.22 (0.08)</td>
<td>4.26 (0.04)</td>
<td>0.62</td>
</tr>
<tr>
<td>Estimated Glomerular Filtrate</td>
<td>74.46 (2.81)</td>
<td>69.87 (2.12)</td>
<td>0.27</td>
</tr>
<tr>
<td>Rate (mls/min/1.73m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Very few differences were observed between symptomatic and asymptomatic patient demographics (Table 3). Patients displayed similar age, BMI, smoking history and alcohol consumptions levels. The only discernible difference within these demographics was the slightly higher number of pack years in the Symptomatic group (34.43±4.17 vs 27.78±3.13, P = 0.32).

Both sub-groups of patients in our study clearly display raised risk factors associated with CAD, mean age in both groups was 70 years old and mean BMI was in the ‘overweight’ classification between 25-29.9. Whilst these factors are likely raised in comparison to a healthy patient cohort, there is very little difference between our sub-groups.

Co-morbidities associated with advanced atherosclerosis were abundant in both subject groups. Hypertension, closely associated with the early developmental pathologies of CAD, showed a prevalence of 70-80% across both symptomatic and asymptomatic patients. High Cholesterol was also highly prevalent in 65-75% of patients. Diabetes presented in approximately a third of both patient groups, another logical occurrence due to the close association between diabetes and Atherosclerosis(162). Perhaps the most interesting data relating to medical history and co-morbidities was related to patients’ previous cerebrovascular symptoms. The percentage of asymptomatic patients reporting previous symptoms was considerably higher than that of the symptomatic group, 39.3% to 16.9% (P = 0.018).

Similar to both demographics and patient co-morbidities, routine blood results between the patient sub-groups were also very similar. These blood results are retrieved from the local hospital laboratory and are not derived from mass spec analysis of serum samples. Noticeable differences between symptomatic and asymptomatic patients were CRP (9.19 ± 1.93 vs 3.8 ± 1.23, P = 0.15) and White blood cell counts (8.55 ± 0.24 vs 7.44 ± 0.34, P = 0.02). Though there was a large difference between mean values of CRP, it did not reach a level of significance. The only routine blood measurement that showed a statistically significant difference between patient groups was the patient white blood cell count, with the symptomatic cohort showing raised levels (8.55 ± 0.24 vs 7.44 ± 0.34, P = 0.02).
7.2 Carotid Plaque Volume

Carotid Plaque Volume was measured using the immersion technique described in section 3.4. The aim of this analysis was to establish differences in plaque volume dependant on patient sub group. The symptomatic group were then differentiated to assess any differences in plaque volume between severity of symptoms. Lastly plaque volume across all patients was compared to degree of stenosis. This comparison allowed us to investigate any relationship between CPV and stenosis, a vital comparison in proving whether CPV can be used as an alternative primary risk predictor for Stroke.

![Carotid Plaque Volume and Symptoms](image)

**FIG.24** A graph to show the relationship between Carotid Plaque Volume and Patient group. Unpaired T-Test showed that Carotid Plaque Volume was significantly higher (P = <0.05) in symptomatic CAD patients (0.836 ± 0.035cm³, n=74) when compared with asymptomatic patients (0.629 ± 0.056cm³, n=20).
Using an unpaired T-test our research shows a significant relationship between symptomatic patients and higher plaque volumes (P <0.05) (Fig 24). Using a one way ANOVA test we could not display a statistically significant link between CPV and symptom severity ($F(2,77) = 2.199, P = 0.08$), however we have shown a general trend of increasing plaque volumes with increasing severity of cerebrovascular symptoms, Mean CPV gradually increased from Amaurosis Fugax (0.721 ± 0.056cm3, n = 12), to TIA (0.844 ± 0.056cm3, n = 43) and again to Stroke (1.041 ± 0.118cm3,
n = 25). We found no significant correlation between carotid plaque volume and degree of stenosis (F(4,87) = 0.3380, P = 0.852) (Fig 26).

7.3 Antiplatelet Resistance

Antiplatelet Resistance was carried out using the Multiplate (Roche, Germany) device. Fifty four patients had blood samples taken for antiplatelet resistance pre-surgery, 9 were resistant, of which 6 were symptomatic and 3 asymptomatic (Fig 27). Antiplatelet resistance prevalence is widely debated within the cardiovascular disease community. We investigated the presence of antiplatelet resistance in CAD patients with the aim of analysing relationships between antiplatelet resistance and parameters such as plaque volume and frequency of micro-emboli.

**FIG.27** Multiplate Antiplatelet resistance levels amongst symptomatic and asymptomatic patients in the study. 54 patients underwent platelet aggregometry measurements with a total of 9 (16%) being resistant.
An unpaired T-test was used to show the relationship between carotid plaque volume and patient response to antiplatelet medication.

There is a close to significant association between carotid plaque volume and antiplatelet resistance ($P = 0.059$) (Fig 28). The difficulty with interpreting these results is that the numbers in the Resistant group ($n=9$) were comparatively low when considered in relation to the Responsive group ($n=45$). With an antiplatelet resistance rate of 16% it is clear that we have not observed the higher percentages of antiplatelet resistance that both our predecessors and fellow researchers have experienced, with meta-analyses showing resistance at a rate of 39% ($^{(89)}$).

7.4 Transcranial Doppler Monitoring – Micro-emboli

Pre-surgery, patients underwent one hour of transcranial Doppler monitoring. We set out to establish the level of micro-emboli in our cohort and compare the frequency of these signals with parameters in the study such as CPV and antiplatelet resistance. We also investigated occurrence of micro-emboli based on patient sub-group and symptomology.
TABLE 4  Transcranial Doppler Monitoring was carried out in 14 patients. The time consuming nature of T.C.D. results in few patients completing the full hour recording. 10 patients presented with cerebral emboli, 9 of which were symptomatic. A total of 37 emboli were observed, 31 emboli were detected in symptomatic patients and 6 emboli in asymptomatic patients.

<table>
<thead>
<tr>
<th>T.C.D and Micro-emboli</th>
<th>No. of patients monitored</th>
<th>No. patients with emboli</th>
<th>Total no. of emboli</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amaurosis Fugax</strong></td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><strong>T.I.A</strong></td>
<td>6</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td><strong>Stroke</strong></td>
<td>4</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td><strong>Asymptomatic</strong></td>
<td>3</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>14</td>
<td>10</td>
<td>37</td>
</tr>
</tbody>
</table>

Few conclusions can be drawn from the micro-emboli data we have collected in this study. As would be expected, the symptomatic group display substantially more micro-emboli than the asymptomatic patients (Symptomatic n=31, asymptomatic n=6). An interesting observation of the data we have collected is the substantially higher level of emboli in the TIA group (n=23) when compared to Amaurosis Fugax (n=4) and particularly, Stroke (n=7) (Table 4). Linear regression of correlation between CPV and Emboli showed no significant correlation between the two measurements ($R^2 = 0.1309$, $P = 0.2244$). Antiplatelet resistant and responsive sub-groups showed no significant difference in numbers of micro-emboli ($P = 0.682$).

7.5 Histology

Plaque samples collected from surgery were dissected into 9 sections, with 3 of these analysed histologically. This histological analysis allowed us to view individual pathologies within the plaque such as intraplaque haemorrhage and macrophage infiltration. Having such detailed histological processing also allowed us to give plaques a ‘stage’ of severity, based on American heart association guidelines, as seen in Table 6.

**Table 5 – Table to show the number of symptomatic and asymptomatic patients with stage V or VI plaques (according to American Heart Association Guidelines)**

<table>
<thead>
<tr>
<th>Plaque Stage</th>
<th>Symptomatic (n=14)</th>
<th>Asymptomatic (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V (a, b or c)</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>VI</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 6 Key features of the stages of plaques seen in both symptomatic and asymptomatic patients. No patient presented with a plaque staged earlier than V.

<table>
<thead>
<tr>
<th>Plaque Stage</th>
<th>Va</th>
<th>Vb</th>
<th>Vc</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Key Features</strong></td>
<td>Large necrotic core</td>
<td>Large necrotic core</td>
<td>Small or absent necrotic core</td>
<td>Large necrotic core</td>
</tr>
<tr>
<td></td>
<td>Thick fibrous cap</td>
<td>Thin fibrous cap</td>
<td>Fibrous throughout</td>
<td>Very thin fibrous cap</td>
</tr>
<tr>
<td></td>
<td>Little to no calcification</td>
<td>Varying levels of calcification</td>
<td>Little to no calcification</td>
<td>Varying levels of calcification</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Structural disruption – IPH, ulceration, rupture.</td>
</tr>
</tbody>
</table>

The most important aspect of the histological analysis is displayed in Table 5. In this study all plaques, whether symptomatic or asymptomatic, were at an advanced stage (Stage V and above according to American Heart Association Guidelines \(^{159}\)). With the high level of severity of all plaques analysed, it is evident that only a small number of end stage pathologies are responsible for plaque transformation from asymptomatic to cerebrovascular symptom inducing.

7.6 Metabolomics – Preliminary Analysis

We used dual mass spectrometry and chromatography techniques to carry out molecular analysis of human and animal tissues. These techniques had not previously been carried out for analysis of human plaque tissue. Preliminary metabolomic analysis of 6 plaques was carried out in order to investigate whether our standard GC-MS/LC-MS protocols could be applied to plaque tissue.
FIG. 29 Principal Component Analysis (PCA) Plot of Preliminary metabolomic Liquid Chromatography - Mass Spectrometry data of 6 carotid plaques. 'Q' represents the quality controls used in the process. Each number represents the subject number relating to the plaque. 3 sections were taken from each plaque, giving 18 samples. Samples highlighted in red, subject 6, belong to an asymptomatic plaque, a relative outlier when compared with other sample sections.

PCA results (Fig 29) showed that plaque tissue was a viable source of metabolomic data and is robust enough to deal with the processing required for mass spec analysis. Of the 6 plaques used, there were 3 sections per plaque, therefore 18 individual sections. The data included in the preliminary analysis was a basic, raw form of data based on Principal Component Values. In simplistic terms, these values indicate how ‘different’ the various parts of each plaque sample are from each other. Of the 6 patients 5 were symptomatic and 1 asymptomatic. Subject 6, highlighted in red, is the asymptomatic patient. It appears that there is a difference in the molecular composition of subject 6’s plaque, as it is a relative outlier when compared to the other samples. These preliminary tests provided important information in regards to the capabilities of GC-MS/LC-MS plaque ‘omic analysis and what could be seen in the analysis of a full patient cohort of plaques.
7.7 Inductively Coupled Plasma Mass Spectroscopy – Trace Metals

FIG. 30A  Ca/Pb

FIG. 30B  Ca/Zn

FIG. 30C  Ca/Na

FIG. 30D  Ca/Mg

Ca/Fe - Corrected to Dry Weight

Ca/Fe - Corrected to Wet Weight

Ca/Cu
**FIG.30A** (Ca/Pb), **FIG.30B** (Ca/Zn), **FIG.30C** (Ca/Na), **FIG.30D** (Ca/Mg), **FIG.30E** (Ca/Fe – Dry), **FIG.30F** (Ca/Fe – Wet), **FIG.30G** (Ca/Cu). Linear Regression of Correlation graphs to show the positive correlation between Lead, Zinc, Sodium, Magnesium and Calcium within both symptomatic and asymptomatic carotid plaque samples.

Linear regression of correlation was carried out on several trace metals detected by ICP-MS. calcium and lead (R² 0.7113; p = <0.0001) (Fig 30A), calcium and zinc (R² 0.6867; P = <0.0001) (Fig 30B), calcium and sodium (R² 0.9499; P = <0.0001) (Fig 30C) and calcium and magnesium (R² 0.9736; P = <0.0001) (Fig 30D) all showed significant positive correlation. There was no significant correlation with between calcium and copper (R² 0.0187; P = 0.448) (Fig 30E) or calcium and iron corrected to both wet (R² 0.0448; P = 0.222) (Fig 30F) and dry weights (R² 0.0003; P = 0.928) (Fig 30G).

![Copper corrected to dry weight](image)

**P = 0.065**

**FIG.31** Scatter plot to show differences in concentration of copper between patient groups.

Overall there was very little difference in trace metal content between symptomatic and asymptomatic plaques. An unpaired T-test showed no statistically significant difference in copper levels between the patient groups (P = 0.065), however the overall trend indicated higher presence of copper in the symptomatic group (Fig.31). Due to discrepancies in weights recorded post-digestion, all concentrations of trace metals were corrected to the wet weight of the sample measured prior to the digestion process. This is less accurate than using the post digestion ‘dry’ weight of each sample, as it is possible to lose some of the sample during the digestion process. This weight loss is not accounted for by the pre-digestion wet weight.
**FIG. 32A** (Iron corrected to Wet Weight) and **FIG 32B** (Iron corrected to Dry Weight). Scatter plots to show the difference in iron concentrations between the patient groups. Both wet and dry weights are presented. Unpaired T-tests showed that there was a significantly higher level of iron present in symptomatic plaques compared with the asymptomatic group ($P < 0.05$) when correcting to wet weight. This significance was lost when using the dry weights of the samples ($P = 0.31$).

The significance of differences of iron levels between the two patient groups presented mixed results. When the pre-digestion wet weight was used there was a clearly significant difference between the two groups (Fig 32A), with symptomatic plaques displaying higher Iron levels. When correcting to the post-digestion dry weight (Fig 32B), which is deemed to be more accurate, mean iron levels were still higher in the symptomatic group, but this was not significant.
8. Discussion

8.1 Demographics, Co-Morbidities and Laboratory Data

Overall very few differences were observed between symptomatic and asymptomatic patient demographics, co-morbidities and laboratory results. Patient displayed similar levels of expected risk factors across demographics and co-morbidities. Slightly higher number of pack years were observed in the symptomatic group (34.43±4.17 vs 27.78±3.13, P = 0.32). Due to the association between smoking and CAD\(^{(163)}\), it would be logical that those with a richer smoking history would be more likely to be symptomatic, however the difference between the figures we have presented is far from significant (P = 0.32).

The percentage of asymptomatic patients reporting previous symptoms was considerably higher than that of the symptomatic group, 39.3% to 16.9% (P = 0.018). There are a number of potential reasons for this. Firstly, after their initial symptoms patients will have both carotid arteries scanned. This will often give incidental findings of stenosis in the asymptomatic carotid, contralateral to the symptom inducing artery. The symptomatic carotid is the clear priority and will be operated on first, however in routine follow up following surgery both arteries will continue to be scanned. This asymptomatic artery, contralateral to the symptomatic artery which was operated upon, may gradually increase to a level of stenosis that requires surgery. By this point the patient will have a history of stroke, but will be classed as an asymptomatic surgical case. Another potential explanation is that these patients have suffered cerebrovascular symptoms but have failed to seek medical help, dismissing the event as a ‘funny turn’ or similar. This can result in a healing of the plaque which may involve pathologies such as incorporation of thrombus and push the level of stenosis towards a level requiring intervention. It maybe some time before it is discovered that the patient has substantial CAD and is medically treated. With these conclusions in mind, it seems unlikely that anything of clinical importance can be drawn from the relationship between asymptomatic patients and previous cerebrovascular symptoms.

Laboratory results were very similar between groups; however two measurements did stand out. Firstly CRP was considerably higher in the symptomatic group (9.19 ± 1.93 vs 3.8 ± 1.23, P = 0.15), although this was not deemed to be significant. Though there was a large difference between mean values of CRP, it did not reach a level of significance. CRP is an acute inflammatory protein and circulatory CRP levels are related to cardiovascular disease as well prediction of heart attack and stroke\(^{(164)}\). CRP levels are also associated with the overall inflammatory state of the patient, with high CRP levels correlating with high levels of systemic inflammation\(^{(165)}\). With CRP known as a mediator of inflammation within atherosclerosis, its relative abundance in symptomatic blood samples when compared with asymptomatic patients is hugely interesting. Although we have
not shown a statistically significant difference between the sub groups, it would be reasonable to investigate this biomarker in larger patient cohorts.

Mean white blood cell counts between the two groups showed a statistically significant difference, with the symptomatic cohort showing raised levels (8.55 ± 0.24 vs 7.44 ± 0.34, P = 0.02). In the literature Grau et al presented evidence that WBC count is an independent predictor of both first time and recurrent vascular events, whilst Raffel et al presented a link between WBC count and vulnerable plaque in coronary artery disease patients\(^{166, 167}\). Monocytes, macrophages and foam cells all play a key role in perpetuating the inflammation within the atherosclerotic plaque\(^{168}\). Some authors propose that macrophage migration into the edge of the fibrous cap is one of the late stage pathologies resulting in plaque rupture. In this location macrophages are releasing a cocktail of inflammatory mediators and proteolytic enzymes into the area of the plaque most vulnerable to the shear stress of the systemic circulation\(^{169}\). Within the core of the plaque, macrophage uptake of localised lipids and cholesterol results in conversion to foam cells. Upon apoptosis these foam cells release their cholesterol back into the necrotic core\(^{168}\). Saturation of free cholesterol in the plaque can lead to cholesterol crystallisation, another late stage pathology associated with plaque rupture\(^{23}\). These pathologies explain why WBC levels may be raised within the symptomatic plaque, as late stage pathologies contribute to symptom inducing rupture of the atheroma. However they do not explain the raised levels in the systemic circulation. It may be that inflammatory mediators released from the cells such as macrophages within the plaque cause a systemic inflammatory cascade in which haematopoiesis is increased and more immature WBCs are released into the blood. A systemic rise in WBCs may also mean that more of these cells are incorporated into the plaque, further perpetuating intra-plaque inflammatory processes. CRP is also known to interact with monocytes and macrophages within the carotid plaque, perhaps giving rise to a link between WBC counts and CRP within patients with extensive atherosclerosis.

Current research shows that routine blood results such as WBC count, and potentially CRP, can be used to indicate patients at higher risk of cardiovascular disease. Our study has built upon this, showing general and significant trends that present CRP and WBC count as measurements that will further differentiate these cardiovascular patients, specifically CAD patients, into those who are likely to go on to suffer stroke. Though CRP and WBC measurements cannot be used in isolation to detect CAD sufferers likely to experience cerebrovascular symptoms, they are certainly a potential parameter for inclusion in any risk prediction model.

### 8.2 Carotid Plaque Volume

Our research shows a significant relationship between symptomatic patients and higher plaque volumes (P = <0.05). These findings are in agreement with other studies investigating the relationship between symptomology and CPV\(^{7, 8, 13, 21}\). Though lacking significance, we have also shown a general trend of increasing plaque volumes with increasing severity of cerebrovascular
symptoms, plaque volumes rising from Amaurosis Fugax to TIA and again to Stroke ($F(2,77) = 2.199, P = 0.08$). Our research continues to cast doubt upon the use of stenosis as the primary risk factor when deciding on suitable treatment methods for CAD patients. We found no significant correlation between carotid plaque volume and degree of stenosis ($F(4,87) = 0.3380, P = 0.852$). Our combined CPV findings lend strong support to the evidence for the use of carotid plaque volume over stenosis when predicting stroke in CAD patients. Larger plaque volumes in symptomatic patients, a general increase in plaque volume correlating with symptom severity and no relationship between plaque volume and stenosis form a solid foundation for CPV to be considered a stronger predictor of stroke than stenosis. No relationship between plaque volume and degree of stenosis indicates that large, vulnerable plaques may exist irrespective of how stenosed the carotid artery is. If there was a significant positive relationship between plaque volume and stenosis it would be unnecessary to pre-operatively measure CPV as it would potentially provide no more information than degree of stenosis. Our research is in agreement with Li et al, who present evidence that CPV has the ability to more accurately represent the overall disease burden in CAD sufferers. It is possible for complex plaques to exist whilst presenting sub-clinical or mild to moderate levels of atherosclerosis (12). These are high risk plaques that would be left to best medical therapy under current guidelines.

**FIG. 33** This graph summarises the contribution of three key plaque components in the carotid plaques of 57 patients. The dashed line represents fibrous tissue. The grey line represents lipid content. The solid black line represents calcification. Taken from (170).

The importance of plaque volume is related to the components that contribute to these large, volatile plaques. Fig. 33 represents three of the main constituent components of the carotid plaque.
plaque, in relation to plaque volume. Plaques with low volume tend to be largely fibrotic, as well as showing very low levels of both lipid and calcification. There is a positive correlation between plaque volume and levels of calcification and lipid content, whilst there is a negative correlation between level of fibrotic tissue and plaque volume. In contrast to low plaque volumes, high plaque volumes consist of minimal fibrotic tissues, but abundant lipid content and calcification. These findings tie in with much of the literature. Fibrosis, although pathological in most tissues, is essentially a stabilising feature of atherosclerosis. Fibrotic tissue is extremely unlikely to rupture, with very low lipid content, even if the plaque does rupture; there is very little intra-plaque material to incur thromboembolism. This greatly contrasts with advanced plaques, with larger volumes. These plaques have very low fibrous content, suggesting a thinning fibrous cap, as well as high lipid content which indicates a large necrotic core. Both of which are features of an unstable atheroma. With high plaque volumes associated with likelihood of a patient becoming symptomatic, it becomes clear that lipid content and potentially calcification are key factors in causing destabilising levels of plaque growth. Ultimately larger atheromas have more abundant intra-plaque pathologies than their smaller counterparts. If a patient presents with high CPV it is likely that their plaque is unstable, volatile and could potentially induce a cerebrovascular attack.

In vivo carotid plaque volume analysis requires an imaging method. In our study this technique was 3D ultrasound (Curefab, Munich). As well as plaque volume, 3DUS is capable of analysing parameters such as plaque ulceration\(^5\). Ulceration of the carotid plaque in CAD is associated with increased occurrence of cardiovascular events\(^5\). Ulceration is often the precursor to full plaque rupture and subsequent thromboembolism; it is therefore a key process to detect in CAD patients. These intra-plaque pathologies are hugely important in analysis of CAD. If ulceration is detectable within a plaque, it is highly likely that other advanced stage plaque intra-plaque pathologies such as IPH and cholesterol crystallisation will be present\(^{20-22}\). Detection of a large plaque volume with ulceration or rupture of the plaque is an indicator that advanced pathologies are present and that the patient should be investigated further or listed for prophylactic surgery.

We have shown, in agreement with existing literature, that plaque volume can be associated with the symptom status of the patient. We have also displayed a general trend in symptomatic patient subclasses, with a steady increase in plaque volume from mild to severe symptoms. Possibly the most important result we have displayed is the lack of any significant relationship between plaque volume and degree of stenosis. This reaffirms evidence that large, symptom inducing plaques can exist at a mild to moderate levels of stenosis. Carotid plaque volume is clearly a solid foundation upon which to build a CAD stroke risk prediction model.

\textbf{8.3 Antiplatelet Resistance}

There is a close to significant association between carotid plaque volume and antiplatelet resistance (\(P = 0.059\)). The difficulty with interpreting these results is that the numbers in the
Resistant group (n=9) were comparatively low when considered in relation to the Responsive group (n=45). Our research indicated a potential relationship between larger plaque volumes and resistance to antiplatelet medications, however the relationship was ultimately not significant (P = 0.059). Our numbers in the resistant group were quite low and it is likely that with increased resistant patient numbers we would witness the difference in plaque volume progress into a significant one; however it is difficult to say for certain. Lack of a response to antiplatelet medications may cause thrombus to be incorporated at the site of the plaque, therefore increasing plaque size \(^\text{(171)}\). If plaques undergo ulceration or rupture, platelets will adhere to the site and build into thrombus. This thrombus may break away and cause symptoms, break away and dissipate asymptotically, or it may be incorporated into the plaque during the healing process following rupture. Systemic thrombotic propensity, one of the three factors of Virchow’s Triad, plays a key role in which of these thrombus related outcomes will occur \(^\text{(171)}\). Those patients responding to their antiplatelet medication will have a far lower thrombotic propensity than those that are resistant. Those with low propensity are unlikely to form any thrombus at the plaque site and if they do it is likely to dissipate quickly due to antiplatelet aided platelet inhibition. Those who have high propensity are more likely to build large thrombi that will be either incorporated into the plaque or breakaway to cause patient symptoms. Incorporation of thrombus can greatly increase the volume of a plaque and this seems a likely explanation for the results we have observed.

Our results indicated a higher percentage of Asymptomatic patients (25%, 3 of 12 patients analysed) with antiplatelet resistance when compared with symptomatic patients (14%, 6 of 42 patients analysed). These results are not in keeping with the literature which indicates that antiplatelet resistance levels are significantly higher in symptomatic CAD cohorts \(^\text{(87-89)}\). Antiplatelet resistance is associated with occurrence of cerebral micro-emboli \(^\text{(71)}\), which in turn are associated with heterogenous plaques and plaque surface disruptions \(^\text{(73-75)}\). Heterogenous plaques and those with ulceration and rupture are significantly linked to patients with symptomatic carotid disease \(^\text{(62, 71)}\). These aspects of CAD pathology tie in well to the association of antiplatelet resistance with symptomatic atheromas, although in our research there was no significant relationship between antiplatelet resistance and emboli. It is possible that asymptomatic patients that are antiplatelet resistant may be progressing towards a symptomatic state. As we proved earlier, symptomatic patients are associated with a high plaque volume. The three asymptomatic resistant patients presented with a mean plaque volume of 1.66cm\(^3\). Plaque volumes of this size are associated with symptomatic patients \(^\text{(3, 4, 7)}\).

With an overall antiplatelet resistance rate of 16% it is clear that we have not observed the higher percentages of antiplatelet resistance that both our predecessors and fellow researchers have experienced. With the multitude of potential intervening factors that affect antiplatelet resistance it is difficult to pinpoint an exact reason why we have seen these lower levels. A most likely explanation is that our subjects were hospital inpatients, virtually guaranteeing patient medication
compliance. Patients that had not taken their antiplatelet medication on the day of testing did not undergo Multiplate analysis. With research indicating that up to 40% of cardiovascular patients do not conform to their antiplatelet medications, these pseudo-resistant patients are largely eliminated from our cohort. Although our levels of resistance are low when compared with other studies, they do fall within the widely varied ranges seen throughout research. Meta-analyses have indicated that aspirin resistance levels can lay between 3% up to 85% and Clopidogrel between a range of 28-44% (91, 92). These hugely varying ranges indicate the difficulty in proving true antiplatelet resistance and the subsequent difficulty of incorporating this parameter into clinical practice.

Overall, our research supports the current evidence that antiplatelet resistance is prevalent amongst cardiovascular patients. Overall prevalence of antiplatelet resistance or low responsiveness is widely varied. In our research antiplatelet resistance was less prevalent than many studies and was linked with patient symptomology and higher plaque volumes. The need for specifically tailored antiplatelet therapy is evident. As highlighted by Neubauer et al, monitoring and actively changing treatment results in a significant reduction in low responsiveness amongst the cardiovascular patient population (172). As part of a risk prediction profile in CAD patients, antiplatelet resistance must be investigated and acted upon. If symptomatic patients with a large CPV represent a significant number of resistant patients, it may be necessary to treat asymptomatic patients with similar characteristics as high risk, monitoring and altering antiplatelet dosage accordingly. Patients at high risk of cardiovascular events will clearly benefit from patient specific tailored therapy.

8.4 Transcranial Doppler Monitoring – Micro-emboli

Few conclusions can be drawn from the micro-emboli data we have collected in this study. As would be expected, the symptomatic group display substantially more micro-emboli than the asymptomatic patients (Symptomatic $n=31$, asymptomatic $n=6$), though there is already abundant literature which provides evidence of this difference between the two groups (67, 71, 73, 74, 173). The aim of this study, whilst continuing to support the evidence of raised micro-emboli in symptomatic patients was to further differentiate this into links between micro-emboli and plaque volume as well as micro-emboli and antiplatelet resistance. Disappointingly, we were not able to link frequency of micro-emboli with antiplatelet resistance ($0.862$), or carotid plaque volume ($R^2 0.1309, P = 0.2244$).

An interesting observation of the data we have collected is the substantially higher level of emboli in the TIA group ($n=23$) when compared to Amaurosis Fugax ($n=4$) and particularly, Stroke ($n=7$). Whilst it might be expected that Stroke, the most severe CAD derived symptom, would show the highest number of emboli, this does not appear to be the case. In those suffering stroke it may be that the majority of the thrombus that has developed at the site of the plaque has broken away
towards the cerebral vasculature, leaving behind very little thrombus that will produce micro-emboli. In those who have suffered TIA it is possible that a much smaller part of the main thrombus has broken off into the cerebral vasculature and therefore a larger thrombus remains at the plaque site, continuing to produce emboli. Alternatively a TIA may be the result of a smaller plaque rupture or ulceration, and the repeated thrombotic process at the rupture site is less severe than that of a thrombus developing at a large, plaque spanning rupture.

Although we displayed raised micro-emboli in the symptomatic group, this did not transfer into a correlation between CPV and micro-emboli. It may be that following cerebrovascular symptoms, the plaque is still ‘active’ due to rupture or ulceration. When a thrombus breaks away from this ruptured plaque, the ruptured area is exposed once again allowing the thrombotic process to be repeated. It is possible that repetitions of this process will create embolic signals as thrombi form and break away from the plaque site repeatedly. Irregular, ulcerated surfaces are associated with a higher frequency of emboli (174). It is possible that rather than the size of the plaque, it is the structure and rupture status of the plaque that is most closely associated with emboli frequency. This would certainly explain why the symptomatic group shows high emboli numbers, whilst no correlation could be shown with plaque volume.

A study carried out by our colleagues on a larger patient cohort focused on platelet aggregation levels in association with patient symptoms as well as micro-emboli (175). Overall they showed mean platelet aggregation levels higher than the resistance threshold for all symptomatic subgroups. Asymptomatic patients showed aggregometry levels that were significantly lower (P = 0.035) than the symptomatic groups (Fig. 34). Our colleagues also displayed a moderate positive correlation ($R^2 0.516$) between patient platelet aggregometry levels and frequency of emboli (Fig. 35). A general trend of higher aggregometry levels correlating with more frequent micro-emboli was observed. Both the research carried out by ourselves and our colleagues indicates that there is potential for micro-emboli and antiplatelet resistance to be incorporated into a stroke risk prediction profile. If symptomatic patients have larger, less stable plaques it appears that these plaques are also likely to cause more frequent emboli and higher levels of antiplatelet resistance.
FIG. 34 Mean platelet aggregation showing significantly lower Au indicating effective platelet inhibitory therapy in asymptomatic patients with CAD compared with those with recent symptoms of cerebral ischemia. Taken from (175)

FIG. 35 Scatter plot demonstrating the correlation between residual platelet aggregation despite aspirin/clopidogrel therapy and the number of pre-operative cerebral emboli/hour detected by TCD in 20 CEA patients with >70% carotid stenosis. Taken from (175)
8.5 Histology

As shown in Table 5 all plaques observed in the histological analysis of our study were highly advanced plaques; Type V, a, b and C, as well as type VI. Type Va plaques are predominantly fibrous in their content. These plaques are fairly stable complex plaques, although the disease is advanced, and the necrotic core abundant, there is a large amount of fibrotic tissue remaining that acts as a protective barrier to the more volatile elements of the plaque. Although in a reasonably stable state as type Va plaques, they remain at risk of progressing towards more volatile states. Type Vb plaques show varying levels of calcification throughout the plaque, it is possible that this calcification can have a destabilising effect on other volatile plaque elements, although some authors argue that calcification can be a stabilising process in the same manner as fibrosis. Overall these plaques appear to be more unstable than those of the type IV, Va and Vc categories. Type Vc plaques are those which are almost entirely fibrotic, with very little lipid content and no clearly defined necrotic core. Absence of the necrotic core, possibly the most dangerous pathology within the plaque, means that type Vc plaques are the most stable category of complex plaque. A plaque that is almost entirely fibrous is extremely unlikely to rupture.

Type VI plaques, as shown in Fig. 36, are effectively type V lesions which present disruption of the plaque surface, intra-plaque haemorrhage or thrombotic deposits. Disruptions of the plaque surface include ulceration and rupture of the fibrous cap. Both of these processes are highly pathological and at this point the plaque becomes likely to cause thrombosis and subsequent cerebrovascular attack. Ulcerations can be miniscule, minor disruptions of the fibrous cap only visible microscopically. However, they can also be large and deeply penetrating, breaking through the entirety of the fibrous cap and exposing the necrotic core to systemic blood flow. These large ulcerations may be visible upon gross examination of a plaque sample. Although thrombosis may occur at the site of the plaque, the thrombus does not always break away into the systemic vasculature, overtime it may become incorporated into the plaque structure, appearing histologically as a thrombotic deposit near the plaque surface. This incorporation of the thrombus is destabilising to the plaque and likely to incur a further thrombotic event in the future. The thrombus contains many biochemical mediators of plaque destabilisation similar to those released during intra-plaque haemorrhage (IPH). IPH occurs as a result of neovascularisation. As a plaque progresses towards a complex state, angiogenesis will occur, with new blood vessels forming and penetrating the plaque. These vessels are weak and prone to rupture. Vessel rupture results in haemorrhage within the plaque. The blood content of the haemorrhage contains a cocktail of biochemical mediators and cells that rapidly perpetuate inflammation within the plaque, causing a more rapid progression of plaque complexity than would be seen otherwise. This haemorrhage event propels the plaque towards potential ulceration and rupture.

What remains unclear is the process by which a type V plaque is destabilised enough that it becomes a type VI plaque, showing pathological processes highly likely to result in CVA. Although
not all plaques in our study have yet been processed for analysis, in the sample we do have \((n = 19)\), 15 were symptomatic and 4 were asymptomatic. All symptomatic plaques were either Type VI \((n=7)\), type Va \((n=4)\) or type Vb \((n=4)\) indicating they are comprised of the most volatile plaque types. Asymptomatic plaques were a mixture of Va \((n=2)\) and Vb \((n=2)\). The results presented are in keeping with the idea that there is a definite pathological difference between asymptomatic plaques and a subset of symptomatic plaques. No asymptomatic plaques were the most severe plaque type; suggesting the pathologies that separate plaque types V and VI play a large role in patient symptomology.

![Fig. 36](image)

*Fig. 36 Double immunostaining visualises smooth muscle of the arterial wall (blue) and dense macrophage infiltration of the fibrous cap. \((L = \text{Lumen}, FC = \text{Fibrous Cap})\). Taken from (Naruko 1996).*

When compared with previous carotid histology studies, our pilot histological results are in keeping with the literature. Asymptomatic plaques have been shown to contain considerably more fibrous and collagen tissue than symptomatic plaques, therefore having a more stable structure\(^{(176)}\). However, fibrosis aside, symptomatic and asymptomatic plaques do show largely similar pathologies between the sub-groups, with multiple studies indicating that all surgical samples analysed are complex plaques, whether asymptomatic or symptomatic \(^{(177}, 178\)\). The main differences between the sub-groups are CVA incurring pathologies such as ulceration and IPH, ulceration is proven to have higher prevalence in symptomatic plaque samples \(^{(179)}\). Incorporation of thrombus into the plaque structure is associated with further ulceration and symptoms \(^{(180)}\). Extent of calcification has not been independently associated with stroke symptoms; perhaps reaffirming the idea that calcification may actually incur a level of plaque stability. One of the most important findings from the literature is that macrophage migration from deep within the plaque to the surface and shoulder areas of the fibrous cap may be a critical step in progression from an asymptomatic plaque to a symptomatic one \(^{(181)}\). Macrophages are key mediators in pathological processes throughout the progression of atherosclerosis. Proteolytic enzymes released by macrophages can rapidly destroy fibrous tissue; they therefore pose a potent threat to the fibrous
cap and degradation of this area of the plaque. A way of imaging macrophage migration through the plaque could be of huge importance in assessing plaque vulnerability in asymptomatic patients and is most definitely an area of research that should be explored. It may be the case that patients are forwarded for surgery upon abundant macrophage infiltration of key plaque areas.

8.6 Metabolomics - Preliminary Analysis

Our preliminary metabolomics work was vital in proving that carotid plaque tissue was a viable biological material for use in advanced mass spectroscopy techniques. Omic analysis of atherosclerotic disease has been carried out in a handful of studies, however none using the advanced techniques that are utilised by CADET. Research so far has focused on proteomic analysis of vascular tissue extracts \(^{(182, 183)}\), the tissue secretome \(^{(184)}\) and proteomic analysis of blood in atherosclerotic disease patients \(^{(185, 186)}\). In these studies, molecular analysis was carried out using a combination of gel electrophoresis, silver staining and mass spectrometry. No studies used the dual technique of Chromatography-Mass Spec and patient cohorts were small, ranging from 17-24 patients. These numbers are considerably smaller than the patient numbers in excess of 100 that have been acquired as part of this research. We believe we are amongst the first research groups to investigate carotid plaques using these techniques.

8.7 Inductively Coupled Plasma Mass Spectroscopy – Trace Metals

Trace metals have various positive and negative effects upon human systemic circulation. Zinc is a known potent anti-inflammatory and anti-oxidant \(^{(187)}\), calcium is known to stabilise plaque tissue but limit function of otherwise healthy arterial tissue \(^{(128, 129)}\) lead is a potent inducer of toxicity \(^{(188)}\) and Magnesium is thought to have a preventative role in early stages of atherosclerosis \(^{(189)}\). Presence of trace metals in atherosclerotic disease has been investigated in a variety of fairly small scale studies. Circulatory levels of trace metals have been related to carotid atherosclerosis in the elderly \(^{(190)}\) and serum trace metals have been correlated with carotid atherosclerosis in haemodialysis patients \(^{(191)}\).

Zinc is known for its anti-oxidant and anti-inflammatory properties, with suggestions that it may provide a protective role within atherosclerosis by stabilising the plaque through inhibition of oxidative free radical production. Iron and Copper are two trace metals with known inflammatory inducing roles within atheromas. Stadler et al investigated the possibility of an inverse correlation between zinc and these two trace metals within the plaque, as zinc can displace these ions in order to reduce oxidation\(^{(192)}\). Results from this research were negative indicating that zinc does not necessarily have the anti-oxidant capabilities predicted. The authors did however find a strongly significant correlation between calcium and zinc \((R^2 0.895; P = <0.005)\) similar to that found as part of our research \((R^2 0.6867; P = <0.0001)\). With calcification acting as a stabilising process in the plaque it may be that zinc does have a protective effect, but not in the anti-oxidant process that would be expected. Edsfeldt et al measured carotid plaque calcium score against presence of
the cytokine Tumour factor-α within the plaque (193). TNF-α is a potent pro-inflammatory mediator and levels of TNF-α were inversely correlated with carotid calcium score ($R^2 = 0.56; P < 0.001$). High levels of calcium and the trace metals that are present in high proportion relative to calcium such as zinc, do appear to act in a protective, stabilising manner, however the processes by which this occurs are unknown.

Calcified tissue is known to readily bind lead within the circulatory system due to lead’s capability to bind calcium receptors (194). Unless exposed through industrial labour, patient lead levels would be expected to be extremely low as lead is highly toxic (195). With calcified tissue within the arteries able to bind lead it is expected that as levels of calcification increase, levels of lead in the plaque may also increase. This is due to more calcium being available to bind with lead, retaining it within the tissue. Our results clearly indicate that this is the case, with calcium levels in the plaque showing strong positive correlation to lead levels within the plaque ($R^2 = 0.7113, P < 0.0001$). Systemic lead levels have previously been associated with prevalence of hypertension (188). Hypertension is a co-morbidity closely associated to CAD; it is therefore possible that those with higher plaque lead concentrations are more likely to have hypertension.

The explanation of the positive correlation between calcium and zinc, magnesium, lead and sodium is largely down to calcification levels in plaque tissue. Multiple studies have positively correlated zinc and calcium levels indicating that both of these metals exhibit protective properties over the plaque. Sodium, magnesium and calcium are all key metals in the process of osteogenesis and calcification itself and therefore it is expected that these metals would be abundant in relative concentrations as calcification develops. Correlation of these 4 trace metals has been presented in various publications (191, 196, 197). In regards to lead, it is evident that trace levels of systemic lead can be bound by calcified tissues within atheromas, the more calcification present the more systemic lead that can be bound by the plaque. The role of lead in intra-plaque pathologies is unknown.

Our results show that the trace metal most likely to show any significance between subject groups is copper. Copper is an inflammatory related trace metal and copper imbalance is associated with a variety of diseases (198). Copper is a pro-oxidant and is capable of causing modification of low density lipoprotein cholesterol (199). Modification of low density lipoprotein can affect plaque composition and influence necrotic core size and stability, a plaque pathology closely associated with symptomatic plaques (200). Further evidence for copper’s role in cholesterol interactions is presented by Svensson, in this work Svensson shows the ability of copper to activate cholesterogenic and LDL receptor genes within macrophages as well as lowering expression of anti-cholesterol CD36 cells and lipid binding proteins (201). Copper is known to facilitate angiogenesis, one of the major pathologies associated with ulceration and rupture in highly advanced plaques (202). Copper has a potent role at a cellular level. Mandinov et al showed that human peripheral mononuclear blood cells use intracellular copper to mediate stress response and
subsequently release cellular stress mediators such as IL-1α, causing a vascular inflammatory cascade (203). Research has suggested that copper chelation can repress this response and lower the inflammatory effects of copper (203). In the murine model, use of copper chelators has attenuated neointimal thickening significantly (203).

Several research projects have presented data linking high copper serum levels with increased disease severity in coronary artery disease, giving evidence for coppers association with atherosclerosis (192, 204, 205). Copper has also been presented at higher levels in the intima of those with vascular disease when compared with healthy controls (192). Clearly excess copper presence in the atherosclerotic plaque is a threat to disease stability. The intra-plaque pathologies associated with copper suggests that symptomatic plaques are more likely to contain copper; however larger patient numbers are required in order to investigate if this trend gains significance in larger patient cohorts.

In regards to Iron, the loss of significance between the dry and wet sample weights is opposed to the literature surrounding concentrations of iron in atherosclerosis. Iron plays a diverse role in the atherosclerotic lesion. Similar to Copper, iron is capable of perpetuating inflammation via oxidative mechanisms, antagonizing potent inflammatory mediators such as metalloproteinases and proteolytic enzymes (206, 207). These groups of enzymes are responsible for extracellular matrix degradation resulting in destruction of the plaque’s fibrous cap (199). Rupture or ulceration of the fibrous cap is one of the key pathologies resulting in cerebrovascular symptoms. High iron levels suggest that degradation of the fibrous cap may be happening at a faster rate than in plaques with lower iron concentrations. Iron may also be present in advanced symptomatic plaques due to its abundant release following intra-plaque haemorrhage (192). Intraplaque haemorrhage is one of the key pathologies seen in the symptomatic plaque and is often cited as a major pre-cursor to plaque rupture and subsequent cerebrovascular symptoms. Stadler et al provide evidence of iron concentrations being raised following IPH due to the uptake of iron complexes by macrophage derived cells local to the atheroma (192). The authors also presented data showing no correlation between iron and calcium, similar to results obtained by our research team, along with an increase in Intima iron levels when compared with healthy controls (192).

Although we have not shown a significant difference in iron and copper concentrations between the patient groups, there is evidence in the literature that these differences do exist. Both iron and copper have been extensively linked with specific plaque pathologies, particularly those seen in the most advanced stage of the plaque when likelihood of cerebrovascular symptoms is extremely high. Significance of copper levels may be achieved through analysis of increased plaque numbers, whilst ensuring weighing accuracies at the dry weight stage of sample processing may also provide insight as to whether iron concentrations truly are significantly raised in the symptomatic group. Both of these modifications to the research appear likely to yield positive results. The correlation
we have shown between calcium and associated trace metals is very similar to that shown in the existing literature, validating our chosen methods and techniques for this analysis.

Further steps in trace metal research associated with atherosclerosis would be to establish a profile of circulatory detectable metals relatable to their concentration within a patient’s atherosclerotic disease. For example a patient showing low calcium and zinc or high iron and copper concentrations may require investigation to establish disease stability and specific intra-plaque pathologies as well as altering of subsequent medical treatment. Interestingly, in the paper based on carotid calcium scores by Edsfeldt et al, CT imaging was used to visualise calcium and biomarkers such as TNF-α, suggesting that circulatory biomarkers may not be necessary where successful in vivo biomarker imaging techniques are available. Similar imaging techniques were carried out by Miralles et al in order to establish calcium levels in relation to patient symptoms (208).

8.8 Summary

It is becoming increasingly clear that stroke risk prediction in CAD patients is multi-factorial. Several varying parameters are linked with stroke risk prediction in these patients, however as of yet none of these have proven to be fully reliable as independent risk predictors. We have shown that CPV is a reliable alternative to stenosis, with higher CPVs linked to symptomatic patients, a general trend of increasing CPV with increasing severity of patient symptomology and importantly no significant association between degree of stenosis and CPV. Plaque volume can be linked to patient antiplatelet resistance. In routine blood tests WBC count and potentially CRP can be linked to symptomatic patients. There are strong correlations between intra-plaque calcium and associated trace metals that may have a protective effect over the plaque. Intra-plaque copper and iron may be two of the key inflammatory mediators within the symptomatic plaque.

The profile required for risk stratification of CAD patients would include the best method for in vivo disease visualisation and analysis, as well as parameters such as blood biomarkers, presence of micro-emboli and presence of vulnerable plaque characteristics. The research conducted as part of this project has contributed to laying the foundations for such a risk profile. Ultimately the 'structure of atherosclerotic plaques causing carotid stenosis is more important than their diagnosis alone (42). There is undoubtedly still room for stenosis in the prediction of stroke in CAD patients; however it should no longer be considered the primary indicator for CEA. Future Research should focus on honing this stroke risk profile for CAD patients, paying particular attention to intra-plaque pathologies and a wider spectrum of systemic biomarkers.
9. Study Limitations

Although the study has been carried out using what we believe to be the best possible methods, adhering to a well-planned protocol, there are inevitably study limitations affecting outcomes and results. The main limitation we have experienced is simply time constraints, which in turn limited patient numbers. According to protocols in hospitals across our region, symptomatic CAD patients who are suitable for surgery must be operated on within two weeks of their vascular referral, however this is often much quicker, frequently within 24-48 hours. Our research is time consuming, with patients undergoing a full medical history and demographic interview, at least an hour of TCD, both 2D and 3D ultrasound scanning and phlebotomy as well as consenting and allowing time for patient consideration of their participation. All of these methods combined can take up to several hours, an amount of time that is not always achievable in urgent surgery scenarios. As a result of this some of our data is fragmented, with not every patient undergoing every aspect of the study. This imposed quite a restraint on our data analysis as numbers for each patient group for each aspect of the study were often quite low. Particularly impacted was the T.C.D aspect of the research, which due to its time consuming and often uncomfortable nature (patients sitting for long periods), resulted in disappointingly low subject numbers for both main sub-groups.

Awareness of suitable patients has also been an issue at times. Due to the scale of the southern sector hospitals in south Manchester, many patients are referred from outlying hospitals to UHSM for their operation. Some patients arrive late in the day and undergo surgery first thing in the morning, with others arriving the morning of their surgery. Without help from auxiliary staff tracing and recruiting these patients was a difficult task. Weekend surgeries were also a cause of missing data as the research team were not available.

As with many other studies involving CAD patients, there is an imbalance in recruitment of symptomatic and asymptomatic patients. Current surgical guidelines mean that asymptomatic patients are significantly less likely to undergo surgery than symptomatic patients (209). Out of the studies reviewed as part of this research, symptomatic patients outnumbered asymptomatic patients at a rate of approximately 5:1. The lack of asymptomatic patients undergoing surgery results in a substantial reduction in readily available asymptomatic data, specifically in regards to surgical carotid plaque samples. A solution to this is the possibility of enlisting other sites to recruit on an asymptomatic only basis, however due to our small research team this was logistically very difficult.

In regards to Transcranial Doppler ultrasound scanning, our main constraint was once again time. Patients may be called to surgery urgently, with the TCD recording being terminated to facilitate this. Lack of temporal window was another major issue in our attempts to carry out TCD in all patients. Lack of temporal window is usually caused by the temporal bone being too thick; this is
an anatomical feature in 20-25% of the population. Ultrasound waves from the Doppler probe cannot penetrate thick temporal bone, therefore ruling out patients who would otherwise be eligible for monitoring. As previously mentioned, discomfort also played a part in reduced T.C.D numbers. Many patients would struggle to remain comfortable and relatively motionless for an extended period of time, resulting in poor quality recordings.

We encountered a minor limitation relating to antiplatelet resistance testing. A proportion of patients undergoing CEA stop their antiplatelet medication on the day of surgery, or in the days prior, in order reduce risk of bleeding intra-operatively. If these patients had not taken their antiplatelet medication in the previous 24 hours we were unable to go ahead with antiplatelet testing. This is because the process of platelet turnover will begin to restore uninhibited platelets into the patient’s system 24 hours after aspirin administration \(^{(210)}\). This can give false positive results, the patient appears to be resistant, but simply hasn’t taken their antiplatelet medication prior to testing. The closest solution we found to tackling both time constraints and patients not being medicated on day of surgery was for patients to attend a single study visit before their surgery. Although with some patients this was feasible as they would attend the hospital for a presurgical outpatients visit, many patients at out-lying hospitals would only attend UHSM for their surgery. As mentioned, these patients would arrive the night before, or morning of, their surgery. This left us very little time with which to complete the research.

**10. Further Research**

Firstly, further research incorporating the elements of this project should be carried out in their entirety. Due to the circumstances at UHSM, it has often been difficult to recruit patients to every element of the study. Certainly during the latter half of this project, deteriorating staffing levels have resulted in many patients attending hospital on the morning of their surgery, giving researchers an impossible time frame in which to carry out the requirements of this project. Expanding this research to a multi-centre level may increase study completion due to differing local hospital protocols for patient attendance pre-surgery. It would be beneficial, where possible, to bring patients in for a single research visit before their surgery allowing a suitable window for Transcranial Doppler Monitoring and ensuring that the patient has taken their antiplatelet medication. Patients frequently do not take Aspirin or Clopidogrel on the morning of surgery and therefore Multiplate testing on these patients is redundant.

In regards to prevalence of antiplatelet resistance, large primary multicentre trials should be carried out. A key issue in antiplatelet resistance research is that the current literature is made up of many inadequately powered studies. These studies use a wide variety of patient demographics and analytical processes. There are currently many ways of measuring antiplatelet resistance; Multiplate Impedance Aggregometry and Light Transmittance Aggregometry are viewed as the gold standards. Large cohorts of patients with cardiovascular disease should be measured
repeatedly over an extended period of time, using both techniques. With ensured compliance, large cohort numbers and an effort to rule out causes of pseudo-resistance, a huge step could be made towards establishing true levels of antiplatelet resistance in the cardiovascular community as well as a single gold standard measurement technique. If testing is carried out repeatedly over a prolonged period, investigators will be able to examine whether antiplatelet resistance is a constant or fluctuating phenomenon.

Visualisation of the disease in the carotid artery is a specific area that requires more attention in vascular research. 3D ultrasound scanning of carotid arteries in order to measure plaque volume is well established in smaller studies and the technique has shown solid repeatability and reproducibility in our study and the wider literature (3-5). Studies have also indicated a reliable level of inter and intra-operator variability (3-5). Proof of the sensitivity and accuracy of this technique as well as evidence that CPV is linked with stroke risk show the viability of integrating this technique into standard clinical care. The next step with 3D ultrasound is the inclusion of contrast to better visual the disease, a trial researching this technique is scheduled to begin at our facility shortly. Aside from 3DUS there are imaging alternatives for atherosclerosis. MRI is used in complex cases but is not hugely time or cost effective, certainly not on any scale wider than its current use in a small subset of cases (211). 3D Computed tomography (3D CT) is not only capable of 3D volume measurements but also of more accurately visualising disease processes within the plaque (212). Future studies should focus on a comparison of all 3D analysis methods, MRI, 3D US and 3D CT. Techniques should be assessed for inter and intra-operator variability as well as repeatability, reproducibility, sensitivity and accuracy. From an economic, primary care standpoint, cost and time effectiveness should also be considered.

In conclusion, this research has collectively highlighted several key differences between symptomatic and asymptomatic CAD patients. Our work has helped contribute to the foundations of a risk prediction profile for currently asymptomatic patients. Those who show traits associated with symptomatic carotid disease should be aggressively monitored with a view to undergoing pre-emptive surgery. The first stage of our follow up research to this project is to complete ‘omic and histological analysis for all plaques and blood samples. This will confirm whether histological findings so far can be applied to a larger patient cohort, whilst also reaffirming our ‘omic findings. The next stage of this project is to roll out similar research across a wider scale, incorporating several other sites, a task that will undoubtedly require funding. Further work in this area of vascular research has the potential to make a huge impact in the Stroke community. A risk prediction profile could allow medics to take aggressive action before symptoms have occurred, rather than after, potentially reducing the numbers of CAD patients who undergo devastating damage following stroke.
11. References


4. van Gijn J, Rinkel GJE. Subarachnoid haemorrhage: diagnosis, causes and management 2001 2001-02-01 00:00:00. 249-78 p.


142. Blann AD, Nadar SK, Lip GYH. The adhesion molecule P-selectin and cardiovascular disease2003 2003-12-01 00:00:00. 2166-79 p.


12.1 – Appendix 1. Transcranial Doppler Monitoring Datasheet
**CPV Study – TCD monitoring**

Subject No. ...........................................  Patient Hospital ID number ...........................................

TCD length ......................  Start time..................  End time ......................

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Validation by primary researcher  Y/N  MES count on validation ............

Validation by researcher 2  Y/N  MES count on validation ............

(Name ...........................................)

**FINAL MES COUNT ........................./hour**
This flow chart shows the progression the patient will make as part of the study. From their initial admission to hospital through to the 3 month follow up after surgery.
### CADET

**Standard Operating Procedure**

#### CADET_SOP_009 _Extraction of small molecules_

*Extraction* of small molecules from biological *tissues* for analysis by GC-MS and LC-MS

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<th>Signature</th>
<th>Date</th>
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<tr>
<td>Prepared by</td>
<td>v1.0</td>
<td>Stephanie Church</td>
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#### Revision History

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<tr>
<th>Revision #</th>
<th>Date</th>
<th>Reason for change</th>
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</table>
1 INTRODUCTION

1.1 CADET’s collaborators collect biological samples and data from patients for use in biomedical research. All material is stored securely and in suitable tubes and containers.

2 Scope

2.1 This protocol describes the extraction of small molecules from biological tissues for analysis by Gas Chromatography Mass Spectrometry (GC-MS) and Liquid Chromatography Mass Spectrometry (LC-MS).

2.2 This SOP applies to all HTA-relevant human sample collections delivered to and stored within the Centre for Advanced Discovery and Experimental Therapeutics (CADET).

3 responsibilities

3.1 The Person Designate is responsible for ensuring all staff are trained in the use of this SOP.

3.2 All CADET laboratory staff are responsible for ensuring they work to this SOP at all times.

4 related documents

4.1 The Human Tissue Act 2004 and associated Codes of Practice
4.2 CADET_SOP_001 – Sample Collection
4.3 CADET_SOP_005 – Sample log-in using ItemTracker
4.4 CADET_SOP_006 – Waste management and disposal of biological material
4.5 CADET_RA009_TissueLyser-1
4.6 CADET_RA015_Pipetting
4.7 CADET_RA006_Fume cupboard
4.8 CADET_RA012_Centrifuges
4.9 CADET_RA014_Vortex mixer
4.10 CADET_RA007_Centrifugal evaporator
4.11 Chloroform COSHH RA
4.12 Methanol COSHH RA
4.13 Succinic d₄ acid COSHH RA
4.14 Stearic acid COSHH RA
4.15 Citric acid COSHH RA
4.16 Benzoic acid COSHH RA

5 health and safety

5.1 Nitrile gloves must be worn when handling any biological samples.

5.2 Cold-resistant gloves must be worn when handling frozen freezer racks.

5.3 SHIELDskin CHEM™ NEO NITRILE™ 300 gloves must be worn when handling chloroform.

5.4 All handling/manipulation of samples must take place in the laboratory. Biological samples must not be brought into designated office space.

5.5 Micro-centrifuge tubes and used tips are disposed of as hazardous chemical waste. Please refer to experimental procedure and COSHH risk assessment forms provided in the laboratories as well as SOP 007 Waste management.

5.6 Chloroform must only be used in the fume cupboard.

6 Equipment/Materials/chemicals

6.1 Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
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<td>Thermo Scientific</td>
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<tr>
<td>Savant Speedvac</td>
<td>Thermo Scientific</td>
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6.2 Materials

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<td>15ml falcon tube</td>
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<td>Chemical</td>
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<td>Cat#</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------------------------</td>
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</tr>
<tr>
<td>3mm Tungsten carbide beads</td>
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<td>TissueLyser Block</td>
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<tr>
<td>8ml Glass vial</td>
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### 6.3 Chemicals

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<th>Chemical</th>
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<tr>
<td>Glycine $d_5$</td>
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<td>Citric acid $d_4$</td>
<td>Cambridge Isotope Laboratories Inc.</td>
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<td>Fructose $^{13}C_6$</td>
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<td>Tryptophan $d_5$</td>
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<td>Leucine $d_{10}$</td>
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<td>LC-MS grade water</td>
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<td>39253</td>
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<tr>
<td>Chloroform</td>
<td>Sigma</td>
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</tbody>
</table>

### 7 Procedure

#### 7.1 Tissue collected by collaborators will be washed immediately after removal and frozen at -80°C until required.

#### 7.2 All HTA relevant material must be logged in Itemtracker on arrival at CADET.

#### 7.3 Remove the tissue from the -80°C freezer.

#### 7.4 If not already done, section samples into 50mg (± 5mg wet weight) blocks using a disposable sterile scalpel. This should be carried out on a cold
block to prevent samples from thawing. Transfer each 50mg block into a
2ml microcentrifuge tube. Store samples at -80°C until required. Clean
work surfaces with 1% Virkon (according to relevant BioCOSHH) and
dispose of the scalpel in a Daniels Sharpsguard 1 litre bin after use.

7.5 (Optional) If not already prepared, make up internal standard mixtures as
follows:

SG- 10mg Succinic acid d₄, 10mg Glycine d₅ in 10ml 50:50 (v/v)
methanol:water
CFT-10mg Citric acid d₄, 10mg Fructose ¹³C₆, 10mg Tryptophan d₅ in 10ml
50:50 (v/v) methanol:water
LA-10mg Leucine d₁₀, 10mg Alanine d₇ in 10ml 50:50 (v/v) methanol:water
SB-10mg Stearic acid d₃₅, 10mg Benzoic acid d₅ in 10ml methanol.

For working stock add 1ml of each (SG, CFT, LA and SB) to 6ml methanol
(final volume 10ml).

7.6 In the fume hood, make up appropriate volume (dependent on number of
samples to be extracted) of 50:50 (v/v) chloroform:methanol in 15ml
falcon tubes. Add internal standard working stock at a dilution of 1 in 16.
Store in the -20°C freezer for toxic chemicals and CMRs. This step should
be completed at least 4 hours before sample extraction (or the day before)
to ensure solution is cold.

7.7 Remove samples from -80°C freezer into TissueLyser block (stored at -
20°C).

For steps 7.8 and 7.9 work in the fume hood.
7.8 To each sample and one empty 2ml microcentrifuge tube (extraction blank) add 800µl 50:50 chloroform:methanol solution prepared in step 7.6.

7.9 Add one 3mm Tungsten carbide bead to each tube.

7.10 Extract samples for 10 minutes at a frequency of 25 Hertz on the TissueLyser.

7.11 Add 400µl of LC-MS grade water to each sample.

7.12 Vortex samples for 10-15 seconds prior to centrifugation at 2400 x g for 15 mins.

For steps 7.13 and 7.14 work in the fume hood.

7.13 From the chloroform phase, transfer 100µl from each sample into a 2ml microcentrifuge tube and 100µl into an 8ml glass vial to create a pool (excluding the extraction blank) from which QC samples will be produced. Gently mix the pool before sampling.

7.14 Transfer 100µl from the QC pool into as many 2ml microcentrifuge tubes as required (this will depend on sample numbers).

7.15 Dry samples using the Speedvac centrifugal concentrator. Ensure ramp 1 is used for chloroform samples.

7.16 Before sampling from the methanol phase, remove any remaining chloroform from the original sample tubes before continuing.

7.17 Centrifuge samples at 2400 x g for 15 mins.

7.18 From the methanol phase, transfer 200µl from each sample into 2ml microcentrifuge tubes and 200µl into an 8ml glass vial to create a pool (excluding the extraction blank) from which QC samples will be produced.

7.19 Transfer 200µl from the QC pool into as many 2ml microcentrifuge tubes as required (this will depend on sample numbers).
7.20 Dry samples overnight using the Speedvac centrifugal concentrator. Use ramp 5 for these methanol/water extracts.

7.21 Once extracts have been dried, store at 4°C until analysis.

7.22 All waste solutions containing chloroform should go into the ‘chlorinated solvent waste’ bottle stored in the fume hood.

7.23 Micro-centrifuge tubes and used tips are then disposed of as hazardous chemical waste in yellow lidded plastic bins.

7.24 If HTA-relevant material, record that these tissue samples have been destroyed in Itemtracker.
12.4 Appendix 4 – Tissue Digestion SOP

CADET

Standard Operating Procedure

CADET_SOP_012 _Tissue Digestion

Digestion of biological tissues with nitric acid for the determination of trace elements by ICP-MS

<table>
<thead>
<tr>
<th>Revision Number</th>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
<th>Approved</th>
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<tbody>
<tr>
<td>Prepared by</td>
<td>v1.0</td>
<td>Stephanie Church</td>
<td></td>
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Revision History

<table>
<thead>
<tr>
<th>Revision #</th>
<th>Date</th>
<th>Reason for change</th>
</tr>
</thead>
</table>
8 INTRODUCTION

8.1 CADET collects biological samples and data from patients for use in biomedical research. All material is stored securely and in suitable tubes and containers.

9 Scope

9.1 This protocol describes the digestion of biological tissue with nitric acid for the determination of trace metals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

9.2 This SOP applies to all HTA-relevant human sample collections delivered to and stored within the Centre for Advanced Discovery and Experimental Therapeutics (CADET).

10 responsibilities

10.1 The Person Designate is responsible for ensuring all staff are trained in the use of this SOP.

10.2 All CADET laboratory staff are responsible for ensuring they work to this SOP at all times.

11 related documents

11.1 The Human Tissue Act 2004 and associated Codes of Practice.

11.2 CADET_SOP_001 – Sample Collection

11.3 CADET_SOP_005 – Sample log-in using ItemTracker

11.4 CADET_SOP_006 – Waste management and disposal of biological material.

11.5 CADET_RA_Acid digestion in a heatblock

11.6 CADET_RA009_TissueLyser-1.

11.7 CADET_RA015_Pipetting

11.8 CADET_RA006_Fume cupboard

11.9 CADET_RA012_Centrifuges
12 health and safety

12.1 Nitrile gloves must be worn when handling any biological samples.

12.2 Cold-resistant gloves must be worn when handling frozen freezer racks.

12.3 SHIELDskin CHEM™ NEO NITRILE™ 300 gloves must be worn when handling chloroform.

12.4 Nitrile gloves must be worn when handling nitric acid.

12.5 All handling/manipulation of samples must take place in the laboratory. Biological samples must not be brought into designated office space.

12.6 Micro-centrifuge tubes and used tips are disposed of as hazardous chemical waste. Please refer to experimental procedure and COSHH risk assessment forms provided in the laboratories as well as SOP 007 Waste management.

12.7 Concentrated nitric acid must only be used in the fume cupboard.

12.8 Any acid contaminated tips or tubes must first be rinsed before disposal in a large beaker of water to dilute any residual acid.

12.9 Dri-block heater is operated at temperatures in excess of 100°C in this protocol. Care must be taken when removing samples from block.

13 Equipment/Materials/chemicals

13.1 Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
<th>Serial Number</th>
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<td>Dri-Block DB3</td>
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13.2 Materials

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<tr>
<th>Material</th>
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<td>Eppendorf</td>
<td>0030 120.094</td>
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### 13.3 Chemicals

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<tr>
<td>2,2,4-trimethylpentane</td>
<td>Fluka</td>
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### 14 Procedure

14.1 **Tissue collected from collaborators will be washed immediately after removal and frozen at -80°C until required.**

14.2 **All HTA relevant material must be logged in Itemtracker on arrival at CADET.**

14.3 **Remove the tissue from the -80°C freezer.**

14.4 **(OPTIONAL) If not already done, section samples into 50mg (± 5mg wet weight) blocks using a disposable sterile scalpel (or ceramic scalpel for dry tissue). Transfer each 50mg block into a 2ml microcentrifuge tube. Clean work surfaces with 1% Virkon (according to relevant BioCOSH) and dispose of the scalpel (if disposable) in a Daniels Sharpsguard 1 litre bin after use. If the ceramic scalpel was used, clean with 1% Virkon after use.**
14.5  (OPTIONAL) Dry tissue to constant weight using the Speedvac centrifugal concentrator.

14.6  (OPTIONAL) IN FUME CUPBOARD AND WEARING SHIELDskin CHEM™ NEO NITRILE™ 300 GLOVES. Defat samples. Add 2ml 50:50 (v/v) Chloroform:2,2,4-trimethylpentane to each sample. Add a 3mm tungsten carbide bead to each tube and extract for 10 minutes at a frequency of 25 hertz on the TissueLyser. Vortex samples for 10-15 seconds prior to centrifugation at 2400 x g for 15 mins. Remove supernatant from remaining tissue and dry to constant weight using the Speedvac centrifugal concentrator.

14.7  For wet tissue, briefly centrifuge sample tubes at 2400 x g to ensure tissue sits at the bottom of the tube.

14.8  Pierce tube lids using the GC septum removal tool to prevent pressure build up during digestion; include two empty 2ml microcentrifuge tubes for digestion blanks.

FOR STEPS 7.9 to 7.12 WORK IN THE FUME CUPBOARD

14.9  Add 1.5ml internal standard mixture to 30ml concentrated nitric acid (or equivalent, depending on volume required) to produce digestion acid.

14.10 Add 200µl digestion acid to each sample and the two digestion blank tubes.

14.11 Put the samples onto the Dri-block heater, ensuring the block is at room temperature before use. Turn the temperature up to 60°C and start the timer. After 30 minutes increase the temperature to the maximum setting and leave samples to digest for a further 3.5 hours.

14.12 Add 20ml of digestion acid to 980ml LC-MS grade water in a polypropylene volumetric flask to produce a 2% v/v dilute nitric acid solution.

14.13 Prepare standards as follows in 15ml metal free centrifuge tubes:
CAL 6 – 10ml 2% nitric acid solution, 50µl environmental calibration standard mixture

CAL 5 – 15ml 2% nitric acid solution, 75µl environmental calibration standard mixture

CAL 4.5 – 4ml 2% nitric acid solution, 1ml CAL 6

CAL 4 – 9ml 2% nitric acid solution, 1ml CAL 6

CAL 3 – 9ml 2% nitric acid solution, 1ml CAL 5

CAL 2.5 – 4ml 2% nitric acid solution, 1ml CAL 4

CAL 2 – 9ml 2% nitric acid solution, 1ml CAL 4

CAL 1.5 – 3ml 2% nitric acid solution, 3ml CAL 2

CAL 1 – 10ml 2% nitric acid solution (blank)

Final solution concentrations for Na, Mg, K, Ca and Fe will be 0, 50, 100, 200, 500, 1000, 2000, 5000 and 10000µg/l. For all other elements final concentrations will be 0, 0.5, 1, 2, 5, 20, 50 and 100µg/l.

14.14 After 4 hours digestion, switch off heatblock. Allow samples to cool before removing samples from the heat block.

14.15 Pulse centrifuge sample tubes at 2400 x g to remove any solution from the lids.

FOR STEPS 7.16 and 7.17 WORK IN THE FUME CUPBOARD
14.16 Add 100µl of each digest to 5ml LC-MS water in 15ml metal free centrifuge tubes.

14.17 Discard used tubes and acid contaminated tips in a large beaker of water. Neutralise using sodium bicarbonate. Keep adding until fizzing stops but take care not to add too much at once or the beaker will overflow.

14.18 Micro-centrifuge tubes and used tips are then disposed of as hazardous chemical waste in yellow lidded plastic bins.

14.19 If HTA-relevant material, record that these tissue samples have been destroyed in Itemtracker.