The Role of Carotid Artery Disease in Ischaemic Stroke

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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<tr>
<td>3D</td>
<td>3 Dimensional</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACAS</td>
<td>Asymptomatic carotid atherosclerosis study</td>
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<tr>
<td>ACES</td>
<td>Asymptomatic carotid emboli study</td>
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<tr>
<td>ACSRS</td>
<td>Asymptomatic carotid stenosis and risk of stroke study</td>
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<td>ACST</td>
<td>Asymptomatic carotid artery trial</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AHA</td>
<td>American heart association</td>
</tr>
<tr>
<td>ARIC</td>
<td>Atherosclerosis risk in community study</td>
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<tr>
<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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<tr>
<td>CAVATAS</td>
<td>Carotid and vertebral artery transluminal angioplasty study</td>
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<tr>
<td>CCA</td>
<td>Common carotid artery</td>
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<tr>
<td>COX-1</td>
<td>Cyclo-oxygenase-1</td>
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<tr>
<td>CPV</td>
<td>Carotid plaque volume</td>
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<tr>
<td>CREST</td>
<td>Carotid revascularisation endarterectomy versus stent trial</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTA</td>
<td>Computed tomographic angiogram</td>
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<tr>
<td>CV</td>
<td>Cardiovascular</td>
</tr>
<tr>
<td>CVA</td>
<td>Cerebrovascular accident</td>
</tr>
<tr>
<td>DLS</td>
<td>Disrupted luminal surface</td>
</tr>
<tr>
<td>ECA</td>
<td>External carotid artery</td>
</tr>
<tr>
<td>ECST</td>
<td>European carotid surgery trial</td>
</tr>
<tr>
<td>ESVS</td>
<td>European society vascular surgeons</td>
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<tr>
<td>FSS</td>
<td>Fluid shear stress</td>
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<tr>
<td>Hs-CRP</td>
<td>High sensitive C-reactive protein</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>ICARAS</td>
<td>Inflammation and carotid artery risk for atherosclerosis study</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>IMT</td>
<td>Intima-media thickness</td>
</tr>
<tr>
<td>IPH</td>
<td>Intraplaque Haemorrhage</td>
</tr>
<tr>
<td>ISD</td>
<td>Interslice distance</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>Lp-PLA₂</td>
<td>Lipoprotein associated phospholipase A₂</td>
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<td>LRNC</td>
<td>Lipid rich necrotic core</td>
</tr>
<tr>
<td>LysoPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MES</td>
<td>Microembolic signals</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRA</td>
<td>Magnetic resonance angiogram</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>NASCET</td>
<td>North American symptomatic carotid endarterectomy trial</td>
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<tr>
<td>Ox-LDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>Ox-NEFA</td>
<td>Oxidised nonesterified fatty acids</td>
</tr>
<tr>
<td>PSV</td>
<td>Peak systolic velocity</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SAPPHIRE</td>
<td>Stenting and angioplasty with protection in patients at high risk for endarterectomy</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TCD</td>
<td>Transcranial doppler</td>
</tr>
<tr>
<td>TH₁</td>
<td>Helper-1 T cells</td>
</tr>
<tr>
<td>TH₂</td>
<td>Helper-2 T cells</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischaemic attack</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor activating peptide</td>
</tr>
<tr>
<td>t-US</td>
<td>Tomographic ultrasound</td>
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<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<tr>
<td>vWF</td>
<td>Von-Willibrand factor</td>
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<tr>
<td><strong>WBC</strong></td>
<td>White blood cell</td>
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<td><strong>WHO</strong></td>
<td>World health organisation</td>
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ABSTRACT

Asymptomatic carotid artery disease (CAD) confers a less than two per cent annual stroke risk for patients on best medical therapy, however a group of patients remain high risk for cerebral ischaemia. There is no current way of identifying such patients. This study evaluated whether carotid plaque volume (CPV) is related to cerebral ischaemia and plaque instability. Given the prominent role of antiplatelet therapy in primary and secondary prevention, antiplatelet resistance amongst patients admitted for a carotid endarterectomy (CEA) was also evaluated. Patients admitted for a primary CEA in Greater Manchester were invited to participate. Following endarterectomy, CPV was calculated using a validated water immersion technique and note made of the stenosis severity, symptom type and time from symptom. A proportion of these patients underwent transcranial Doppler (TCD) insonation of the middle cerebral artery to detect microemboli, 3D tomographic ultrasound (t-US) to measure CPV, histological analysis of the plaque along with blood sampling for antiplatelet resistance and measurement of lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$), P-Selectin and high sensitive C-reactive protein (Hs-CRP). Data from a carotid surveillance clinic was explored to evaluate the effectiveness of 2D arterial duplex in carotid surveillance. Mean CPV was significantly increased in symptomatic patients and the rate at which it decreased was similar to the rate at which the benefit from CEA decreases. CPV was not related to the degree of stenosis and could be measured accurately by 3D t-US. Mean CPV was significantly increased in those patients with a histologically unstable plaque and mean Lp-PLA$_2$ was significantly increased in those with a marked inflammatory plaque. Mean platelet aggregation was significantly increased in symptomatic patients and correlated significantly with the number of cerebral emboli. The use of 2D duplex surveillance alone is not sufficient in identifying those patients who will benefit most from CEA. CPV is a potential marker of high risk plaques with provisional results indicating it can be measured accurately using 3D t-US. Routine antiplatelet resistance testing should be considered in patients with CAD, especially those who have suffered previous CV events.
DECLARATION

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I would like to thank my supervisory team, Professors Charles McCollum and Garth Cooper, for their support, guidance and commitment to my research. I would also like to thank Dr Elizabeth Byrne for providing her histopathological expertise. I am grateful to the surgeons and clinicians for collaborating and allowing their patients to take part in the study, and indeed the patients themselves for volunteering, along with the technicians of independent vascular services and the pathology department at CMFT for help with the processing and staining of my histological slides.

I would like to thank the Manchester Surgical Research Trust for affording me the opportunity to pursue my research through their funding and support.

In addition I would like to thank all members of the academic surgical unit based at UHSM for their support during my studies and for providing many fond memories, especially my fellow research fellows.

Finally, all of this would not have been possible without the love and support from my friends and family. I would like to especially thank my wife Alka and three daughters Tia, Ava and Nina for their support and understanding during this time.
DEDICATION

For Alka, Tia, Ava & Nina
SECTION 1: INTRODUCTION
Introduction to research studies and thesis structure

This thesis is submitted in journal format with the results section presented as a series of manuscripts. The research was clinically orientated and such that a series of manuscripts around a common theme would be produced. To meet both the requirements of the research programme and speciality training it was agreed by my supervisory team that the journal format would be appropriate and was approved by the university.

This thesis includes a series of studies focusing on the role carotid artery disease has in ischaemic stroke. The evidence to justify carotid intervention in patients with asymptomatic carotid artery disease is weak but some patients will become symptomatic. Hence this research was designed to identify certain features of carotid artery disease, particularly carotid plaque volume, which could be used as an indicator for carotid intervention in such patients.

The thesis is split into 4 sections each containing chapters. The results chapters are written in journal format with one journal already published and the others written in preparation for submission.

Section one contains two chapters. The first chapter briefly covers the basic anatomy and pathophysiology of the carotid artery and atherosclerosis. The second chapter focuses on carotid artery disease and the evidence surrounding the investigation and management of carotid artery disease, both symptomatic and asymptomatic, and the literature surrounding what constitutes an unstable plaque.

Sections two contains two chapters with subchapters detailing the various methods employed in this research.

Sections three is the results section containing four chapters presented in manuscript format. Chapter 5 details the outcomes for patients with asymptomatic carotid disease enrolled into a carotid artery surveillance clinic. Chapter 6 details how CPV relates to symptom status and how it can be measured using 3D-tUS. Chapter 7 details the results from histological analysis of the plaques along with the measurement of plasma Lp-PLA2, P-Selectin and Hs-CRP. Chapter 8 details the
prevalence and relevance of antiplatelet resistance amongst patients admitted for a CEA. The patients included in chapters 7 and 8 are sub-groups from those enrolled into the main project detailed in chapter 6.

Section four is the overall discussion and conclusion including limitations to the work and suggestions for further/ongoing work.

Section 5 contains the references.
CHAPTER 1: ANATOMY AND PHYSIOLOGY

1.1 Cerebral Anatomy

The brain is composed of three main structures; the cerebrum, cerebellum and brainstem. It is covered by three concentric tissue layers termed pia mater, arachnoid mater and dura mater from inner to outer.

The cerebrum is the largest part of the brain and is responsible for higher functions such as movement, touch, vision, hearing, speech and emotion. As well as being split into left and right hemispheres, it is split into 4 lobes; frontal, parietal, temporal and occipital. Each hemisphere controls the contralateral (opposite) side of the body with each lobe being responsible for specific functions. (Figure 1-1) (1)

The cerebellum sits beneath the cerebrum and co-ordinates muscle movement, posture and balance. The brainstem, consisting of the midbrain, pons and medulla, connects the cerebrum and cerebellum to the spinal cord. It relays information from the cerebrum and cerebellum, controls autonomic functions such as breathing and heart rate and is the origin of 10 cranial nerves. (1)
1.1.1 Blood Supply to the Brain

The brain is a highly vascular organ with a high metabolic rate receiving 15% of the cardiac output and consuming 25% of the body’s total oxygen consumption. The blood supply to the brain is via the anterior and posterior circulation, supplied by the internal carotid and vertebral arteries respectively. The anterior circulation supplies the anterior portion of the brain including the frontal, parietal and temporal lobes. The posterior circulation primarily supplies the posterior portion of the brain including the occipital lobes, cerebellum and brainstem while contributing to the blood supply of the temporal and parietal lobes via the posterior cerebral branch. The anterior and posterior circulations are connected via the circle of Willis. 

Figure 1-1 Gross Anatomy of the brain together with the specific functions of each lobe
The internal carotid artery (ICA), along with its branches, forms the anterior circulation. It arises as the common carotid artery (CCA); it bifurcates into the ICA and external carotid artery (ECA) at the level of the 4th cervical vertebra in the neck. The left CCA arises directly from the aortic arch and the right CCA from the brachiocephalic trunk. The ECA supplies the facial tissues while the ICA ascends superiority, within the carotid sheath, to enter the skull through the carotid canal of the temporal bone. Once within the cranial cavity it passes anteriorly through the cavernous sinus, distal to which it gives off the ophthalmic, anterior meningeal, anterior choroidal and posterior communicating arteries before terminating into two branches; the anterior cerebral artery and middle cerebral artery (MCA). (Figure 1-3) The ophthalmic artery supplies the orbit, the anterior cerebral artery supplies the anteromedial part of the cerebrum, the MCA supplies the lateral cerebrum and the posterior communicating artery connects the anterior and posterior circulation. \(^{(1)}\)
The vertebral arteries supply the posterior circulation. They arise from the subclavian arteries and ascend the posterior neck, entering the skull through the foramen magnum. Before continuing within the cranial cavity to formulate the basilar artery they give off the anterior and posterior spinal arteries, meningeal branches and posterior inferior cerebellar artery. The basilar artery then branches into the posterior cerebral arteries.

The circle of Willis (Figure 1-2) consists of three paired arteries; the ICA, the anterior cerebral artery and the posterior cerebral artery and connects the anterior and posterior circulations via 2 connecting arteries;

- Anterior communicating artery – connecting the two anterior cerebral arteries

*Figure 1-3 Path of the internal carotid artery*
• Posterior communicating artery – connecting the ICA and posterior cerebral arteries

1.2 Arterial Wall Anatomy

Arteries transport oxygenated blood to the tissues along with other blood components. The arterial wall consists of three layers; tunica intima, tunica media and tunica adventitia. (Figure 1-4) \(^{(1)}\)

The tunica intima is the innermost layer and is lined by endothelial cells which are in direct contact with arterial blood. Beneath this lies the subendothelial layer which consists of connective tissue and deep to this layer is the internal elastic lamina which consists of elastic fibres.

The tunica media is a smooth muscle layer which is responsible for constriction and relaxation of the artery. It contains elastin fibres which aid in recoil and collagen which provides strength to the artery. Deep to this lies the external elastic lamina which separates the media from the adventitia.

The tunica adventitia is the outermost layer consisting of connective tissue and collagen, further strengthening the artery.
1.3 Role and Function of Blood Cells

Plasma contains the cellular elements white blood cells (WBCs), red blood cells (RBCs) and platelets. RBCs transport oxygenated haemoglobin to the tissues. WBCs form part of the immune system and platelets aid in haemostasis.

WBCs consist of granulocytes, lymphocytes and monocytes. Together they form part of the body’s immune system through the triggering of inflammation in response to antigens. Lymphocytes are key components of the immune system and can be split into T-Cells and B-Cells. Four varieties of T cells exist, all with specific functions within the immune response; helper T-Cells, suppressor T-Cells, killer T-Cells and memory T-Cells. Helper cells are split into helper 1 (TH1) and helper 2 (TH2) cells and together these cells interact in regulating the immune response. They interact with macrophages and other cells involved in the immune response through the release of hormone like chemical messengers called cytokines.

Platelets are anucleate cells whose main function is to aid clotting through the formation of a platelet plug secondary to platelet aggregation. They are produced in the bone marrow and have a half-life of 4 days. Within the blood vessel they flow close to the endothelium, monitoring its integrity. They express glycoprotein receptors for collagen and von Willibrand factor (vWF); GPIIa-IIla, GPVI, GPIb-IX-V and GPIIb-IIIa. Damage to the endothelial lining exposes collagen and vWF with

Figure 1-4 Anatomy of the arterial wall (2)
subsequent platelet adherence. Platelets first bind to collagen via the GPIa-IIa and GPVI receptors but binding to vWF via the GPIb-IX-V receptor is essential for platelet adherence in areas of high shear stress (fast blood flow). Platelets then change shape and become activated, inducing a series of reactions which result in platelet aggregation. Platelet activating factor is released causing diacylglycerol formation secondary to phospholipase C activation. This in turn causes the alpha and dense granules, located within platelets, to discharge their contents causing an increase in intracytoplasmic calcium which triggers the release of adenosine diphosphate (ADP). Activated platelets also stimulate the production of thromboxane A$_2$ (TXA$_2$) from Arachidonic Acid (AA) via the AA pathway that is catalysed by the enzymes cyclo-oxygenase-1 (COX-1) and thromboxane synthase. ADP and TXA$_2$, together with locally produced thrombin, bind to platelet receptors triggering a change in the GPIIb-IIIa receptor causing it to become activated to which vWF and fibrinogen bind to. Fibrinogen is divalent and therefore binds to GPIIb-IIIa receptors on adjacent platelets causing aggregation and the subsequent formation of a platelet plug. (Figure 1-5)

*Figure 1-5 Schematic representation of platelet aggregation* (2)
1.4 Pathophysiology of Atherosclerosis

Atherosclerosis is a chronic inflammatory process causing hardening of the arteries secondary to atheromatous plaques.

In the normal arterial wall, endothelial cells have anti-inflammatory properties that prevent the attachment of WBCs as they pass by. In atherosclerosis, the endothelial cells are disturbed causing them to express cell adhesion molecules to which white blood cells, specifically monocytes, attach. At the same time as the endothelial cells express adhesion molecules their permeability changes allowing low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) to enter the intima and become retained as the amount exceeds the intima’s ability to eliminate them. LDL is then oxidised, releasing phospholipids that activate the endothelial cells further and hence further expression of adhesion molecules, perpetuating the process.

Endothelial cells are disturbed secondary to conditions such as hypertension, hypercholesterolaemia and smoking. Once monocytes attach to the endothelial cell they enter the intima and mature into a macrophage in response to locally produced macrophage-colony stimulating factor (M-CSF). The macrophages then express scavenger receptors which capture modified LDL particles causing them to become engorged with lipids. These engorged macrophages are called foam cells which release inflammatory mediators that amplify and sustain the inflammatory process. (Figure 1-6) It is this processes that begins the development of atherosclerotic plaques. (4-7)
1.4.1 Plaque Development

Fatty streaks, consisting predominantly of foam cells are precursors to atheroma development. The plaque then develops through a cascade of reactions causing an accumulation of immune cells, mostly macrophages and T-cells, and the activation and proliferation of smooth muscle cells (SMCs).

Two types of macrophages are found within atherosclerotic lesions. One that is induced by hypercholesterolaemia and leads to the development of foam cells and the other which has pro-inflammatory properties responsible for the release of the cytokines interleukin-1β (IL-1β) and tumour necrosis factor (TNF). Macrophages become activated when their pattern recognition receptors, such as Toll-Like Receptor 4, become initiated by LDL. Cholesterol also accumulates within the
macrophage and activates the enzyme inflammasome which causes the release of IL-1β, perpetuating the inflammatory process. T-Cells accumulate following attachment to adhesion molecules and become activated by LDL. Many of the T Cells are of the TH1 subtype whose role is to activate macrophages and regulate inflammation through release of the pro-inflammatory cytokines, Interferon –γ and TNF, which in turn stimulate macrophage activation. In addition to T-cells and macrophages, other immune cells are present including mast cells, neutrophils and B-cells.

The most abundant cells in the atherosclerotic lesion are SMCs which not only reside in the intima but also migrate from the media and proliferate in response to platelet derived growth factor. The role of the SMC is to produce an extracellular matrix consisting of collagen and elastin which forms a fibrous cap that covers the atherosclerotic plaque. During this process, some of the foam cells die forming a lipid rich necrotic core comprising of lipids and cellular debris that has not been cleared. As the plaque grows it encroaches into the lumen causing a stenosis. (4-7)

In summary, the plaque is an accumulation of immune cells, particularly macrophages and T cells with a lipid rich necrotic core covered by a fibrous cap.

1.4.2 Shear Stress

Force applied by the flow of blood also has a role to play in the initiation of atherosclerosis. There are three forces at play in blood flow; fluid shear stress (FSS), cyclic stretch and hydrostatic pressure. It is these forces which account for atherosclerosis occurring at certain sites within the vascular tree i.e. bifurcations. FSS in blood vessels is the force exerted on the endothelial cells by the flow of blood. It is proportional to the velocity of blood flow and inversely related to the cube of the vessel radius. Therefore, endothelial cells act as sensors to regulate FSS and preserve vessel wall homeostasis through vasoconstriction and vasodilatation. At certain sites along the vascular tree, such as the carotid bifurcation, the arteries are exposed to oscillatory shear stress. These arteries then undergo remodelling to preserve luminal diameter and maintain normal FSS. Over time the intima thickens and these sites become preferential for atherosclerotic formation. Disturbed flow
can also cause endothelial cells to become permeable and release pro-inflammatory mediators. \(^{9,10}\)

### 1.4.3 Unstable Plaque and Thrombus Formation

Most plaques progress slowly, causing stenosis while remaining asymptomatic. While this stenosis may cause end organ ischaemia, major CV events, such as stroke and MI, are caused by thrombus formation on a ruptured plaque leading to either occlusion at that site or embolisation distal to this. Thrombus formation occurs when the fibrous cap of the atherosclerotic plaque is disturbed to expose collagen and vWF, causing the adherence of platelets and subsequent thrombus formation as described above. (Figure 1-7) Plaques rupture as their fibrous caps thin out. This occurs as the amount of collagen, which gives the cap its strength, decreases secondary to a decreasing number of SMCs and the release of proteolytic enzymes from activated macrophages and T-cells that breakdown collagen. Consequently, as the amount of collagen decreases the cap thins and is prone to fissure formation or ulceration secondary to the turbulent blood flow and shear stress. The ulceration thus exposes the pro-coagulant material triggering platelet aggregation. \(^{4}\)
Figure 1-7 Thrombus formation on an unstable plaque (B)
CHAPTER 2: CAROTID ARTERY DISEASE

2.1 Introduction

The World Health Organization (WHO) defines stroke as ‘rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin’. Transient Ischaemic Attack (TIA) is a brief period of focal neurological dysfunction lasting less than 24 hours but typically less than 1 hour. Amarousis Fugax, a type of retinal TIA, is a painless, transient loss of vision that is often described as a curtain descending over the eye caused by decreased retinal blood flow. Stroke can either be ischaemic or haemorrhagic and it is not possible to determine the type on clinical grounds. They both have the common endpoint of hypoxia induced cerebral ischaemia.

The clinical signs and symptoms experienced by the patient are determined by the region of the brain affected. Symptoms as a result of an anterior circulatory stroke, as supplied by the ICA, can include any of the following:

- Hemiparesis/Hemisensory loss
- Facial Droop
- Speech disturbance

They can either occur alone or in combination and will be experienced on the contralateral side of the body to the cerebral hemisphere affected and hence the contralateral ICA.

Carotid Artery Disease (CAD) is caused by the formation of an atherosclerotic plaque causing disturbed blood flow. Stroke is the third leading cause of death in the western world behind CV disease and cancer of all types, being responsible for 11% of all deaths. Stroke is the leading cause of disability in the UK with 152 000 sufferers per year, having a major impact on the economy, costing around £7 billion per year. 80% of strokes are ischaemic, of which 30% are caused by
atherosclerotic emboli originating from CAD.\(^{(14)}\) As explained in the previous chapter, thrombus formation occurs on a ruptured atherosclerotic plaque and part of this thrombus can dislodge. In the ICA, this thrombotic material travels upstream into the cerebral circulation and occludes an artery, this phenomenon is termed embolisation. Thus, each patient who presents with symptoms of cerebral ischaemia, whether it is a stroke or TIA, is assessed for CAD through the use of an arterial duplex. If found to have CAD, they are referred to vascular surgeons for consideration of carotid intervention if deemed appropriate.

### 2.2 Epidemiology of Carotid Artery Disease

Subclinical CAD disease is common; the cardiovascular health study consisted of both men and women aged over 65 and detected carotid stenosis in 75% of men and 62% of women with maximal stenosis increasing with age and being greatest in males.\(^{(15)}\) However, population-based studies have estimated the prevalence of moderate carotid artery stenosis (\(\geq 50\%\)) in the population aged 60-69 to be 2.3% in men and 2% in women. The prevalence of severe stenosis (\(>70\%\)) was 0.8% for men and 0.2% for women, rising to 2% and 1% in those aged 70-79.\(^{(16)}\) CAD was more prevalent in individuals with CV risk factors (smoking, hypertension, diabetes and hypercholesterolaemia). The first major randomised trials on intervention for CAD quoted an annual stroke risk of 2% for patients with an asymptomatic carotid artery stenosis, significantly less than those with symptomatic disease.\(^{(17)}\)

In addition to being a causative factor in ischaemic stroke, CAD is also a marker of general atherosclerotic disease. A carotid bruit is an abnormal sound heard when auscultating the carotid artery and can be indicative of an underlying carotid stenosis. In the Framingham study it was detected in 7% of persons aged 65-79 and women aged 50-84 had a significantly increased risk of stroke and MI if one was detected.\(^{(18)}\) Whilst it is accepted to be neither sensitive nor specific for CAD it is accepted as being a marker of generalised atherosclerotic disease with a predictive value of 85%.\(^{(19)}\) Results from prospective studies have shown that carotid intima-media (IMT) thickness is a significant predictor of incrementally increased risks for both stroke and MI.\(^{(20-23)}\)
2.3 Pathology of Carotid Artery Disease

The development of CAD, secondary to atherosclerosis, is multifactorial with environmental and familial factors at play. In addition to the classical risk factors for atherosclerosis such as smoking, hypertension, hypercholesterolaemia and diabetes, haemodynamic and genetic factors also have a role.

2.3.1 Genetics

It is well know that CV disease has a familial role; a positive family history of CV disease and MI are independent risk factors for atherosclerosis.\(^{(24, 25)}\) A large meta-analysis of genomic studies on coronary artery disease identified a number of genes which contributed to atherosclerosis.\(^{(26)}\)

Familial studies have shown higher degrees of carotid stenosis in persons with a family history of parental premature coronary artery disease and younger parental age at death.\(^{(27, 28)}\) When measuring the internal carotid IMT in 1886 men and women from the offspring cohort of the Framingham heart study, it was found to have a heritability of 0.35, indicating that genetics may have a role to play in the development of CAD.\(^{(29)}\)

Genetic linkage has been reported in three familial studies suggesting there are chromosomal segments that predispose to increased carotid IMT.\(^{(30-32)}\) The Framingham heart study found a link between chromosome 12 and internal carotid IMT.\(^{(31)}\) Chromosome 12 is where the gene for the macrophage scavenger receptor is located and as mentioned earlier is integral in atherosclerosis.

2.3.2 Carotid Plaque Development

Chapter 1 explained the basic principles involved in atherosclerosis and plaque development. Carotid plaque development follows all the same basic principles with the addition of flow related initiating factors. As mentioned, shear stress has a role to play in disturbing endothelial function and turbulent flow at bifurcations, such as the carotid, provides an atherogenic environment. (Figure 2-1) This turbulent flow disturbs the endothelial cell causing the release of pro-inflammatory mediators such as platelet-derived growth factor and vascular endothelial growth
factor making the endothelium more permeable and triggering the inflammatory atherosclerotic process.

Carotid plaques are classed as symptomatic when they cause symptoms of cerebral ischaemia. This is due to plaque rupture and subsequent thrombus formation. During this process either atherosclerotic material from the plaque or thrombotic material embolises to the brain where it travels within the cerebral circulation until it lodges in a vessel, causing occlusion. The degree of cerebral ischaemia caused depends not only on the size of the emboli but also on the extent and timing of subsequent reperfusion; the extent and distribution of collaterals; and autoregulatory capacity.

*Figure 2-1 Turbulent flow at the carotid bifurcation* (8)
2.3.3 Histological Classification of Carotid Plaques

Before the American Heart Association (AHA) classification of atherosclerotic plaques, there was simply two categories; the fatty streak - consisting of foam, inflammatory and SMCs, and the atheromatous plaque - a continuation of the fatty streak to include a raised lesion with a lipid rich necrotic core and fibrous cap. The AHA classification classifies the lesion according to its stage of development. (Figure 2-2) While this classification is based on coronary atherosclerotic plaques, there is no recognised classification for carotid plaques rather suggested adaptions of the AHA. In summary the AHA classification is as follows;

- Type I lesions represent initial early changes with an increase in macrophages and foam cells
- Type II lesions are fatty streaks, as described above, predominantly SMCs and foam cells
- Type III lesions are considered intermediate consisting of pools of extracellular lipid
- Type IV lesions are referred to as atheroma characterised by a larger lipid core
- Type V lesions are referred to fibroatheromas where the lipid core remains separated from the lumen by fibrous connective tissue and can be split into types Va, Vb or Vc depending on calcification present (Va) or not (Vb) or minimal lipid (Vc).
- Type VI lesions, referred to as complicated, are type V lesions that demonstrate disruption of the fibrous cap, haematoma or thrombus formation.

Type IV, V and VI lesions are regarded as advanced lesions.
### AHA classification of atherosclerotic plaques according to their stage of development

<table>
<thead>
<tr>
<th>Nomenclature and main histology</th>
<th>Sequences in progression</th>
<th>Main growth mechanism</th>
<th>Earliest onset</th>
<th>Clinical correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I (initial) lesion isolated macrophage foam cells</td>
<td>I</td>
<td>growth mainly by lipid accumulation</td>
<td>from first decade</td>
<td>clinically silent</td>
</tr>
<tr>
<td>Type II (fatty streak) lesion mainly intracellular lipid accumulation</td>
<td>II</td>
<td>growth mainly by lipid accumulation</td>
<td>from third decade</td>
<td></td>
</tr>
<tr>
<td>Type III (intermediate) lesion Type II changes &amp; small extracellular lipid pools</td>
<td>III</td>
<td>from third decade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IV (atheroma) lesion Type II changes &amp; core of extracellular lipid</td>
<td>IV</td>
<td>from fourth decade</td>
<td></td>
<td>clinically silent or overt</td>
</tr>
<tr>
<td>Type V (fibroatheroma) lesion lipid core &amp; fibrotic layer, or multiple lipid cores &amp; fibrotic layers, or mainly calcific, or mainly fibrotic</td>
<td>V</td>
<td>accelerated smooth muscle and collagen increase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type VI (complicated) lesion surface defect, hematoma-hemorrhage, thrombus</td>
<td>VI</td>
<td>thrombosis, hematoma</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 2-2 AHA classification of atherosclerotic plaques according to their stage of development**

### 2.3.4 Carotid Plaque Morphology

#### 2.3.4.1 The Vulnerable Carotid Plaque

Much of the work surrounding plaque instability originates from studies of coronary arteries at autopsy from people who had sudden fatal MIs. This work has extended into carotid artery plaques and the main features of an unstable plaque mentioned in the literature are; thin/ruptured fibrous caps, presence of intraplaque haemorrhage (IPH) and lipid rich necrotic cores (LRNC) with inflammation playing a pivotal role. All three of these features seem to be related and shown to be associated with an increased risk of stroke.

As previously mentioned, atherosclerotic plaques are covered by a fibrous cap made up predominantly of SMCs and collagen. Plaques with a thin cap are weakened and more susceptible to rupture when exposed to increased haemodynamic forces such as the oscillatory sheer stresses at the carotid
bifurcation. The thinning of the cap is secondary to collagen degradation caused by proteolytic enzymes released in the inflammatory process. Matrix metalloproteinases (MMP) released by macrophages have been shown to play a role in this along with interferon-γ. Interferon-γ is secreted by activated T cells causing a decrease in the ability of SMCs to express collagen genes. Several histological studies have demonstrated a link between cap rupture and inflammation, none more so than the Oxford plaque study who found that cap thickness was independently associated with cap rupture and strongly associated with plaque inflammation. Marked inflammation within the cap, as determined by macrophage content, was independently associated with cap rupture as was IPH with LRNC demonstrating a strong positive association. IPH and LRNC are inter-related with many proposing that IPH contributes to increasing LRNC volumes. The membranes of RBCs contain free cholesterol along with macrophages. Kolodgie et al, through the use of an antibody to glycophorin A, demonstrated the association between IPH, size of the LRNC and plaque instability. Glycophorin A is localised in erythrocyte membranes and the amount of glycophorin A and Iron levels not only corresponded with the size of the necrotic core but also matched the macrophage content. This erythrophagocytosis not only contributes to the LRNC through the release of cholesterol but perpetuates the inflammatory process as it enhances the macrophages ability to oxidise LDL. Kockx et al strengthened this theory when reporting on finding clusters of macrophages with ingested red cell fragments close to microvessels in atherosclerotic plaques. IPH is thought to be due to rupture of microvessels which form secondary to neovascularisation within the plaque. Takaya et al demonstrated the association between LRNC and IPH in their MRI study over 18 months. Those with IPH had larger LRNC volumes and if present at baseline had an accelerated atherosclerotic progression.
Clinical Studies

Plaques referred to as ulcerated, irregular or with a disrupted luminal surface can be considered the same as those referred to as having a thin/ruptured fibrous cap.

The North Manhattan Stroke Study \(^{(50)}\) demonstrated an 8.5% increased 5 year risk of stroke in patients found to have an irregular plaque compared to 3% in those with a smooth plaque. This was in line with previous work from Japan reporting an increased stroke risk in such patients \(^{(51)}\) and Gao et al who concluded that ulcerated plaques were significantly correlated with neurological events in their meta-analysis including 2839 plaques. \(^{(52)}\) Madani et al found that the presence of 2 or more ulcers, as detected by 3D ultrasound, was associated with a significantly higher risk of Stroke, Death or TIA within 3 years, despite best medical therapy. \(^{(53)}\)

Gupta et al \(^{(54)}\) performed a systematic review and meta-analysis concluding the presence of IPH, LRNC, and thinning/rupture of the fibrous cap, detected by MRI, was associated with an increased risk of future stroke or TIA, irrespective of the level of carotid stenosis. Thinning/rupture of the fibrous cap had the highest hazard ratio at 5.93 (2.65-13.2). Takaya et al also found a significant association between thin or ruptured fibrous caps detected by MRI at baseline in those that went on to suffer a cerebrovascular event. \(^{(55)}\) Whilst IPH and a large percentage LRNC were also associated, the greatest association was for thin fibrous caps.

In the largest histological study of 1640 carotid plaques, Howard et al \(^{(56)}\) demonstrated that predicted stroke risk significantly increased as the number of plaque features associated with plaque instability increased (p=0.002). Plaque inflammation and a large lipid core were also significantly correlated with predicted stroke risk in those patients operated on within 30 days of symptom onset.

Teng et al \(^{(57)}\) agreed that large lipid cores are associated with stroke risk when they found carotid plaques with a large LRNC and thin fibrous cap were high risk, complementing the work by both Howard and Underhill et al. \(^{(58)}\) They devised a carotid atherosclerosis score to predict high risk plaques based on the percentage of LRNC as a precursor to cap rupture. Xu et al \(^{(59)}\) expanded their work and used this scoring system to predict high risk plaque features. They looked specifically for
which plaque features were associated with IPH or a disrupted luminal surface (DLS) in a prospective longitudinal study of 120 asymptomatic patients with an ICA stenosis between 50-70%. All new DLS were found in those with a higher baseline score with a trend for developing a new DLS with an increasing score. This is in line with the conclusion drawn by Underhill et al. who found those with DLS had a significantly larger LRNC. When comparing the groups of patients with DLS to those without, the DLS group had a larger percentage wall volume and larger percentage LRNC volume. The increase in percentage LRNC was also found to be significantly quicker in those who developed a new DLS.

2.3.4.2 Carotid Plaque Volume

Carotid Plaque Volume (CPV) is the actual volume of atherosclerotic plaque present within the carotid artery and is a representation of the plaque burden.

The evidence to support that carotid artery stenosis is not the best predictor of stroke risk is based on the work by Glagov et al. into human atherosclerotic coronary arteries. He found that human coronary arteries enlarge in relation to plaque area i.e. the artery undergoes outward remodelling to preserve the luminal diameter. Luminal stenosis only occurred once 40% of the internal elastic lamina was occupied by plaque. This theory is further strengthened by the work of Barbiarz, Saam and Dong. They each conducted studies on asymptomatic carotid arteries with minimal or no stenosis. Dong et al found substantial plaque burden in angiographically normal arteries (0% stenosis) while Saam et al frequently found complicated atherosclerotic lesions (American Heart Association (AHA) Type VI Carotid Atherosclerotic Lesions) in carotid arteries with ≤ 50% stenosis.

Greg Stone found comparable results when looking at the natural history of coronary atherosclerosis in the PROSPECT Study. Through the use of intravascular ultrasound they were able to identify high plaque burden as a feature of an unstable plaque. Historic angiograms of patients returning with recurrent coronary events, that were not secondary to the initially treated lesion, were reviewed to evaluate the extent of disease in the now symptomatic lesions. Over
50% of these plaques had thin caps and were frequently classed as mild on the previous angiogram based on the degree of stenosis.

Rozie et al advocate evaluating the role of CPV as an additional parameter in assessment of stroke risk. (65) They demonstrated an association between increasing CPV and change in plaque composition – the proportion of calcium and lipid content increases as the amount of fibrous tissue decreases. We know that as lipid content increases so too does likelihood of fibrous cap rupture.

Wannarong et al (66) measured the progression of IMT, total plaque area and TPV in the carotid arteries of 349 patients over a 5 year period using 3D USS. They found progression of TPV predicted stroke, death or TIA and any CV event. A change in IMT did not predict stroke, death, TIA or any CV event.

### 2.3.5 Cerebral Microemboli

Cerebral microemboli are often subclinical and asymptomatic although can be detected by transcranial Doppler (TCD) insonation of the MCA, referred to as microembolic signals (MES). (67) Given stroke secondary to CAD is thromboembolic and that silent infarcts detected on neuroimaging increases the risk of ipsilateral stroke, it is believed these silent embolic events may be precursors to the larger embolisation’s associated with stroke. (68)

The asymptomatic carotid emboli study (ACES) demonstrated that the presence of MES independently predicts 2 year stroke risk. (69, 70) Additionally MES have been shown to be associated with echolucent plaques, one of the ultrasound features of an unstable plaque. (71, 72)

MES are seen in 40% of recently symptomatic CAD patients with research suggesting that the presence of MES within 48 hours post stroke is associated with an increased likelihood of the recurrence of cerebral ischemic events. (73)

Despite the strong evidence between the presence of MES and future stroke risk it is not practical to perform routine TCD on patients with asymptomatic disease due to its timely nature and the need for manual detection of the emboli.
2.4 Investigation of Carotid Artery Disease

2.4.1 Arterial Duplex

Arterial duplex is the first line investigation in detecting CAD due to its quick, non-invasive and low cost nature. It uses a combination of B-mode imaging and flow velocity on continuous wave doppler to grade stenosis and image the plaque. Duplex reliably detects stenosis greater than 50%, with a positive predictive value >95% compared with angiography. A joint working group between the vascular and technology societies of GB and Ireland developed criteria for grading carotid stenosis based on the NASCET criteria (Table 2-1). (74)

As well as determining the degree of stenosis, arterial duplex can also comment on the echolucency of the plaque and grade it according to the Grey-Weale classification (75),

- Type 1 – Echolucent,
- Type 2 - Predominantly echolucent,
- Type 3 - Predominantly echogenic,
- Type 4 – Echogenic.

Research has shown that echolucency is associated with an increased risk of stroke. (76, 77)
Table 2-1 Grading of carotid stenosis according to the joint working group recommendation based on NASCET criteria (74)

<table>
<thead>
<tr>
<th>%Stenosis (NASCET)</th>
<th>ICA PSV (cm/s)</th>
<th>ICA/CCA PSV Ratio</th>
<th>ICA&lt;sub&gt;PSV&lt;/sub&gt;/CCA&lt;sub&gt;EDV&lt;/sub&gt; Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>&lt;125</td>
<td>&lt;2</td>
<td>&lt;8</td>
</tr>
<tr>
<td>50-59</td>
<td>&gt;125</td>
<td>2-4</td>
<td>8-10</td>
</tr>
<tr>
<td>60-69</td>
<td></td>
<td></td>
<td>11-13</td>
</tr>
<tr>
<td>70-79</td>
<td>&gt;230</td>
<td>&gt;4</td>
<td>14-21</td>
</tr>
<tr>
<td>80-89</td>
<td></td>
<td></td>
<td>22-29</td>
</tr>
<tr>
<td>&gt;90 but less than near occlusion</td>
<td>&gt;400</td>
<td>&gt;5</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Near Occlusion</td>
<td>High, low – string flow</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Occlusion</td>
<td>No Flow</td>
<td>Not Applicable</td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>

2.4.2 Angiography

Catheter angiography used to be the ‘gold standard’, however with evolving CT and MR techniques it is now only used when deploying carotid stents.

MRA has been shown to have comparable sensitivity to duplex for accurately detecting stenoses of 70-99% but is recommended to be used in conjunction with arterial duplex. While it is non-invasive and lacks ionising radiation, gadolinium contrast is used which has been identified as a cause of nephrogenic systemic fibrosis, a condition causing fibrosis of the skin and organs in patients with pre-existing renal failure exposed to gadolinium. (78)

CTA allows for rapid acquisition of cross-sectional data and has been shown to be highly specific, although again comparable to arterial duplex. Its advantages over MRA lie in the fact it is quicker and better tolerated by the patient with the thinner slices used negating any movement artefact. However, it is a form of ionising radiation that uses nephrotoxic contrast. It is for these reasons that arterial duplex
remains the first line investigation of choice, with the use of MRA and CTA for further evaluation of CAD. (78)

2.4.3 Three Dimensional Duplex

Three dimensional ultrasound has been widely used to assess plaque volume and been proven to be reliable. (79) Much of the work concerns TPV which is the total amount of plaque visible along the CCA and ICA. The inter- and intra-observer variability is low (79-83) but increases with decreasing amounts of TPV. Plaque volume has been reported to be consistent for interslice distances (ISD) between 1 to 3mm. More than 3mm, plaque volume variability increases. Pollox et al (84) revealed the potential increased sensitivity using 3D ultrasound to measure TPV and various studies have validated CPV measurements. (85) Fenster et al (79) revealed a small mean error which decreased as volumes increased when using 3D ultrasound to measure plaque volume in 48 simulated carotid artery plaques. The actual plaque volumes were determined using a water displacement technique and using similar methods Palombo et al (86) demonstrated excellent correlation between 3D measured volumes and those measured by the water displacement technique ($r=0.99, P<0.01$) with a mean (sd) difference of 3.12 (15.1) mm$^3$. It was also reproducible with a mean (sd) difference between 2 observers measurements of 0.6 (11.2) mm$^3$. Walker et al confirmed this on animal models. (87)

From the literature it is evident that CPV can be accurately measured using 3D ultrasound.

2.4.4 Biomarkers

It is known that 10-20% of persons with coronary artery disease do not possess the traditional CV risk factors and 50% of MIs occur in patients who have normal lipid levels. (88, 89) Hence the search for a biomarker to identify such patients has been and continues to be researched. Whilst many biomarkers have been studied in relation to the atherosclerotic process and CV risk in general, including stroke, there is very little regards CAD and as yet no biomarker is regularly used in the clinical assessment of such patients. (90)
High sensitive C-reactive protein (Hs-CRP) and P-Selectin have long been studied in their relation to atherosclerosis, of late lipoprotein-associated phospholipase A$_2$ (LP-PLA$_2$) has shown potential as a marker of plaque instability.

Lp-PLA$_2$ is a calcium independent member of the phospholipase A$_2$ family that mediates the hydroxylation of oxidised phospholipids in low density lipoproteins (LDL). This oxidation leads to the production of pro-inflammatory compounds, mainly lysophosphatidylcholine (LysoPC), which have both pro-inflammatory and pro-atherogenic properties. $^{(91)}$ Hence, Lp-PLA$_2$ has been the focus of many studies as a potential marker for atherosclerotic disease, independent of the traditional CV risk markers.

Hs-CRP is a very sensitive marker of CRP activity. CRP is an acute phase protein synthesized by the liver and is an indicator of underlying inflammation. It has been shown that the level of inflammatory activity within a plaque determines how quickly atherosclerosis progresses and its vulnerability to rupture. It is proposed Hs-CRP, along with other inflammatory mediators, regulate this inflammatory activity and so the level of Hs-CRP represents the level of inflammatory activity taking place. $^{(92-94)}$

P-Selectin is a protein located on the surfaces of endothelial cells and platelets and believed to be involved in the initiation of atherosclerosis through its role as a cell adhesion molecule.

**2.4.4.1 Lipoprotein Associated Phospholipase A$_2$**

As previously mentioned, atherosclerosis is a chronic inflammatory process and elevated levels of oxidised LDL (Ox-LDL) have been found to be associated with plaque instability. $^{(95, 96)}$ During initiation of atherosclerosis, LDL particles are oxidised causing them to change from their natural form through alteration to the phospholipid and apoliprotein B components. This is secondary to a series of reactions mediated by lipid hydroperoxidase and aldehydes causing a change to the physical and chemical properties of LDL. $^{(91)}$ It was initially thought this oxidative modification of LDL was the final step in promoting atherosclerosis and studies report increased levels of Ox-LDL in acute coronary syndrome and CAD. $^{(95, 96)}$
et al \(^{(96)}\) evaluated the levels of circulating Ox-LDL in patients with acute coronary syndrome finding those who had suffered an acute MI had significantly higher levels of Ox-LDL (4 times) compared to those with stable angina. Histologically, patients with unstable angina were found to have macrophage derived foam cells densely filled with Ox-LDL compared to those with stable angina.

The same has been reported in carotid plaques. Nishi has previously reported on macrophage infiltration being a landmark for plaque instability. \(^{(97)}\) Based on this they evaluated the relation between Ox-LDL, vulnerable plaques and macrophage content. \(^{(95)}\) Carotid plaques were classed histologically as macrophage rich or macrophage poor together with measurements of plaque and plasma Ox-LDL. Plasma Ox-LDL was not only significantly higher in patients compared to controls but also significantly (70x) higher in plaque compared to plasma. Plaque Ox-LDL correlated strongly with macrophage content and was significantly increased in those classed as having a macrophage rich plaque. Given this strong association and increased macrophage counts being a landmark for instability, they concluded that Ox-LDL was associated with plaque instability.

More recent work on Lp-PLA\(_2\) has demonstrated that oxidation of LDL is not the final step in promoting atherosclerosis rather this oxidation renders LDL susceptible to hydroxylation by Lp-PLA\(_2\). This hydroxylation causes a series of pro-inflammatory reactions secondary to the production of several pro-inflammatory compounds, mainly LysoPC and oxidised nonesterified fatty acids (Ox-NEFA). (Figure 2-3) Both these products have pro-inflammatory and pro-atherogenic properties (Table 2-2) through the attraction of inflammatory cells into the atherosclerotic lesion and the local release of inflammatory mediators and vascular adhesion molecules. They also have a pro-apoptotic effect on macrophages which accounts for the lipid rich necrotic core commonly found in unstable atherosclerotic plaques. \(^{(91, 98, 99)}\)

Lp-PLA\(_2\) can be synthesised de-novo by plaque inflammatory cells namely monocytes, macrophages, T cells and mast cells and 70-80% of it is transported bound to LDL, with a preference for the pro-atherogenic parts LDL-4 and LDL-5. \(^{(88)}\)
Figure 2-3 Pathway of how Lp-PLA₂ is involved in atherosclerosis producing LysoPC and oxNEFA\textsuperscript{(100)}
<table>
<thead>
<tr>
<th>Mediator</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC</td>
<td>Endothelial dysfunction</td>
</tr>
<tr>
<td></td>
<td>Increased membrane permeability</td>
</tr>
<tr>
<td></td>
<td>Induction of leucocyte adhesion molecule expression</td>
</tr>
<tr>
<td></td>
<td>Chemoattractant for T-Lymphocytes and monocytes</td>
</tr>
<tr>
<td></td>
<td>Macrophage and smooth muscle cell proliferation</td>
</tr>
<tr>
<td></td>
<td>Pro-apoptotic effect</td>
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<tr>
<td></td>
<td>Release of Arachidonic Acid</td>
</tr>
<tr>
<td></td>
<td>Release of Myeloperoxidase</td>
</tr>
<tr>
<td></td>
<td>Increased expression of IL-1β, IL-6, VCAM-1, ICAM-1, MCP-1, TNF-α, IFN-γ, PDGF, heparin-binding EGF-like proteins</td>
</tr>
<tr>
<td>Ox-NEFA</td>
<td>Increased membrane permeability</td>
</tr>
<tr>
<td></td>
<td>Pro-apoptotic effect</td>
</tr>
<tr>
<td></td>
<td>Chemoattractant for monocytes</td>
</tr>
<tr>
<td></td>
<td>Increased expression of VCAM-1</td>
</tr>
</tbody>
</table>

Table 2-2 Proinflammatory and pro-atherogenic properties of Lyso-PC and Ox-NEFA


2.4.4.1.1 Lp-PLA₂ and Stroke Risk

The west of Scotland coronary prevention study \(^{(107)}\) was one of the first studies to report an association between increasing levels of plasma Lp-PLA₂ and the risk of future coronary events. The Veterans Affairs HDL Intervention Trial \(^{(101)}\) then followed, finding a significant association between increased Lp-PLA₂ and increase in all CV events which was most significant for stroke. This led to further studies on Lp-PLA₂ as a marker of CV risk, including two studies that trialled an inhibitor of LP-
PLA$_2$: 330 patients with angiographically proven coronary artery disease taking an Lp-PLA$_2$ inhibitor demonstrated a halt in the increase of the necrotic core compared to the placebo group $^{(102)}$ and similar results were found in an animal study reporting a decrease in plaque area and necrotic core resulting in fewer unstable lesions following inhibition of Lp-PLA$_2$. $^{(103)}$

Various studies have also looked at Lp-PLA$_2$ and the risk of stroke. The atherosclerosis risk in communities (ARIC) study $^{(104)}$ found Lp-PLA$_2$ to be a significant predictor of stroke when following up 12,773 healthy middle aged men over 6-8 years. Elevated blood pressure readings potentiated the risk with systolic readings above 130mmHg increasing the risk by 3.5 times compared to readings below 113mmHg. Interestingly, lipid levels did not predict stroke. The same applied to women in the women’s health initiative study $^{(105)}$ but only applied to those suffering large vessel strokes as opposed to small vessel strokes. The Rotterdam study $^{(106)}$ complemented these findings.

Lp-PLA$_2$ has also been found to predict recurrent stroke $^{(110, 111)}$ with elevated levels associated with a 2.1 fold increase in recurrent stroke and predictive of stroke, MI or vascular death. $^{(107)}$ This risk has been shown to remain following adjustment for diabetes, hypertension, hypercholesterolaemia and smoking and levels of Lp-PLA$_2$ did not change significantly with time negating any potential effects of the acute stroke on Lp-PLA$_2$ levels. $^{(108)}$
2.4.4.1.2 Lp-PLA\textsubscript{2} and Carotid Artery Disease

Despite there being vast amounts in the literature evaluating the role of Lp-PLA\textsubscript{2} as a potential marker of CV risk there is little looking specifically at Lp-PLA\textsubscript{2} in relation to CAD. In their histological study, Mannheim et al \textsuperscript{(99)} found expression of Lp-PLA\textsubscript{2} was significantly increased in symptomatic plaques and those with pre-existent coronary heart disease, correlating significantly with LysoPC. Lp-PLA\textsubscript{2} was most abundant in the necrotic core, co-localizing with macrophages and Ox-LDL hence strengthening the argument that Lp-PLA\textsubscript{2} is involved in plaque instability. Symptomatic plaques had lower levels of collagen that were inversely correlated with TUNEL+CELLS which are markers of apoptosis. LysoPC correlated with TUNEL+CELLS suggesting that LysoPC is involved in apoptosis. The relation of the necrotic core to plaque instability has already been covered and since LysoPC is a by-product of the Lp-PLA\textsubscript{2} induced hydroxylation of Ox-LDL then it would be fair to conclude that Lp-PLA\textsubscript{2} has a contributory role in plaque instability secondary to its ability to potentiate the inflammatory response and increase the necrotic core.

Sarlon-Bartoli et al \textsuperscript{(109)} found circulating levels of Lp-PLA\textsubscript{2} were significantly increased in patients with high grade stenosis and unstable plaques when analysing the plaques of 42 patients undergoing CEA. Median plasma Lp-PLA\textsubscript{2} was significantly higher in patients with unstable plaques compared to stable plaques and remained true when analysing only asymptomatic patients.

2.4.4.2 High Sensitive CRP

There are several theories as to how CRP is related to atherosclerosis centred around whether it merely reflects the level of inflammatory activity within atherosclerotic plaque , has a role at a cellular level promoting atherosclerosis or a pro-coagulant effect. (Figure 2-4) Some postulate that raised levels of Hs-CRP reflect an increased level of systemic inflammatory activity within the body. Since atherosclerosis is an inflammatory process, this raised level of generalised inflammatory activity accelerates the atherosclerotic process. \textsuperscript{(92)} Others postulate that as the level of inflammatory activity taking place within the plaque increases, hence its ability to become unstable, then so too does Hs-CRP. \textsuperscript{(93, 94)}
Several studies report that CRP is actively involved in atherosclerosis through endothelial cell activation, expression of cellular adhesion molecules and the uptake of LDL into macrophages. \textsuperscript{(110-115)} Hatanaka et al, \textsuperscript{(116)} in an early experimental study, found CRP binding activity in macrophages and postulated that CRP promoted lipid intake and foam cell development along with cytotoxic effects. Others point to a pro-coagulant effect of CRP through its ability to stimulate monocyte tissue factor production which is a known activator of the extrinsic coagulation cascade therefore contributing to the thrombotic events associated with plaque rupture. \textsuperscript{(117-120)}
2.4.4.2.1  Hs-CRP and Cardiovascular Disease

Since CRP is an acute phase reactant many believed that the increased levels merely represented the body’s inflammatory response to the CV event rather than a prognostic factor. This led Ridker et al (121) to measure baseline CRP in 1086 apparently healthy men taking part in the physicians health study. Baseline CRP was significantly increased in those men who suffered an MI or ischaemic stroke, independent of traditional CV risk factors with the relative risk increasing as CRP increased. That there was no relation to venous thrombosis implies CRP’s role is limited to atherosclerotic disease and has a long term effect given the increased relative risks at greater than 6 years follow-up. These findings were then replicated in apparently healthy post-menopausal women. (89)

The Rotterdam study (122) demonstrated a clear link between increased levels of CRP and progression of atherosclerosis over a six year period at various points in the arterial tree, including the carotid artery, independent of age, gender and smoking. Interestingly, CRP was not associated with progression of carotid atherosclerosis in those patients with no sonographic evidence of carotid atherosclerosis at baseline. The inflammation and carotid artery risk for atherosclerosis study (ICARAS) (123) replicated these findings and by measuring Hs-CRP at follow-up demonstrated that not only were elevated levels of baseline Hs-CRP significantly associated with progression so too were increased levels at follow-up.

Much of the early work surrounding CRP centred on coronary heart disease and a number of studies have not only shown CRP to be increased in patients with symptomatic CV disease but that it can also predict recurrent CV events. The Framingham study (124) reported that both men and women with increased concentrations of CRP had increased risks of both ischaemic stroke and TIA over a 12-14 year period which remained following adjustment for traditional risk factors. The ARIC study (125) found comparable results with improvement in risk prediction of ischaemic stroke when Hs-CRP was added to a model based on traditional risk factors.
2.4.4.2 Hs-CRP and Carotid Artery Disease

Many papers have found a relation between Hs-CRP and progression of CAD both symptomatic and asymptomatic. (126-128)

Rerkasem et al (129) in his cross sectional study found Hs-CRP levels were significantly increased in symptomatic compared to asymptomatic patients admitted for CEA (3.9ng/ml vs 2.1ng/ml, p=0.04). When adjusted for time from neurological event the significance remained and the type or severity of neurological event had no relation to the level of Hs-CRP meaning the body’s systemic inflammatory response to stroke was not involved.

Alvarez et al (130) evaluated Hs-CRP levels in patients undergoing CEA and compared their levels not only to neurological status but to plaque instability as determined histologically. Hs-CRP was significantly increased in symptomatic patients and those classed as having an unstable plaque. Hs-CRP also correlated significantly with macrophage and T cell counts. Interestingly, they also reported that Hs-CRP increased with time from neurological event.

2.4.4.3 P-Selectin

Stimulation of P-Selectin results in its expression on the surface of endothelial cells where it aids in leucocyte rolling, attachment and migration through the endothelial cell wall, initiating the atherosclerotic process. (131-134) P-Selectin has been shown to have a preference for endothelial cells overlying atherosclerotic plaques. (134)

The early studies involving P-Selectin focused on coronary heart disease and demonstrated a significant increase in those patients with unstable angina or recent MI. (135-137) However they also demonstrated P-selectin levels decreased with time from the event making it difficult to interpret if the increase was reflective of the body’s systemic response to the ischaemic injury or related to the unstable plaque. Its association with ischaemic stroke has also been reported. (138-140)

Ridker et al (141) demonstrated baseline plasma P-selectin was significantly higher in apparently healthy women who went on to suffer a CV event with the risk
increasing as P-selectin increased, independent of other risk factors. P-Selectin also had a moderate but significant correlation with Hs-CRP. Bielenski et al.\textsuperscript{[142]} later went on to replicate this association in the multi-ethnic study on atherosclerosis, reporting a positive linear association, independent of traditional risk factors, between P-selectin and coronary events. However, this was only evident in non-Hispanic white Americans.

Frijns et al.\textsuperscript{[138]} were the first to report on P-Selectin being significantly increased in patients with ICA stenosis and Koyama et al.\textsuperscript{[143]} replicated these results with findings of P-Selectin being significantly and positively correlated with CAD as measured by carotid IMT and stiffness.

However, while it is evident that P-Selectin is involved in the atherosclerotic process Blann et al.\textsuperscript{[144]} raised doubts about its additional prognostic value over traditional CV risk factors in their comprehensive review of the literature.
2.5 Management of Carotid Artery Disease

Best medical therapy is the mainstay of treatment for patients with CAD together with intervention if deemed appropriate. Patients are classed as having a symptomatic ICA stenosis if they suffer symptoms of cerebral ischaemia corresponding to the cerebral hemisphere supplied by the diseased ICA.

2.5.1 Medical Management

All patients diagnosed with CAD should receive best medical therapy and lifestyle advice to optimise their CV risk factors for both primary and secondary prevention. The main risk factors for atherosclerosis are hypertension, hypercholesterolaemia, diabetes and smoking. The European Stroke Initiative recommended a range of interventions that constitute best medical therapy including dietary and exercise advice together with smoking cessation aids.

Antiplatelet and statin therapy are the mainstays of medical therapy along with optimisation of blood pressure. Blood pressure readings below 140/90 should be sought with the aid of medications if needed. Research has demonstrated that a reduction in systolic and diastolic pressure readings of 10mmHg and 5mmHg respectively are associated with a 41% reduction in the risk of stroke. \(^{(145)}\)

2.5.1.1 Statin Therapy

Several studies have demonstrated the effectiveness of statin therapy in primary and secondary stroke prevention with the European stroke initiative recommending that all patients with CV disease take statin therapy. \(^{(146-152)}\)

The benefits of statin therapy in stroke prevention seem less to do with the lowering of cholesterol levels and more related to their anti-inflammatory and anti-oxidant properties inhibiting monocyte adhesion and intimal migration. Animal studies have demonstrated a decrease in lipid core size along with decreased macrophage content and metalloproteinase production causing an increase in collagen. This has been reflected in the histological assessment of endarterectomised plaques in patients receiving statins. \(^{(153-155)}\)
2.5.1.2 Antiplatelet Therapy

In patients with CAD, slowing plaque progression and inhibiting platelet induced thrombus formation reduces the risk of embolic infarcts.\(^{(156)}\)

Aspirin and Clopidogrel impact on platelet activation and aggregation via their own distinct mechanisms but share the same endpoint of reducing atherosclerotic progression and platelet associated thrombus formation.\(^{(157)}\) Aspirin inhibits the action of COX-1, inhibiting the synthesis of \(\text{TXA}_2\) and subsequent platelet activation.\(^{(158)}\) Since platelets are anucleate, enzymatic activity cannot be restored hence they remain inactive for the remainder of their life cycle.\(^{(158)}\)

Clopidogrel is an Adenosine Diphosphate receptor antagonist, blocking ADP P2Y12 receptors on the surface, thereby stopping activation of P2Y1 receptors and subsequent platelet aggregation.\(^{(159)}\)

The UK antiplatelet trialists’ collaboration published a series of meta-analyses highlighting the benefits of antiplatelet therapy in significantly reducing stroke by 25%.\(^{(160}, 161\)\)

2.5.1.3 Antiplatelet Resistance

Research over the past several years indicates a widespread ‘resistance’ or ‘non-responsiveness’ to antiplatelet therapy. In the U.S. alone, 185,000 recurrent strokes occur each year, with a third of these occurring in patients receiving antiplatelet therapy.\(^{(162)}\)

Since aspirin resistance was first described in the 1980s,\(^{(163)}\) resistance to antiplatelet therapy has been widely reported. Grotemeyer et al first reported an association between aspirin resistance and vascular events with 40% of CAD patients with aspirin resistance experiencing a serious vascular event compared with 4.4% of those sensitive to aspirin therapy.\(^{(164)}\)

Antiplatelet resistance can be referred to as ‘laboratory’, proven by multiplate assays\(^{(162)}\) or ‘clinical’ when CV events occur despite antiplatelet therapy.
True drug resistance needs to be distinguished from poor compliance, inadequate dosage, medication interactions or increased platelet turnover;\(^{(162, 165, 166)}\) up to 40% of patients with CV disease fail to comply with their antiplatelet therapy.\(^{(162)}\) Medication interaction is another key cause of perceived resistance;\(^{(167-169)}\) non-steroidal anti-inflammatory agents block the effect of aspirin through competitive binding of COX-1.

The prevalence of antiplatelet resistance varies widely in the literature between 5.5%-61% for aspirin\(^{(162)}\) and 4-30% for clopidogrel.\(^{(170)}\) This variability is largely explained by the number of methods used to measure platelet function. The variability in the prevalence of aspirin resistance was demonstrated by Harrison et al\(^{(171)}\) who screened for aspirin resistance in patients receiving low dose aspirin after TIA or ischaemic stroke using four different methods: Verify Now-Aspirin Assay, PFA-100 assay, optical aggregometry and a combination of these tests; resistance was found in 17%, 22%, 5% and 2% respectively.\(^{(167)}\)

The causes of antiplatelet resistance are thought to include polymorphisms of the Cyclo-oxygenase 1/2 and thromboxane synthase genes for aspirin and the cytochrome P450 family for clopidogrel.\(^{(162, 166, 167, 172)}\)

The association between enhanced platelet production in diabetes and antiplatelet resistance is well documented.\(^{(170, 172, 173)}\) Aspirin resistance is also associated with poor glycaemic control and obesity in type 2 diabetics.\(^{(173)}\) A progressive decline of platelet inhibition has been reported in patients on long term aspirin despite being initially responsive to aspirin.\(^{(174, 175)}\)

There has been little research on resistance to antiplatelet therapy in stroke patients. In a study of 281 patients with cerebrovascular disease, aspirin resistance was associated with poor clinical outcomes but was not an independent risk factor for future ischaemic events.\(^{(176)}\) However, a meta-analysis including 6450 patients reported that aspirin resistance was significantly associated with CV events despite platelet inhibitory therapy.\(^{(177)}\)
2.5.2 Carotid Intervention

Current European Society Vascular Surgeons (ESVS) guidelines recommend CEA for a symptomatic severe carotid stenosis (>70%). For patients with asymptomatic disease, they now recommend that CEA should be considered in the ‘average surgical risk’ patient with a 60-99% in the presence of one or more imaging features of increased risk, provided their peri-operative mortality and stroke rate is <3% and have at greater than 5 year life expectancy.

We know that the degree of carotid artery stenosis is a poor predictor of stroke risk. Specifically, in asymptomatic patients with >70% carotid stenosis, the risk of ipsilateral stroke is around 2% a year. Therefore in these patients the benefit of intervention with CEA is minimal, in simple terms around 32 CEA s would need to be undertaken to prevent just one stroke in CAD patients over a five year follow up period.

Historically, the risk of stroke following TIA was quoted as 1-2% at 7 days rising to 2-4% at 30 days but these figures grossly underestimate the true risk when you look at data from further reviews. One review of 524 stroke patients who had suffered a preceding TIA found that the TIA occurred on that day of stroke in 17%, on the day before in 9% while 43% occurred within 7 days of the TIA.

2.5.2.1 Carotid Endarterectomy

CEA is routinely performed under general anaesthesia through an oblique neck incision anterior to the sternocleidomastoid muscle. The CCA, ICA and ECA are exposed by sharp dissection with attention paid to identifying and avoiding injury to the vagus and hypoglossal nerves. (Figure 2-5)
Following administration of intravenous heparin the arteries are clamped sequentially and an arteriotomy performed starting on the CCA and extending along the ICA until healthy intima is seen. Most surgeons elect to place a shunt at this point to maintain cerebral perfusion while the endarterectomy is performed. The arteriotomy is routinely closed with the use of a patch, commonly bovine, to preserve luminal diameter although some surgeons may opt for a primary closure should the diameter of the ICA be deemed large. (Figure 2-6) Others may perform an eversion endarterectomy but the merits/disadvantage of each closure is outside the scope of this project and so is not discussed.
2.5.2.2 Carotid Artery Stenting

Despite the CAVATAS\(^{(182)}\) and SAPHIRE\(^{(183)}\) studies reporting comparable 30-day outcomes between CEA and carotid stenting the ESVS\(^{(178)}\) suggest CEA is the best option for symptomatic patients based on a meta-analysis by the Cochrane Collaboration. Stenting can be offered to those patients at high risk from CEA but should be performed in high volume centres with low peri-procedural risks.

2.5.3 Outcomes of Carotid Intervention

The operative mortality rate for CEA in symptomatic patients is less than 1%. The peri-operative risk of stroke is reported to be around 1-2%. Much of the data concerning outcomes post CEA quotes a 30-day endpoint of stroke/death. The first major trials, ECST\(^{(184)}\) and NASCET\(^{(185)}\), reported a 30-day stroke risk of 6-8%, but more recent trials, the international carotid stenting study \(^{(186)}\) and carotid revascularisation endarterectomy versus stenting trial (CREST),\(^{(187)}\) have shown a decrease in this to 3-5%. The risk of peri-operative MI is quoted as 0.5-1%, however very few trials included MI as a 30 day endpoint. In the CREST trial it was reported as 2.3%. In addition to stroke/death/MI there is also the risk of cranial nerve injury (5-9%) and wound complications (3%).
With regards asymptomatic patients, their 30-day outcomes are significantly better with the risk of stroke being 1-2%, death 0.5-1% and MI 1%. (185, 188)

Based on the two major trials concerning symptomatic patients, the characteristics associated with increased risk of peri-operative stroke are; female gender, elevated peri-operative blood pressure, hemispheric vs retinal symptoms, contralateral occlusion and an irregular plaque.

2.5.4 Follow-up Following Carotid Intervention

There is debate as to whether patients should be routinely followed-up following CEA and if so for what length of time. The main reason for follow-up is detection of re-stenosis. Based on evidence from the major randomised trials, the risk of developing a significant restenosis (>70%) is around 2%/year.

It is accepted that re-stenosis occurring within 2 years is the result neo-intimal hyperplasia and thus carries a low risk of stroke. Results from the SPACE (189) trial observed no association between restenosis greater than 70% and increased risk of ipsilateral stroke. However, the CREST trial found a significant increase in the risk of stroke in those who developed a severe re-stenosis compared to those who didn’t at 2 years (8.9% Vs 1.2%, P<0.05). (187) Despite this, routine follow-up of patients post CEA is neither recommended nor routinely performed in most vascular units. This is in part due to a lack of evidence supporting re-intervention in asymptomatic re-stenosis and that re-stenosis was detected in the majority of patients at the time of symptoms and not preceding this.

2.6 Asymptomatic Carotid Artery Disease

There is much debate how best to treat patients with asymptomatic CAD and the ESVS have recently updated their guidelines regards this (see chapter 2.5.2). The annual risk of stroke in patients with asymptomatic CAD receiving best medical therapy is decreasing. Clinicians and surgeons have traditionally quoted around a 2% annual risk based on the 5 year data from the ACAS (190) and ACST (17) trials. These were published in 1995 and 2004 respectively quoting annual stroke risks of 2.2% and 1.1% respectively. By the time the 10 year results of ACST (191) had been
published this risk had fallen to 0.7% with further reviews and studies confirmed the decreased risk.\textsuperscript{[69, 192-196]} This decreased risk is attributable to improved medial therapy, both pharmacological together with better public awareness and monitoring/treatment of CV risk factors.\textsuperscript{[150, 196, 197]}

Despite these documented low risks, there is still a group of patients with high risk asymptomatic disease who progress to become symptomatic given that 30% of ischaemic stroke are secondary to CAD. Whether these patients progress despite best medical therapy or whether the presentation with symptomatic CAD is their first indication of CV disease is difficult to assess. Because of this there is great interest in researching what makes CAD become symptomatic after such a long quiescent period and if this can be detected by either imaging or blood profiling. If this was to be proven then the question of carotid surveillance would become more pertinent.

While it has been documented that the detection of cerebral microemboli by TCD of the MCA, the presence of silent infarcts on CT or MRI and the echolucency of a plaque on ultrasound are all associated with an increased risk of stroke, (see chapter 2.3) it is not feasible to perform routine TCD on patients with asymptomatic CAD nor CT or MR. Therefore some surgeons opt to follow-up such patients with serial duplex’s quoting the outcomes from the ACSRS\textsuperscript{[198]} study of a 16% 8 year risk of stroke in the presence of disease progression, however, 68% of the patients who suffered strokes in this study showed no evidence of disease progression.
OVERVIEW OF THESIS AIMS

The main aim of this thesis was to advance our understanding of the role carotid artery disease has in ischaemic stroke. Particularly, does CPV have any relation and can it be measured accurately in-vivo? The ultimate aim would be to produce a scoring system based on CPV to better predict patients with asymptomatic disease who are high risk. It is evident that a marker of high risk plaques in carotid artery disease is needed and this project aims to gather the information for this to lead toward a multi-centre cohort study regarding asymptomatic carotid artery disease. The specific questions asked were;

1. Does 2D duplex have a role in routine carotid artery surveillance in identifying patients at high risk of stroke/ischaemic events?

2. Is carotid plaque volume a predictor of unstable plaques?

3. Can CPV be measured accurately in-vivo by 3D t-US?

4. Is there any relation between plaque instability histologically and inflammatory activity with CPV?

5. Can we detect a biomarker that reflects an unstable carotid plaque?

6. Does increased platelet aggregation have relate to cerebral emboli?
CHAPTER 3: PRE-OPERATIVE INVESTIGATIONS

3.1 Introduction

Local ethics committee approval was obtained for the study (11/NW/0308) which included recruitment of patients from three hospitals within Greater Manchester;

- University Hospital South Manchester (UHSM)
- Central Manchester University Hospital (CMFT)
- Royal Oldham Hospital (ROH)

Patients admitted for a primary CEA were invited to take part unless they met one of the exclusion criteria;

- Carotid artery Occlusion
- Diagnosis of Atrial Fibrillation
- Diagnosis or treatment for cancer within the last 6 months
- Lack capacity to give informed consent
- Physically unable to take part in the study

Each patient received a patient information sheet together with a verbal explanation of the study and written informed consent was obtained. A detailed medical history was obtained from each patient together with their clinical case notes and entered onto a data entry form. If time allowed, patients underwent a series of pre-operative investigations in addition to their routine standard clinical investigations;

- 3D t-US to calculate CPV
- TCD insonation of the MCA to detect cerebral emboli
- Blood tests for;
- Platelet aggregometry to detect antiplatelet resistance
- Lp-PLA₂
- P-Selectin
- Hs-CRP

The endarterectomised carotid plaque specimen was collected and CPV calculated prior to freezing for subsequent histological analysis.

The plaque specimen had to be frozen together with the blood samples within 20 minutes of collection. Hence, to standardise practice and ensure uniformity only those patients recruited from UHSM had blood tests and their carotid plaque frozen for analysis.

3.1.1 Data Collection

A detailed medical history was taken from each patient including their CV history together with their medications. Specific attention was paid to the timing and nature of their symptoms of cerebral ischaemia. Symptoms divided into stroke, TIA and amaurosis fugax using established criteria. Patients were classified as symptomatic if symptoms had occurred in the previous 6 months.

3.2 3D Tomographic Ultrasound to Measure Carotid Plaque Volume

In addition to the 2D arterial duplex that each patient received as part of routine clinical care, a 3D t-US of the affected carotid artery was also performed to measure CPV. This was performed by a trained operator in the UHSM vascular studies unit using a Philips iu22 ultrasound system attached to the Curefab 3D system. An L12-5 probe with a central frequency of 8.5 MHz was used in the composite imaging (SonoCT) mode. The Curefab system consists of an elector magnetic field emitter, tracking sensors and the Curefab 3D computer software. The system works with the standard ultrasound machine with tracking sensors attached to the ultrasound probe tracking precisely the orientation and position of the probe in time and space. Multiplanar reconstructions were produced by the 3D software through the
use of pattern recognition algorithms to transform the 2D ultrasound image slices into a single 3D volume of the observed area. (Figure 3-1)

![Figure 3-1 3D representation of an atherosclerotic plaque affecting the ICA](image)

CPV was calculated using manual planimetry. The 3D image was repeatedly sliced transverse to the vessel axis with a predefined ISD of one millimetre. Multiple slices were produced along the length of the plaque and in each slice the plaque-lumen and plaque outer vessel boundaries were traced and the area measured automatically. (Figure 3-2) The plaque volume was then calculated by summing these areas and multiplying them by the ISD of one millimetre, as validated in the literature. ([68, 80, 81, 85, 86, 200, 201]) All measurements were made from the bifurcation proximally along the CCA and distally along the ICA according to the length of the specimen as measured post-operatively. The measurements were performed on three separate occasions, once by me and twice by the vascular scientist, blinded to each other’s results, to enable intra- and inter-observer reliability to be assessed.
3.3 Transcranial Doppler to Detect Cerebral Emboli

The ipsilateral MCA was insonated for one hour pre-operatively, within 24 hours of surgery, to detect cerebral emboli. An Acuson Multiprobe JH-6007 TCD machine was used together with a 2 MHz pulsed, range gated Doppler.

TCD insonates the cerebral arteries through foramina or “windows” within the skull that can be penetrated with the ultrasonic beam. The main TCD approach to insonate the MCA is via the transtemporal window.

A bracket, with the probe attached, is placed on the patients head and the probe positioned on the temporal aspect. (Figure 3-3)
The probe is gently manipulated into the correct position to obtain the optimal signal from the MCA. Once found, the probe is fixed in place and the patient asked to refrain from any sudden head movements and talking. The transducer is attached to a laptop and a recording produced. (Figure 3-4)

The participant and the recording were observed for the full duration making note of any potential embolic signals or movements that could account for artefact. The recording was then analysed thoroughly to identify the presence of MES as defined by international consensus criteria; a signal duration of <300 ms at least 3 dB higher than the background flow, during random periods in the cardiac cycle, being present on one side only of the baseline and accompanied by a typical chirping sound. This was then repeated by a second observer blinded to the results.
3.4 Platelet Aggregometry

Antiplatelet resistance was measured using multiplate impedance aggregometry, a robust and reproducible technique.\(^\text{203, 204}\) The electrical impedance generated by platelet aggregation upon the administration of a known platelet agonist is measured to evaluate residual platelet activity in patients on antiplatelet therapy.\(^\text{205}\) The patient’s blood is placed in cells containing electrical micro-rods. An agonist is added to the cells to stimulate platelet activity with activated platelets attaching to the electrical rods, to produce a signal. The more platelets that are activated and attach, the greater the signal produced. (Figure 3-5) Multiplate analysers are widely used for the measurement of intraoperative platelet function during cardiac surgery.\(^\text{204, 205}\)
Three millilitres of venous blood was taken using an 18 gauge needle into a double wall Hirudin blood tube and gently inverted three times to ensure adequate mixing of the anticoagulant. The samples were transported by hand to avoid excessive shaking, stored at room temperature and analysed 90 minutes post venepuncture according to manufacturer guidelines.

300ul of hirudinated blood was pipetted into each multiplate test cell and diluted 1:1 with 0.9% saline before being incubated for three minutes. 10ul of each agonist was then added to each individual cell; AA, Adenosine diphosphate (ADP) and Thrombin Receptor Activating Peptide (TRAP). Aggregation was recorded over 6 minutes in aggregation units (Au) and an aggregation curve plotted against time was produced. (Figure 3-6) Patients were classed as resistant to Aspirin or Clopidogrel if the area under the curve was greater than 40Au or 47Au respectively. (206, 207)
3.5 Blood Biomarker Testing

Venous blood was taken using an 18 gauge needle into a lithium heparin bonded tube and inverted 3 times. Plasma was prepared within 20 minutes of collection by centrifuging at 3000rpm for 20 minutes at 4°C. During centrifugation 4 cryovials were labelled with the date and subject ID. Following centrifugation, 1ml of plasma was aliquoted into each vial and frozen to -80°C. Commercial ELISA kits were used to measure Lp-PLA₂ and P-Selectin. Hs-CRP was measured in the biomedical laboratory at UHSM using the Abbott architect system.

3.5.1 Lipoprotein-Associated Phospholipase A2

Lp-PLA₂ was measured using a commercial ELISA kit from R&D system UK; Human PLA2G7/PAF-AH/Lp-PLA₂. Plasma was thawed and then immediately analysed according to the manufactures guidelines as below.
**Preparation of Reagent**

20ml of wash buffer concentrate was added to deionized water to prepare 500ml of wash buffer solution.

Colour reagents A & B were mixed together in equal volumes. 20ml calibrator diluent RD5-17 was added to 60ml of deionized water to prepare 80ml of Calibrator Diluent RD5-17 (diluted 1:4)

Human PLA2G7 Standard was reconstituted with calibrator diluent RD5-17 giving a stock solution of 100ng/ml. This was then left to stand for 15 minutes.

500μL of calibrator diluent RD5-17 was pipetted into 7 test tubes and diluted with the stock solution to produce a dilution series as below.

![Figure 3-7 Dilution series](image)

**Preparation of Sample**

Plasma samples were diluted 20-fold by adding 10μl of sample to 190μl of Calibrator Diluent RD5-17

**Assay Procedure**

A 96 well microplate was used together with a schematic representation of the microplate noting which subject ID corresponded to each well along with the standards and controls. 2 microplates were used with 2 samples for each patient to
ensure quality control. Following successful preparation of the samples and reagents the below sequence was followed to perform the assay procedure.

1. 100µl of Assay Diluent RD1-9 was added to each well of the microplate
2. 50µl of the standard, control and sample was then added to each well. The microplate was covered and placed on a horizontal orbital microplate shaker and incubated at room temperature for 2 hours with the shaker set at 500±50rpm
3. Each well was aspirated and washed with wash buffer using a squirt bottle. This was repeated four times and following the last wash care was taken to ensure all the wash buffer was removed. The plate was then inverted and blotted against clean paper towels.
4. 200µl of Human PLA2G7 Conjugate was added to each well. The plate was covered and incubated as above on the microplate orbital shaker for 2 hours.
5. Step 3 was then repeated ensuring a thorough wash of all the wells.
6. 200µl of substrate solution was then added to each well and then incubated at room temperature for 30 minutes on the benchtop ensuring protection from light.
7. 50µl of stop solution was added to each well observing for a colour change from blue to yellow. If this did not occur then the plate was gently tapped to ensure thorough mixing.
8. The optical density of each well was determined using a microplate reader set to 450nm with a wavelength corrected to 540nm and 570nm.

Quantitative analysis of the samples using a four parameter logistic curve was used to calculate the results.

3.5.2 P-Selectin

P-Selectin was measured using a commercial ELISA kit from R&D system UK; Human P-selectin/CD62P. Plasma was thawed and then immediately analysed according to the manufactures guidelines as below.

Sample Preparation
Samples, along with the control, were diluted 20-fold by adding 15µl of sample to 285µl of sample diluent.

**Reagent Preparation**

20ml of wash buffer concentrate was added to deionized water to prepare 500ml of wash buffer solution.

1ml of deionized water was added to each standard and mixed gently until all the contents had dissolved. They were then left at room temperature for 10 minutes.

500ul of deionized water was mixed with the P-selectin control, mixed gently and left at room temperature for 10 minutes. Following this it was diluted 20-fold by adding 15ul to 285ul of sample diluent.

250ul of conjugate concentrate was added to the bottle of conjugate diluent and mixed gently.

**Assay Procedure**

A 96 well microplate was used together with a schematic representation of the microplate noting which subject ID corresponded to each well along with the standards and controls. 2 microplates were used with 2 samples for each patient to ensure quality control. Following successful preparation of the samples and reagents the below sequence was followed to perform the assay procedure.

1. 100µl of standard, control and sample was added to each well
2. 100µl of diluted P-Selectin Conjugate was added to each well
3. The plate was covered and incubated at room temperature for 1 hour
4. Each well was washed using 300µl of wash buffer three times ensuring all wash buffer was removed following each wash. Following the last wash the plate was inverted and blotted against tissue paper.
5. 100µl of Substrate was added to each well and the plate covered and incubated at room temperature for 15 minutes
6. 100µl of stop solution was added to each well
7. The optical density was then recorded using a microplate reader set to 450nm with a wavelength correction set to 620nm
Quantitative analysis of the samples using a four parameter logistic curve was used to calculate the results.

3.5.3 High Sensitive CRP

Hs-CRP was measured in the biomedical laboratory at UHSM on the Abbott Architect system using the CRP VARIO assay. The MULTIGENT CRP Vario is a latex immunoassay. Latex microbeads are coated with an anti-CRP antibody. Plasma is then mixed with the latex beads and CRP, if present, will react with the antibody causing agglutination. This agglutination is detected as an absorbance change (572 nm), with the rate of change being proportional to the quantity of CRP in the sample.
CHAPTER 4: CAROTID PLAQUE SPECIMEN

At surgery the atherosclerotic plaque was removed en-bloc where possible. Immediately following endarterectomy, the plaque was placed in a dry pot and immediately on ice. The specimen was transported to the laboratory by hand for calculation of CPV and frozen to -80°C ready for subsequent histological analysis.

4.1 Measurement of Carotid Plaque Volume

The total length of the plaque was recorded along with the bifurcation length. (Figure 4-1)

![Figure 4-1 Measurement of bifurcation and total length of the endarterectomised carotid plaque](image)

If needed, the ECA was removed by sharp dissection. The plaque was then placed on an electric balance and weight noted in grams. The CPV was calculated using a water suspension technique - a variation of the hydrostatic weighing technique used for measuring volume, based on Archimedes’ principle. It has been shown to have a mean difference of 0.03 ± 0.45% between actual and measured volumes with good reproducibility. (208)

A 150ml polythene beaker, filled with 110 ml of normal saline, was placed on an electric balance and zeroed. A suture was tied around the endarterectomised plaque which was then suspended beneath the normal saline and weight noted in
The volume was then calculated by dividing the suspended weight with the density of normal saline which is known to be 1.0046 cm$^3$.

Once CPV had been calculated the plaque was divided into 9 equal segments and placed into cryovials numbered one to nine (proximal to distal). The portion of the plaque demonstrating the most macroscopically active disease i.e. most ulcerated was noted and the corresponding cryovial numbers recorded. The plaque samples were frozen to -80°C prior to histological analysis.

4.2 Histology

Preparation and staining of the slides was performed at the adult histopathology department in the clinical sciences building at CMFT. The plaque samples demonstrating most active disease, as demonstrated above, were thawed and then fixed immediately in 10% formalin for 24 hours. Following fixation the samples underwent decalcification to enable embedding.

Once the samples had been decalcified they were trimmed and placed in cassettes ready for processing. The samples were dehydrated in a graded ethanol series through 70% to 100% ethanol and then cleared using Xylene. The samples were then embedded in paraffin wax to create a mould. Adjacent 5µm transverse slices were taken using a microtome and stained as below.

4.3 Histological Staining

The following stains were used;
• Haematoxylin and eosin (H&E) for grading according to AHA
• Elastin van gieson (EVG) for grading according to AHA
• Monoclonal antibody CD3 to detect T-Cells
• Monoclonal antibody CD68 to detect macrophages

The staining process was performed according to set laboratory protocols.

4.3.1 H&E Staining

H&E staining was performed on the Leica XL Autostainer using an automated process as outlined in table 4-1;
4.3.2 EVG Staining

VWR Miller’s Elastin stain was used along with the reagents 2% Oxalic acid and 0.5% potassium permanganate.

Staining Process

1. Sections placed in xylene to remove wax for up to 3 minutes
2. Sections washed in industrialised methylated spirit (IMS) to remove xylene for 1 minute.
3. Sections transported to water
4. Treated with 0.5% potassium permanganate solution for 5 minutes
5. Wash in distilled water
6. Sections bleached with 2% Oxalic acid until section decolours
7. Sections washed in tap water
8. Slides rinsed in Industrial methylated spirit (IMS)
9. Sections stained in VWR Miller’s elastin stain for up to 3 hours
10. Slides rinsed in IMS to remove excess stain
11. Slides washed in tap water and checked microscopically that elastin fibres are stained – if not then process is repeated from step 8 and sections left in Elastin stain until the fibres are stained.
12. Counter stained with van Gieson solution for 2 minutes
13. Sections then mounted and ready for analysis

4.3.3 CD3 Staining Protocol

A monoclonal rabbit antibody (Anti-CD3 (2GV6) was used.

Summary

Antibody: Anti-CD3 (2GV6) Rabbit
Manufacturer: Vantana (Cat# 790-4341)
System: Automated Ventana BenchMark ULTRA
Detection Kit: UltraView DAB Detection System with Amplification
Antigen Retrieval: 36 minute heat in CC1 (Tris based pH8.4 buffer)
Antibody concentration: RTU
Antibody Incubation: 32 minutes at room temperature.
Counterstain: Haematoxylin 12 minutes, bluing reagent 4 minutes.
Positive Tissue Control: Tonsil

Staining Process

The automated Ventana BenchMark ULTRA IHC/ISH Staining Module (Ventana Co., Tucson, AZ, USA) was used together with the Ultraview 3, 3’ diaminobenzidine (DAB) version 3 detection system (Ventana Co.). Tissue sections (5 µm) were deparaffinised and incubated in EZPrep Volume Adjust (Ventana Co.). At intervals between steps the slides were washed with a TRIS-based Reaction Buffer, pH 7.6. A
heat-induced antigen retrieval protocol set for 36 minutes was carried out using a TRIS–ethylenediamine tetracetic acid (EDTA)–boric acid pH 8.4 buffer (Cell Conditioner 1). The sections were incubated with ultraviolet inhibitor blocking solution for 4 minutes, then with antibody to CD3 for a set time of 32 minutes at room temperature. This was followed by incubation with horseradish peroxidase-linked secondary antibody (8 minutes), followed by DAB chromogen and substrate (8 minutes), and copper enhancer for 4 minutes. Counterstain (haematoxylin II) was applied for 12 minutes before an incubation of 4 minutes with bluing reagent.

4.3.4 CD68 Protocol

A monoclonal mouse antibody Anti-CD68 (2GV6) was used.

**Summary**

**Antibody:** Anti-CD68 (2GV6) Mouse  
**Manufacturer:** Dako (Cat# M0876)  
**System:** Automated Ventana BenchMark ULTRA  
**Detection Kit:** UltraView DAB Detection System with UltraWash  
**Antigen Retrieval:** 64 minute heat in CC1 (Tris based pH8.4 buffer)  
**Antibody concentration:** 1+50  
**Antibody Incubation:** 32 minutes at room temperature.  
**Counterstain:** Haematoxylin 12 minutes, bluing reagent 4 minutes.  
**Positive Tissue Control:** Tonsil

**Staining Process**

The automated Ventana BenchMark ULTRA IHC / ISH Staining Module (Ventana Co., Tucson, AZ, USA) was used together with the Ultraview 3, 3' diaminobenzidine
(DAB) version 3 detection system (Ventana Co.). Tissue sections (5 µm) were deparaffinised and incubated in EZPrep Volume Adjust (Ventana Co.). At intervals between steps the slides were washed with a TRIS-based Reaction Buffer, pH 7.6. A heat-induced antigen retrieval protocol set for 64 minutes was carried out using a TRIS– ethylenediamine tetracetic acid (EDTA)–boric acid pH 8.4 buffer (Cell Conditioner 1). The sections were incubated with ultraviolet inhibitor blocking solution for 4 min, then with antibody to CD68 for a set time of 32 minutes at room temperature. This was followed by incubation with horseradish peroxidase-linked secondary antibody (8 minutes), followed by DAB chromogen and substrate (8 minutes), and copper enhancer for 4 minutes. Counterstain (haematoxylin II) was applied for 12 minutes before an incubation of 4 minutes with bluing reagent.
4.4 Histological Analysis

The slides were analysed by myself, and a histopathologist, blinded to each other’s results. In addition to the basic histology training I had received during medical school, I underwent further in-house training regards the analysis of atherosclerotic plaques by an experienced histopathologist. In cases of discrepancy between the two observers those samples were re-assessed and the histopathologist grading used. The stability of the plaque was graded according the American Heart Association classification of atherosclerotic plaques (Table 4-2). The type of lesion was graded and type VI lesions were classed as unstable as they demonstrate disruption of the fibrous cap with haematoma or thrombus formation.

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Increase in macrophages and foam cells</td>
<td>Stable</td>
</tr>
<tr>
<td>II</td>
<td>Predominantly smooth muscle and foam cells with more lipid accumulation</td>
<td>Stable</td>
</tr>
<tr>
<td>III</td>
<td>As above with detection of extracellular lipid pools</td>
<td>Stable</td>
</tr>
<tr>
<td>IV</td>
<td>As above with formation of a lipid core</td>
<td>Stable</td>
</tr>
<tr>
<td>V</td>
<td>Lipid core with a fibrotic layer</td>
<td>Stable</td>
</tr>
<tr>
<td>VI</td>
<td>As above but with disruption of the fibrous cap, haematoma or thrombus formation.</td>
<td>Unstable</td>
</tr>
</tbody>
</table>

*Table 4-2 AHA classification of atherosclerotic plaques*

Together with the above the degree of inflammation was also graded using a grading system developed by the Oxford plaque study group (Table 4-3). Those given a score of 3 were regarded as having marked inflammation.
<table>
<thead>
<tr>
<th>Inflammation Score</th>
<th>CD3 (T-cells)</th>
<th>CD68 (Macrophages)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No CD3-stained cells</td>
<td>No CD68-stained cells</td>
</tr>
<tr>
<td>1</td>
<td>Occasional scattered cells or 1 group of &gt;20 cells</td>
<td>Occasional scattered cells or 1 group of &gt;50 cells</td>
</tr>
<tr>
<td>2</td>
<td>Several groups (&lt;5) of &gt;20 cells</td>
<td>Several groups (&lt;5) of &gt;50 cells</td>
</tr>
<tr>
<td>3</td>
<td>Many groups (&gt;5) of &gt;20 cells or 1 group of &gt;100 cells</td>
<td>Several groups (&gt;5) of &gt;50 cells</td>
</tr>
</tbody>
</table>

Table 4-3 Table used to grade the level of inflammation as produced by the Oxford plaque study group (44)
SECTION 3: RESULTS
CHAPTER 5: ROUTINE SURVEILLANCE FOR ASYMPTOMATIC CAROTID ARTERY DISEASE AND POST ENDARTERECTOMY IS NOT INDICATED

S Ball, L Ngu, N Jamal, C McCollum

Contributions and role:

**S Ball:** Conception, data collection and analysis, manuscript writing

**L Ngu:** Assistance in data collection

**N Jamal:** Assistance in data collection

**Prof C McCollum:** Conception, Supervisor
5.1 Abstract

**Background:** Patients with an asymptomatic carotid stenosis carry an annual stroke risk of less than 2% when receiving best medical therapy. However, a percentage of these patients will become symptomatic and require intervention. Some surgeons therefore opt for surveillance, by way of arterial duplex, in such patients despite there being no guidance for this.

**Aims:** To ascertain whether carotid surveillance can predict which patients are more likely to progress their stenosis and if this is related to symptom development.

**Methods:** This retrospective cohort study included patients with asymptomatic carotid disease involved in a carotid surveillance clinic. Demographic and relevant clinical data was recorded from patient’s notes including duplex reports. Kaplan-meier survival analysis was applied.

**Results:** 231 internal carotid arteries (ICAs) were surveilled over a mean of 4.3 years. Stenosis progression was significantly associated with a history of myocardial infarction (MI), (p=0.001), stroke or transient ischaemic attack (TIA) (p=0.035) and hypercholesterolaemia (p=0.046). Symptom development was not related to progression to a severe stenosis (p=0.070) but was related to the severity of stenosis at the time they entered the surveillance programme (p=0.024). A severe stenosis at baseline and evidence of progression to a severe stenosis were significantly associated with undergoing a carotid endarterectomy (CEA) (p<0.001 and p=0.003 respectively).

**Conclusion:** The results in this paper do not support routine surveillance of asymptomatic carotid artery disease by arterial duplex given progression to a severe stenosis was not related to symptom development.
5.2 Introduction

The prevalence of moderate carotid artery stenosis (>50%) in men aged 60-69 years is 2.3% and women 2%, rising to 6.0% and 3.6% respectively in those aged 70-79. The prevalence of severe carotid artery stenosis (>70%) in men aged 60-69 years is 0.8% and women 0.2% rising to 2.1% and 1.0% respectively in those aged 70-79. Stroke accounts for 1.1 million deaths annually in Europe and half of stroke survivors remain dependent on others for their activities of daily living hence having a massive social and economic impact on healthcare. Carotid artery disease (CAD) accounts for 30% of ischaemic strokes secondary to thromboembolic infarcts from a ruptured/unstable atherosclerotic carotid plaque. Current ESVS guidelines recommend CEA be performed for a symptomatic severe carotid stenosis (>70%) within two weeks for maximal patient benefit. For asymptomatic disease, ESVS recently updated their guidelines and state that CEA should be considered in the average surgical risk patient with a 60-99% stenosis demonstrating one or more imaging features associated with increased risk of stroke provided their life expectancy is at least 5 years with a peri-operative stroke risk less than 3%.

The benefit of CEA in symptomatic patients is clear, with an absolute risk reduction of 23% when performed within two weeks. However, regards asymptomatic disease, the need for carotid intervention is still debated. Those with an asymptomatic severe stenosis have an annual stroke risk of under two per cent. With improving medical therapy, better monitoring of CV risk factors and greater public awareness many report this risk to be less than 1%. Therefore the question of what to do with patients with asymptomatic carotid disease continues to be asked. There is no risk model or single way of determining which patients will benefit most from intervention hence it is very much based on the individual surgeons practice. While certain USS features, the detection of cerebral emboli and the presence of cerebral infarcts on neuroimaging are associated with increased risks of stroke, it is not feasible to perform routine TCD nor neuroimaging on all patients with asymptomatic disease. Therefore many rely on USS features, such as echolucency, or progression of disease. The asymptomatic carotid stenosis and risk of stroke study (ACRSRS) is the largest prospective study on patients receiving
best medical therapy alone for asymptomatic CAD and provided evidence that progression of CAD, as measured by arterial duplex, is a risk factor for future stroke. The 8 year cumulative ipsilateral stroke risk was 16% in those with disease progression and was highest at 25% in those with a baseline stenosis of 90-99%. This confirmed previous published findings of disease progression being significantly linked with symptom development \(^{(15)}\) and that it not only increased the risk of stroke but also all major adverse CV events. \(^{(16)}\)

The main aims of this study are to ascertain if surveillance of an asymptomatic carotid stenosis, by way of arterial duplex, is beneficial in identifying patients who are likely to demonstrate disease progression. Also, whether progression to a severe stenosis is associated with the development of symptoms of cerebral ischaemia.
5.3 Methods

This retrospective study at a single tertiary vascular unit was undertaken with electronic and case note analysis being performed for all patients attending a carotid artery surveillance clinic. This clinic involved patients attending for surveillance of an asymptomatic carotid stenosis. Patients were included if they had two scans at least one year apart. Patients were excluded if they were found to have a mild carotid stenosis (<50%) or an occluded carotid artery. As data was collected retrospectively with no additional investigations performed as part of a research study and all patients received care according to their clinical needs, ethics approval was not deemed to be necessary.

In addition to a clinical review by either a nurse specialist or junior doctor, each patient underwent a 2D arterial duplex to grade the severity of carotid stenosis based on the NASCET criteria as recommended by the joint working group of the vascular and vascular technology societies of GB and Ireland. \(^{(17)}\) The level of stenosis was graded in ranges of 50-59%, 60-69%, 70-79%, 80-89% or 90-99%. Those with a stenosis between 50-69% were classed as moderate and scanned annually and those with a stenosis between 70-99% were classed as severe and scanned bi-annually. Progression of CAD was defined as an increase in the range of stenosis. For this study, only those patients who demonstrated progression from a moderate to severe stenosis were analysed.

Case notes were reviewed to obtain a detailed medical and drug history at baseline and at each clinic appointment, specifically if they had suffered any cerebral ischaemic events. The electronic database of carotid duplexes was accessed to record the degree of stenosis.

Records of clinic attendance were kept from 2008 and the clinic stopped running in 2015. Those who had not been scanned in 2014, according to the clinic template, were deemed to be lost to follow-up. New patients entered the surveillance clinic each year, therefore, patients were followed-up for differing lengths of times.

Statistics
Analysis was split into 3 sections; patients demonstrating stenosis progression from a moderate to a severe stenosis; patients who became symptomatic and patients who underwent a carotid endarterectomy during the surveillance period. For each group, Kaplan-Meier survival analysis \(^{18}\) was conducted to compare the effect of known CV risk factors, along with the presence of contralateral disease, the laterality of the disease, the degree of stenosis at baseline (i.e. when they entered the programme) and evidence of disease progression. A log rank test was conducted to determine if there were any differences in survival distributions. Termination of follow-up was reached when one of the following happened; 1) evidence of progression: 2) development of symptoms: 3) CEA performed. In the group undergoing CEA, analysis was only performed for those patients undergoing CEA for asymptomatic disease. Interaction analysis was not performed as the number of events in each group was too small.

Continuous variables are displayed as mean (sd). Continuous outcomes were compared using student’s \(t\)-test, and categorical outcomes with either \(\chi^2\) test or Fisher’s exact tests, as appropriate. A p-value of <0.05 was considered significant and all analyses were performed using SPSS version 22.
5.4 Results

231 ICAs were being surveilled over a mean period of 4.3 years. 115 (49.7%) were male with a mean (sd) age of 76 (8.8). The left ICA was being surveilled in 118 (51%) and 198 (85%) were graded as a moderate stenosis at their baseline scan. There were no statistically significant differences regards known CV risk factors in those patients graded as moderate compared to severe at baseline. (Table 5-1)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Moderate (n=198)</th>
<th>Severe (n=33)</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>75.9 (8.6)</td>
<td>77.2 (9.4)</td>
<td>0.466†</td>
</tr>
<tr>
<td>BMI (kg/m^2)*</td>
<td>27.1 (7.2)</td>
<td>26.0 (3.6)</td>
<td>0.249‡</td>
</tr>
<tr>
<td>Sex ratio (M : F)</td>
<td>100 : 98</td>
<td>15 : 18</td>
<td>0.610</td>
</tr>
<tr>
<td>Diabetic</td>
<td>50 (25)</td>
<td>10 (30)</td>
<td>0.551</td>
</tr>
<tr>
<td>Hypertension</td>
<td>154 (78)</td>
<td>27 (82)</td>
<td>0.581</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>108 (55)</td>
<td>17 (52)</td>
<td>0.363</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>70 (35)</td>
<td>14 (42)</td>
<td>0.604</td>
</tr>
<tr>
<td>Previous myocardial infarct</td>
<td>39 (20)</td>
<td>4 (12)</td>
<td>0.235</td>
</tr>
<tr>
<td>Previous Stroke or TIA</td>
<td>95 (48)</td>
<td>16 (48)</td>
<td>0.702</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td>0.970</td>
</tr>
<tr>
<td>Current</td>
<td>41 (21)</td>
<td>7 (21)</td>
<td></td>
</tr>
<tr>
<td>Ex</td>
<td>70 (35)</td>
<td>11 (33)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>87 (44)</td>
<td>15 (45)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-1 Characteristics of patients in the surveillance programme. Values in parentheses are percentages unless otherwise indicated; *values are mean(s.d.). †χ^2 test, except ‡Student’s t test.
Of the 231 ICAs surveilled, 34 (15%) demonstrated disease progression to a severe stenosis, 19 (8%) underwent a prophylactic CEA, 8 (4%) developed symptoms of cerebral ischaemia and 32 (14%) were lost to follow-up. (Figure 5-1)

Figure 5-1 Flowchart depicting the flow of patients through the surveillance programme.

**Stenosis Progression**

34 (15%) ICAs demonstrated progression from a moderate to severe stenosis. Of these seven underwent a CEA and three became symptomatic. A log rank test was run to determine if there were differences in those with progression of a moderate to severe stenosis compared to those without according to known CV risk factors, including the presence of contralateral disease and laterality of disease. The survival distributions were statistically significant for those that had a history of MI, CVA or TIA and hypercholesterolaemia. The mean time to progression for those with a history of MI was 2151 (95% CI: 1923, 2379) days compared to 2642 (95% CI: 2530, 2755) days for those without (\(x^2(1)=11.98, P=0.001\), Figure 5-2). The mean time to progression for those with a history of CVA or TIA was 2318 (95% CI: 2181, 2457) days compared to 2671 (95% CI: 2531, 2812) days for those without (\(x^2(1)=4.42, P=0.035\), Figure 5-3). The mean time to progression for those with a history of hypercholesterolaemia was 2448 (95% CI: 2286, 2670) days compared to 2502 (95% CI: 2381, 2623) days for those without (\(x^2(1)=3.98, P=0.046\), Figure 5-4).
Figure 5-2 Kaplan-Meier Survival curve for event survival for those with and without a history of MI. Numbers of patients at risk of stenosis progression listed for every 500 days. \( P=0.001 \)

Figure 5-3 Kaplan-Meier Survival curve for event survival for those with and without a history of CVA/TIA. Numbers of patients at risk of stenosis progression listed for every 500 days. \( P=0.035 \)
Symptom Development

Eight (3.5%) patients developed symptoms during surveillance (5 TIAs, 2 CVAs and 1 amaurosis fugax). None of the traditional CV risk factors were associated with symptom development, however those patients who had a severe stenosis at baseline were significantly more likely to develop symptoms with a mean time to symptom of 2190 (95% CI: 1955, 2425) days compared to 2811 (95% CI: 2758, 2864) days for those with a moderate stenosis ($\chi^2(1)=5.1, P=0.024$, Figure 5-5). Of the eight who developed symptoms, seven underwent a CEA with the remaining one demonstrating an occlusion. Three of the eight symptomatic patients demonstrated progression from a moderate to a severe stenosis but did not reach significance ($\chi^2(1)=3.29, P=0.070$) and the progression was identified at the time of symptom, not before.
Carotid Intervention

19 (8%) patients underwent a prophylactic CEA during their surveillance. As explained previously, analysis was only performed in relation to those who underwent a CEA for asymptomatic disease. None of the traditional CV risk factors demonstrated any significance between those that underwent CEA and those who didn’t. A severe stenosis at baseline was significantly related to those who underwent a CEA with a mean time to CEA of 1674 (95% CI: 1343, 2005) days compared to 2660 (95% CI: 2567, 2753) days for those classed as moderate ($\chi^2(1)=17.6$, $P<0.001$, Figure 5-6). Those who demonstrated progression from a moderate to a severe stenosis were statistically more likely to undergo a CEA with a mean time to CEA of 2294 (95% CI: 1997, 2592) days compared to 2634 (95% CI: 2533, 2736) days for those without ($\chi^2(1)=8.68$, $P=0.003$, Figure 5-7).
Figure 5-6 Kaplan-Meier Survival curve for event survival based on the severity of stenosis on entering the surveillance programme. Numbers of patients at risk of CEA listed for every 500 days. (P<0.001)

Figure 5-7 Kaplan-Meier Survival curve for event survival based on stenosis progression. Numbers of patients at risk of CEA listed for every 500 days. (P=0.003)
5.5 Discussion

Routine surveillance of an asymptomatic carotid stenosis is debated with varying clinical practice amongst vascular surgeons. This paper identified CV risk factors associated with progression of carotid stenosis, namely MI, CVA/TIA and hypercholesterolaemia but progression of a moderate to severe stenosis was not associated with symptom development nor was there any association with known CV risk factors. The relationship between stenosis progression and history of MI would be expected given the strong association between CAD and coronary artery disease.\textsuperscript{(19)}

Interestingly, the three patients who became symptomatic with evidence of stenosis progression were only found to have progression at the time of symptoms. Hence, the stenosis was not a progressive one that ultimately became symptomatic. Neither the ACSRS study\textsuperscript{(14)} nor the independent study from Boston\textsuperscript{(15)} stipulated at what point the progression of stenosis was noted in relation to symptoms. If this was noted at the time of symptoms then it is difficult to justify routine surveillance based on these findings. The ACSRS study quoted a cumulative stroke risk of 16\% over 8 years which in effect means an annual stroke risk of 2\%. Also, in Conrad et al’s study,\textsuperscript{(15)} 60\% of the patients who developed a stroke had a moderate stenosis and in the multivariate model, progression of carotid disease was not an independent predictor of cerebral symptoms. This finding compliments the finding of the current study.

In terms of those who became symptomatic the severity of stenosis at the time they entered the surveillance programme was significantly related. However, if the ESVS guidelines are to be followed then all patients found to have a severe stenosis should have been considered for CEA and if the decision was not for CEA then one wonders what the rationale behind surveillance was. Perhaps this highlights the uncertain nature of how such patients should be managed.

In terms of those who were offered a CEA then the severity of stenosis at baseline was significantly related as too was evidence of progression from a moderate to severe stenosis. However, this finding offers little to aid in the management of such
patients as the decision for CEA is very much dependent on the surgeon and patient.

Whilst it would seem logical that disease progression while on best medical therapy is a bad prognostic sign it is difficult to justify routine surveillance based on the results in this paper as progression was not related to symptom development and none of the symptomatic patients in our study were identified as having progressive disease prior to their symptoms.

Only 7 (20%) of the patients demonstrating progression of a moderate to severe stenosis underwent a prophylactic CEA. Based on the evidence in the literature, the main purpose of such a surveillance programme is to identify and offer CEA to those patients demonstrating progression from a moderate to severe stenosis. The fact only 20% of such patients underwent CEA, together with disease progression not relating to symptom development, there seems to be little justification for the programme based on the results in this study. Perhaps, the low CEA rate reflected more the uncertainty of what to do with such patients.

In comparison to other studies of a similar nature the numbers involved were relatively small and the numbers of events for each group were low rendering interaction analysis not possible. The retrospective nature of the study meant the authors relied on the correct information being recorded in the patient’s notes meaning it was not always possible to gather the full data regards co-morbidities. As has been mentioned, the decision to operate on patients with asymptomatic disease is not only dependent on the surgeons practice but also patient related factors need to be taken into account. Due to the nature of this study it was not possible to ascertain why some patients demonstrating disease progression underwent CEA and others didn’t. Another limitation is that 32 (14%) patients were lost to follow-up. Whilst this is below the generally accepted level of 20% it is still on the higher end hence the authors acknowledge the potential for attrition bias. Because the surveillance clinic stopped on a specific date we had to assume that those patients who had not been scanned in 2014 were lost to follow-up which is not wholly accurate. Again this is a weakness of a retrospective study and should be
addressed in further longitudinal prospective studies and the authors acknowledge that the results should be interpreted in clinical context.

Given the current trend towards best medical therapy for asymptomatic carotid disease then surveillance by arterial duplex alone seems obsolete. The focus should move towards developing methods of identifying features of high risk plaques which could guide which patients will benefit from intervention.
5.6 References


CHAPTER 6: CAROTID PLAQUE VOLUME IN PATIENTS UNDERGOING CAROTID ENDARTERECTOMY

S Ball, S Rogers, K Kanesalingam, R Taylor, E Katsogridakis, C McCollum

Contributions and role:

S Ball: Patients recruitment, data collection and analysis, analysis of 3D t-US to measure CPV, manuscript writing

S Rogers: 3D t-US scanning and analysis

K Kanesalingam: Patient recruitment

R Taylor: Patient recruitment

E Katsogridakis: Manuscript preparation

Prof C McCollum: Conception, Supervisor

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6.1 Abstract

**Background:** The main indication for carotid endarterectomy (CEA) is severity of carotid artery stenosis, even though most strokes in carotid disease are embolic. The relationship between carotid plaque volume (CPV) and symptoms of cerebral ischaemia, and the measurement of CPV by minimally invasive tomographic ultrasound imaging, were investigated.

**Aims:** To explore the relationship between CPV and symptoms of cerebral ischaemia in patients undergoing CEA.

**Methods:** The volume of the endarterectomy specimen was measured using a validated saline suspension technique in patients undergoing CEA. Time from last symptom and severity of stenosis measured by duplex ultrasonography were recorded. Middle cerebral artery emboli were counted using transcranial Doppler (TCD) in a subset of patients.

**Results:** Some 339 patients were included, 270 with symptomatic and 69 with asymptomatic carotid stenosis. Mean (s.d.) CPV was higher in symptomatic than in asymptomatic patients (0.97(0.43) versus 0.74(0.41) cm$^3$; $P < 0.001$). CPV did not correlate with severity of carotid stenosis ($P = 0.770$). Mean CPV was highest at 1.03(0.46) cm$^3$ in the 4 weeks following cerebral symptoms, declining to 0.78(0.36) cm$^3$ beyond 8 weeks. Among 33 patients who had TCD, mean CPV was 1.00(0.48) cm$^3$ in the 27 patients with ipsilateral cerebral emboli compared with 0.67(0.16) cm$^3$ in those without ($P = 0.142$). There was excellent correlation between CPV measured by tomographic ultrasound imaging and the endarterectomy specimen in 34 patients ($r = 0.93, P < 0.001$).

**Conclusion:** CPV correlated with symptoms of cerebral ischaemia, but not carotid stenosis. It could be a potential indicator for CEA.
6.2 Introduction

Stroke is the third leading cause of death in the Western world behind ischaemic heart disease and cancer. It is the leading cause of disability in the UK, affecting over 150 000 people per year and costing the UK economy around £7 billion (€7.7 billion; exchange rate 14 August 2017) each year.\(^1,2\) Carotid disease causes 30 per cent of ischaemic strokes\(^3\), although most strokes are caused by atheroembolism. The principal indication for operation on carotid disease remains the severity of stenosis.\(^4\–8\) To confer maximal benefit, CEA should be performed as soon as possible following symptoms of cerebral ischaemia, and certainly within 2 weeks as 43 per cent of patients suffering stroke had a TIA in the preceding 7 days. Patients with 70–99 per cent stenosis have an absolute risk reduction of 23 per cent when CEA is performed within 2 weeks.\(^4,5,9,10\) Although recent symptoms of cerebral ischaemia are a clear indication for early carotid surgery, severity of stenosis alone is a poor predictor of stroke risk as asymptomatic patients with greater than 70 per cent carotid stenosis on best medical care have an ipsilateral stroke risk of under 2 per cent per year.\(^6,11\)

CPV is the equivalent of atherosclerotic burden and is measured as the volume of atherosclerotic material within a defined length of artery. That stenosis is not inevitable in severe atherosclerotic arterial disease has been known for many years.\(^12\) The importance of atherosclerotic burden has been emphasized in studies\(^13–15\) reporting substantial plaque burden in angiographically normal arteries; American Heart Association type VI (severe complex) lesions were frequently found in carotid arteries with less than 50 per cent stenosis. The PROSPECT study\(^16\) of coronary atherosclerosis demonstrated that cardiac events were related to high atherosclerotic burden rather than the severity of coronary artery stenosis. It is now accepted that atherosclerotic burden in coronary arteries is more important than stenosis in predicting subsequent CV events.\(^17–21\)

Two recent MRI studies\(^22,23\) of carotid plaque burden have underlined the importance of CPV in patients undergoing CEA within days of acute ischaemic stroke.\(^24\) Patients randomized to early surgery after acute stroke had large-volume
unstable plaques, often discharging atherosclerotic material and very different from plaques removed at elective CEA.\(^{(24)}\)

The aim of this study was to explore the relationship between CPV and symptoms of cerebral ischaemia in patients undergoing CEA. A method to measure CPV accurately in CEA specimens was developed, and the relationship between CPV, the severity of carotid stenosis and recent symptoms of cerebral ischaemia was explored in patients undergoing CEA. The relationships between CPV measured in the endarterectomy specimen and CPV measured by new tomographic ultrasound imaging (tUS) technology and middle cerebral artery emboli counts before surgery were also explored in subgroups of these patients.
6.3 Methods

Patients undergoing primary CEA in Greater Manchester over a three year period were recruited. Local ethics committee approval was obtained, with informed consent in writing obtained from all patients. Patients with atrial fibrillation, a diagnosis or treatment for cancer within 6 months, or unable to give informed consent were excluded.

A detailed medical history was taken, recording CV risk factors and the timing and nature of any symptoms of cerebral ischaemia. Patients were classified as symptomatic if they had symptoms of cerebral ischaemia in the previous 6 months. Symptoms were further divided into stroke, TIA or amaurosis fugax using established criteria. For symptomatic patients, the time between the onset of the most recent symptom and CEA was also recorded; patients were subdivided into groups with an interval of less than 2, 2–4, 4<8 and more than 8 weeks. The severity of carotid stenosis was measured using peak systolic flow velocity (PSV) on duplex Doppler ultrasound imaging, based on the NASCET criteria.

In addition to routine preoperative duplex imaging, a subset of patients underwent tUS within 24 h before surgery, performed by an experienced vascular scientist blinded to the clinical details. A magnetically tracked freehand three-dimensional (3D) ultrasound system (Curefab, Munich, Germany) was attached to a Philips iu22 duplex machine (Philips, Bothwell, USA). Sensors attached to the transducer tracked the transducer orientation and position in time and space. Multiplanar reconstructions were computed to produce 3D ultrasound volumes. System accuracy had been proven previously with phantom studies registered with CT and MRI. CPV was calculated by tracing the luminal surface and the adventitia at an interslice distance of 1 mm. Multiple slices were created along the length of the carotid plaque, over the same length subsequently measured following endarterectomy, and a CPV calculated automatically. There was no attempt to differentiate between plaques mainly involving the bifurcation and those solely within the internal carotid artery. All measurements were repeated by a vascular laboratory scientist and the research fellow; both were trained in the technique, and were blinded to the patient’s symptoms and each other’s results.
Preoperative TCD insonation of the ipsilateral middle cerebral artery to count microemboli over 1 h was undertaken in a subgroup of patients less than 24 h before CEA. MES were counted by two trained and blinded observers using the 1995 international consensus criteria. $^{(29)}$ Transient, unidirectional signals occurring within the Doppler spectrum, at least 3 dB higher than the background blood flow and lasting less than 300 ms, were counted as emboli.

During CEA, the surgeon was asked to pay particular attention to ensure that the entire carotid plaque specimen was removed en bloc, where possible. A member of the research team was present to collect the plaque in a dry pot on ice immediately after endarterectomy to minimize disruption before measuring CPV within the next hour. The full length of the endarterectomy plaque was recorded before the plaque was weighed while suspended below the surface of 110 ml normal saline in a 150-ml polythene beaker placed on an electronic balance. The volume was calculated by dividing the suspended weight by the density of saline at 23°C. Intraobserver agreement on the measurement of CPV, using data from the first 81 patients, demonstrated a mean bias of only 0.01 (95 per cent limits of agreement $-0.11$ to $0.14 \text{ cm}^3$). Interobserver agreement for CPV had a mean bias of 0.004 $\text{cm}^3$ (95 per cent limits of agreement $-0.18$ to 0.19 $\text{cm}^3$). These demonstrate good reliability and repeatability for the measurement of CPV.

**Statistical Analysis**

Normality of variables was assessed on the basis of skewness and kurtosis measures. Descriptive statistics for CPV are presented as mean (s.d.). Student’s $t$ tests and $\chi^2$ tests were used to compare patient characteristics and CPV values between asymptomatic and symptomatic patients. Differences in CPV between the symptom subgroups and at each time interval following the most recent symptoms of cerebral ischaemia were assessed using ANOVA, followed by Scheffé’s multiple comparison test. Interobserver and intraobserver agreements for CPV measurement using the suspension hydrostatic weighing technique and tUS images were investigated using the Bland–Altman method of agreement. The conventional two-sided 5 per cent significance level was used for all analyses.
6.4 Results

A total of 345 patients were recruited to the study but the endarterectomy specimens in six were removed piecemeal, rendering CPV measurements impossible. This left a total of 339 patients, all with carotid stenosis over 50 per cent (270 symptomatic, 69 asymptomatic) (Table 6-1). Symptomatic patients were more likely also to have had a previous episode of cerebral ischaemia ($P = 0.035$). There were no significant differences in CV risk factors between asymptomatic and symptomatic patients.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Asymptomatic (n = 69)</th>
<th>Symptomatic (n = 270)</th>
<th>P†</th>
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<tbody>
<tr>
<td>Age (years)*</td>
<td>69.6(8.7)</td>
<td>70.6(8.7)</td>
<td>0.370‡</td>
</tr>
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<td>BMI (kg/m²)*</td>
<td>27.2(4.1)</td>
<td>27.1(4.9)</td>
<td>0.905‡</td>
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<td>Sex ratio (M : F)</td>
<td>47 : 22</td>
<td>177 : 93</td>
<td>0.661</td>
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<td>Diabetes</td>
<td>13 (19)</td>
<td>50 (18.5)</td>
<td>0.953</td>
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<td>Hypertension</td>
<td>54 (78)</td>
<td>201 (74.4)</td>
<td>0.597</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>55 (80)</td>
<td>188 (69.6)</td>
<td>0.177</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>14 (20)</td>
<td>63 (23.3)</td>
<td>0.556</td>
</tr>
<tr>
<td>Previous myocardial infarct</td>
<td>12 (17)</td>
<td>39 (14.4)</td>
<td>0.567</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>22 (32)</td>
<td>52 (19.3)</td>
<td>0.035</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td>0.979</td>
</tr>
<tr>
<td>Smoker</td>
<td>16 (23)</td>
<td>62 (23.3)</td>
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</tr>
<tr>
<td>Ex-smoker</td>
<td>38 (55)</td>
<td>138 (51.1)</td>
<td></td>
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<tr>
<td>Never smoked</td>
<td>13 (19)</td>
<td>47 (17.4)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2 (3)</td>
<td>23 (8.5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6-1 Characteristics of patients undergoing CE. Values in parentheses are percentages unless otherwise indicated; *values are mean(s.d.). †χ² test, except ‡Student’s t test.

Total mean (s.d.) CPV was significantly greater in men than in women (1.01(0.45) versus 0.76(0.34) cm³ respectively; P < 0.001). CV risk factors were not otherwise significantly related to CPV (Table 6-2). CPV was not associated with the severity of carotid stenosis in these patients, in whom the severity of stenosis was the indication for CEA (P = 0.770) (Figure 6-1).
<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>n</th>
<th>Volume*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>221</td>
<td>1.01(0.45)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>F</td>
<td>118</td>
<td>0.76(0.34)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>62</td>
<td>0.95(0.42)</td>
<td>0.772</td>
</tr>
<tr>
<td>Hypertension</td>
<td>252</td>
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<td>0.573</td>
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<td>Hypercholesterolaemia</td>
<td>240</td>
<td>0.94(0.43)</td>
<td>0.728</td>
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<td>Ischaemic heart disease</td>
<td>76</td>
<td>0.97(0.49)</td>
<td>0.456</td>
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<td>Previous myocardial infarct</td>
<td>50</td>
<td>0.97(0.49)</td>
<td>0.490</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>72</td>
<td>0.88(0.42)</td>
<td>0.251</td>
</tr>
<tr>
<td>Smoker</td>
<td>78</td>
<td>0.93(0.51)</td>
<td>0.650</td>
</tr>
</tbody>
</table>

Table 6-2 Relationship between carotid plaque volume and CV risk factors. *Values are mean(s.d.). †Versus carotid plaque volume in absence of risk factor (Student’s t test).
Mean CPV was significantly greater in the 270 symptomatic patients than in the 69 patients with no symptoms of cerebral ischaemia (0.97(0.43) versus 0.74(0.41) cm$^3$; $P < 0.001$). The difference remained significant following adjustment for sex ($P < 0.001$). The mean CPV was 1.03(0.49) cm$^3$ in 93 patients following recent stroke, compared with 0.95(0.42) cm$^3$ for the 141 patients who had a TIA and 0.91(0.28) cm$^3$ for the 34 patients with amaurosis fugax ($P = 0.254$).

Among the 270 symptomatic patients, mean CPV was highest in patients undergoing CEA shortly after symptoms of cerebral ischaemia and declined as the interval between symptoms and surgery increased ($P < 0.001$) (Figure 6-2). Mean CPV for patients undergoing CEA within 2 weeks of symptoms (1.039 cm$^3$) was very similar to that among patients undergoing CEA within 2–4 weeks (1.035 cm$^3$), so these patients were grouped together for statistical analysis. Mean CPV within 4 weeks following symptoms of cerebral ischaemia was 1.03(0.46) cm$^3$ compared with 0.95(0.24) cm$^3$ between 4 and 8 weeks, and 0.78(0.36) cm$^3$ more than 8 weeks after symptoms. Those who underwent CEA more than 8 weeks following
symptoms of cerebral ischaemia had a CPV almost identical to that of asymptomatic patients, but significantly lower than that in patients undergoing CEA less than 4 weeks after symptoms ($P = 0.001$, Scheffé’s multiple comparison test).

Using PSV as a measure of the haemodynamic severity of stenosis, there were no equivalent differences. Mean (s.d.) PSV was 3.49(1.84) cm/s within 4 weeks of symptoms, 3.88(2.13) cm/s between 4 and 8 weeks, and 3.47(2.09) cm/s more than 8 weeks after the onset of the most recent symptom of cerebral ischaemia ($P = 0.570$). Mean PSV was higher in asymptomatic patients undergoing CEA than it was in symptomatic patients (3.99(1.77) versus 3.47(1.94) cm/s respectively; $P = 0.056$).

Cerebral emboli were counted in the ipsilateral middle cerebral artery over 1 h by TCD before CEA in 33 symptomatic patients. They were detected in 27 of these patients, the mean (s.d.) number of emboli during the hour of monitoring being 3.9(2.7). Mean CPV was 1.00(0.48) cm$^3$ in the 27 patients with cerebral emboli compared with 0.67(0.16) cm$^3$ among the six patients in whom no cerebral emboli
were detected \((P = 0.142)\). There was a significant but weak correlation between number of cerebral emboli per h and CPV \((r = 0.36, P = 0.045)\). There was a negative but insignificant correlation between number of cerebral emboli per h and PSV \((r = -0.255, P = 0.190)\).

Of 50 patients who underwent preoperative tUS, results from ten were omitted owing to extensive acoustic shadowing rendering interpretation impossible, and six did not have corresponding surgical specimens. The CPV measured by tUS in the remaining 34 patients correlated closely with the CPV measured in the endarterectomy specimen \((r = 0.93, P < 0.001)\), with minimal bias \((0.20 \text{ (95 per cent c.i. -0.29 to 0.69) cm}^3)\) (Figure 6-3). Inter-rater analysis demonstrated minimal bias \((-0.23 \text{ to 0.25) cm}^3\)) between the CPV measurements calculated by the two observers, again with excellent correlation \((r = 0.98, P < 0.001)\) (data not shown). Intrarater analysis demonstrated minimal bias \((0.07 \text{ (0.25 to 0.39) cm}^3\)) between the CPV measurements calculated when repeated by the primary observer, again with excellent correlation \((r = 0.97, P < 0.001)\).

\[\text{Figure 6-3 A Scatter plot demonstrating correlation between in vivo measurements of carotid plaque volume by three-dimensional ultrasonography and ex vivo measurements using the immersion technique (} r = 0.93, P < 0.001). \text{ B Bland-Altman plot (mean(s.d.) bias 0.20 (95 per cent c.i. -0.29 to 0.69) cm}^3)\]
6.5 Discussion

CPV in the endarterectomy specimen was associated with recent symptoms of cerebral ischaemia in patients undergoing CEA. Perhaps more importantly, CPV was markedly higher in the first few weeks following symptoms of cerebral ischaemia, when the risk of stroke is also known to be high. Rothwell and colleagues\(^{(10)}\) clearly showed that CEA had the greatest impact on stroke risk if undertaken within days of cerebral ischaemia, and no more impact than it had for asymptomatic patients if undertaken more than 12 weeks following symptoms.

The results suggest that the healing of carotid plaques, following what is presumed to be a discharge of atherosclerotic material at the time of cerebral ischaemia, may be faster than previously recognized. The decline in plaque volume over time following symptoms of cerebral ischaemia was not associated with any significant change in the severity of carotid stenosis. There was no significant relationship between the severity of stenosis and CPV, although this is a population of patients in whom the severity of carotid stenosis was sufficient to justify CEA. It was hardly surprising that the severity of carotid stenosis was marginally higher in the asymptomatic patients as many surgeons are reluctant to undertake CEA in asymptomatic patients unless there is stenosis exceeding 80 per cent.

Middle cerebral artery emboli in patients with carotid disease are known to be associated with recurrent ischaemic events\(^{(30)}\). The Asymptomatic Carotid Emboli Study\(^{(31)}\) demonstrated that the annual risk of stroke in patients with embolic signals was 3.6 per cent compared with 0.7 per cent in those without. The finding that CPV was significantly higher in patients who had ipsilateral cerebral emboli also suggests that CPV may be a measure of stroke risk. The absence of a correlation between cerebral emboli and severity of stenosis underlines the low risk of stroke in asymptomatic severe carotid stenosis. Although there has been little previous research on CPV, when measured by ultrasound imaging in 349 patients it was associated with the frequency of subsequent stroke, TIA and death.\(^{(32)}\) Both CPV and changes in plaque composition were also reported to be indicators of stroke risk.\(^{(33)}\)
The concept of the vulnerable plaque was first reported in coronary artery disease, where ruptured, inflammatory and thin cap plaques were associated with acute coronary syndrome.\(^{(34–36)}\) The same was assumed to be true for carotid plaques, leading to many attempts to identify carotid plaque characteristics that relate to the subsequent risk of stroke.\(^{(22,23,37–39)}\) The present results raise the intriguing possibility that intraplaque haemorrhage\(^{(37)}\) and measures of plaque perfusion\(^{(38,40)}\) may be the consequence of plaque rupture, rather than risk factors for future stroke. The finding that CPV declines with time following symptoms of cerebral ischaemia suggests a healing process measured in weeks, which may be much shorter than that suggested in the literature. It is possible that, following the discharge of atherosclerotic material from a carotid plaque, the space is filled with blood which has been interpreted as intraplaque or subplaque haemorrhage. Subsequently this haematoma is lysed and progressively replaced by granulation tissue as part of the healing process, with resulting increases in plaque perfusion.\(^{(38)}\) The fact there is no difference between CPV within 2 weeks of symptoms and that at 2–4 weeks is consistent with the time required for a haematoma to start to resolve by lysis, and for the resulting inflammation to settle.

Previous studies\(^{(33,41,42)}\) measuring CPV by both MRI and CT suggested that CPV was associated with CV risk factors and symptoms of cerebral ischaemia. If the accuracy of tUS measurement of CPV is confirmed in larger studies, it is possible that the measurement of CPV could replace severity of stenosis as the principal indication for CEA. The ultimate objective could be population screening for carotid disease. 3D ultrasound techniques for the measurement of CPV need to be explored with this in mind.\(^{(43–45)}\)

Limitations of this study were that only 33 patients of the intended 50 underwent preoperative TCD to detect middle cerebral artery emboli. Neither CT nor MRI of the brain is routine in patients undergoing CEA in Manchester, precluding any investigation of the relationship between CPV and cerebral infarction. The need for urgent CEA following symptoms of cerebral ischaemia has been established by two major meta-analyses\(^{(46,47)}\) demonstrating that the risk of stroke is highest immediately following TIA and then declines over a similar timescale. These studies
showed that the stroke risk within 1 week of TIA was approximately 10 per cent and that this risk declined progressively, such that the risk was no higher than in patients with asymptomatic carotid disease by 12 weeks. As CPV declines following symptoms of cerebral ischaemia at a rate remarkably similar to the reduction in stroke risk, this is consistent with CPV being associated with stroke risk. Once a minimally invasive method for measuring CPV accurately in patients has been established, the relationship between CPV and stroke risk needs to be explored in a definitive cohort study.
6.6 References


15 Saam T, Underhill HR, Chu B, Takaya N, Cai J, Polissar NL et al. Prevalence of American Heart Association type VI carotid atherosclerotic lesions identified by magnetic resonance imaging for different levels of stenosis as measured by duplex ultrasound. J Am Coll Cardiol 2008; 51: 1014–1021.


CHAPTER 7: CAROTID PLAQUE VOLUME IS RELATED TO PLAQUE INSTABILITY

S Ball, E Byrne, S Church, P Begley, G Cooper, C McCollum

Contributions and role:

S Ball: Conception, Patient recruitment, data collection and analysis, preparation of histological slides and analysis, performing ELISA for Lp-PLA₂ and P-Selectin.

E Byrne: Histopathological analysis

Prof G Cooper: Co-Supervisor

Prof C McCollum: Supervisor
7.1 Abstract

**Background:** Patients receiving best medical therapy for asymptomatic carotid artery disease (CAD) have an annual stroke risk of less than 2%. The authors have published on the potential importance of carotid plaque volume (CPV) in symptomatic CAD, being significantly increased in patients with recent symptoms of cerebral ischaemia.

**Aims:** To explore the relationship between CPV and histological markers of plaque instability. The relationship between CPV and plasma concentrations of high sensitive C-reactive protein (Hs-CRP), lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) and P-Selectin were also explored.

**Methods:** Patients undergoing a primary carotid endarterectomy (CEA) were included. Their plaques were removed en-bloc to calculate CPV by Archimedes’ principle and histologically assessed according to the American Heart Association. Plasma was also taken for determination of Lp-PLA$_2$, Hs-CRP and P-selectin levels.

**Results:** Mean (sd) CPV was significantly increased in patients with symptomatic disease ($0.91(0.35)$cm$^3$ vs $0.72(0.31)$cm$^3$, $p=0.012$) and significantly increased in males, those diagnosed with hypercholesterolaemia, and those who had suffered a previous episode of cerebral ischaemia. Mean (sd) CPV was also significantly increased in those with unstable plaques ($0.90(0.34)$cm$^3$ vs $0.71(0.26)$cm$^3$, $p=0.004$). Mean (sd) Lp-PLA$_2$ was increased in patients with marked plaque inflammation ($141.2(45)$ ng/ml vs $105.3(20.4)$ ng/ml, $p=0.001$), and in those with an unstable plaque.

**Conclusion:** CPV is associated with plaque instability and symptomatic status. These findings further evidence the potential for CPV to serve as an indicator for carotid intervention in future.
7.2 Introduction

Stroke is a major economic and healthcare burden to the UK costing the economy around £7 billion per year. Carotid disease currently accounts for 30% of ischaemic strokes\(^{(1)}\) and despite substantive evidence that the mechanism is thromboembolic, the current guidelines for intervention are still based on the degree of stenosis.\(^{(2-6)}\) With improving medical therapy, public awareness and better monitoring of CV risk factors, the annual stroke risk from asymptomatic CAD is now less than 2%.\(^{(4,7)}\) This has led surgeons to adopt a more conservative approach when treating such patients, however, a certain percentage of these patients will still suffer cerebral ischaemia so a need to better identify such at-risk patients has emerged.

We have previously published on the importance of CPV in relation to symptomatic carotid disease.\(^{(8)}\) Symptomatic patients had significantly increased CPV compared to those with asymptomatic disease, which decreased with time from symptom onset at a rate similar to that of the accepted stroke risk, and was not related to the degree of stenosis. Preliminary results on measuring CPV in vivo using t-US were encouraging, with excellent correlation between CPV measured by ex-vivo and in-vivo methods (\(r=0.93, p<0.001\))

As well as traditional CV risk factors, Hs-CRP and P-selectin have been studied in relation to atherosclerosis and the development of CV events. Not only is elevated Hs-CRP postulated to be associated with future CV events\(^{(9-11)}\) and symptomatic peripheral arterial disease,\(^{(12)}\) it has also been shown to be associated with progression of atherosclerosis at various points in the arterial tree, including the carotid artery.\(^{(13-16)}\) While P-selectin has been shown to be involved in atherosclerosis and represents an increased risk for future vascular events,\(^{(17-20)}\) a later review questioned the additional prognostic value it has over traditional risk factors.\(^{(21)}\)

Of late, Lp-PLA\(_2\) has shown promise as a potential marker of symptomatic CV disease. It is a calcium-independent member of the phospholipase A\(_2\) family that catalyses hydroxylation of oxidised LDL (Ox-LDL) stimulating the release of lysoPC, amongst others, which has both pro-inflammatory and pro-atherogenic properties,
thereby perpetuating the inflammatory process and contributing to plaque instability.\(^{(22)}\) One of the first studies to report on Lp-PLA\(_{2}\) and CV events was the west of Scotland coronary prevention study (WOSCOPS) who found an association between increasing levels of plasma Lp-PLA\(_{2}\) and the risk of future coronary events.\(^{(23)}\) This led to studies looking at the potential of Lp-PLA\(_{2}\) in relation to coronary disease and CV events in general with many reporting on the positive association between increasing levels of Lp-PLA\(_{2}\) and increased stroke risk along with all CV events.\(^{(11, 24-28)}\) A trial of an Lp-PLA\(_{2}\) inhibitor in 330 patients with angiographically-proven coronary artery disease demonstrated a relative reduction in increase of the necrotic core compared to the placebo-treated group.\(^{(29)}\) A similar response was also reported in an animal study where decrease in plaque area and necrotic core resulted in fewer unstable lesions following inhibition of Lp-PLA\(_{2}\).\(^{(30)}\) However, few studies have looked specifically at Lp-PLA\(_{2}\) in CAD.

The main aim of this study as to explore the relationship between CPV and markers of plaque instability as assessed by the American heart association grading: specifically if unstable plaques had an increased CPV. Plasma concentrations of Hs-CRP, Lp-PLA\(_{2}\) and P-Selectin were measured with the aim of finding a biomarker that complemented CPV.
7.3 Methods

Patients undergoing a primary CEA at the University Hospital of South Manchester were invited to participate. The patients included were a sub-group of the patients described in chapter 6. Local ethics committee approval was granted and written informed consent gained from all patients. Patients were excluded if they had a history of atrial fibrillation, a diagnosis or treatment for cancer within the last 6 months or were unable to give informed consent.

A detailed medical history relating to traditional CV risk factors was taken from all patients. Patients were classed as symptomatic if they had suffered an episode of cerebral ischaemia within 6 months and further subdivided into CVA, TIA and amaurosis fugax using established criteria. As part of their clinical work-up, each patient underwent a 2D duplex to grade the severity of carotid stenosis. On the day of surgery, 5ml of venous blood was taken into a heparin bonded tube and spun at 3000rpm for 20 minutes within 20 minutes of collection. Plasma was then aliquoted into 4 vials, labelled appropriately, and stored immediately at -80°C for subsequent analysis. Lp-PLA₂ and P-selectin levels were determined using commercial ELISA kits (Human PLA2G7/PAF-AH/ Lp-PLA2 and Human P-selectin/CD62P, R&D Systems, UK). Hs-CRP was measured using the Abbott Architect (CRP VARIO 6K26-30 and 6K26-41).

Following removal of the carotid plaque, en bloc where possible, it was collected in a dry container and transported to the laboratory where CPV was determined by fluid displacement (Archimedes' principle). The method for calculating CPV has been previously published and validated. Following CPV measurement, the plaque was divided into segments and note made of the most diseased i.e most ulcerative segment. The plaque segments were then frozen to -80°C for future histological analysis.
**Histological Analysis**

Plaque segments selected for histological analysis were thawed and fixed in 10% formalin, then embedded in paraffin wax, and 5µm slices made and stained with haematoxylin and eosin, elastic van Gieson, mouse monoclonal anti-CD68 antibody and rabbit monoclonal anti-CD3 antibody; the latter two stains were employed for macrophage and t-cell detection, respectively. A histopathologist and a trained researcher each performed histological analysis; they were blinded to the clinical case details and each other’s experimental findings. Criteria reported in the American Heart Association’s classification of coronary atherosclerosis was used to grade the lesions as either stable or unstable, with unstable plaques being those that demonstrated disruption of the fibrous cap, with haematoma or thrombus formation.\(^{(32)}\) The level of inflammation was graded according to the published protocol by Redgrave et al\(^{(33)}\) as part of the Oxford Plaque Study based on the number of macrophages and T-cells present within the plaque and graded as marked or mild. In cases of discrepancy between the two observers those samples were re-assessed and the histopathologist grading used.

**Statistics**

Statistical analysis was performed using SPSS 22. Normality of variables was assessed on the basis of histograms and Q-Q plots. CPV, Lp-PLA\(_2\), and P-Selectin were deemed to be normally distributed whereas Hs-CRP was not normally distributed. Descriptive statistics for CPV, Lp-PLA\(_2\), P-selectin and Hs-CRP are presented as mean (sd) and median (IQR) as appropriate; \(\chi^2\) tests, student’s t-tests and Mann-Whitney U tests were used to compare patient characteristics to CPV, Lp-PLA\(_2\), P-selectin and Hs-CRP levels as well as between symptomatic and asymptomatic patients; stable and unstable plaques; and marked inflammatory and mildly inflammatory plaques. Differences in CPV, Lp-PLA\(_2\), Hs-CRP and P-selectin between the symptom subgroups were assessed using ANOVA, followed by Scheffé’s multiple comparisons test, and the Kruskal-Wallis H test. Two-tailed tests were applied and p-values of < 0.05 were considered significant.
7.4 Results

Patient Characteristics

Of the 150 patients recruited, 92 were male (61.3%) and 114 (76%) had suffered recent symptoms of cerebral ischaemia. Of the 114 patients with recent symptoms of cerebral ischemia, 40 (35.1 %) had a stroke, 58 (50.9 %) a TIA, and 16 amaurosis fugax (14.0 %). 115 patients had their CPV measured, 109 plaques underwent histological analysis and 79 patients had plasma tested for Lp-PLA2, Hs-CRP and P-Selectin. 50 patients had their CPV calculated, plaque histologically analysed and plasma tested. 100 patients had both their CPV calculated and plaque histologically analysed.

85 of the patients classified as symptomatic were operated on within 4 weeks of symptoms, of which 62 (72.9 %) were treated within 2 weeks of symptoms. Of the 36 classed as asymptomatic, 26 had a carotid stenosis of > 80% with 9 of the remaining 10 > 70%. Other than a history of a previous episode of cerebral ischaemia (p=0.007), there was no statistically significant between-group difference of known CV risk factors and symptom status (Table 7-1).
<table>
<thead>
<tr>
<th></th>
<th>Asymptomatic (n = 36)</th>
<th>Symptomatic (n = 114)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>70.8</td>
<td>70.9</td>
<td>0.950‡</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>28.1</td>
<td>27.6</td>
<td>0.631‡</td>
</tr>
<tr>
<td>Sex ratio (M : F)</td>
<td>24 : 12</td>
<td>68 : 46</td>
<td>0.451</td>
</tr>
<tr>
<td>Diabetes</td>
<td>10 (28)</td>
<td>26 (23)</td>
<td>0.598</td>
</tr>
<tr>
<td>Hypertension</td>
<td>30 (83)</td>
<td>86 (75)</td>
<td>0.454</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>30 (83)</td>
<td>75 (66)</td>
<td>0.069</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>8 (22)</td>
<td>18 (16)</td>
<td>0.438</td>
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<tr>
<td>Previous myocardial infarct</td>
<td>8 (22)</td>
<td>12 (11)</td>
<td>0.083</td>
</tr>
<tr>
<td>Previous CVA/TIA</td>
<td>14 (39)</td>
<td>19 (17)</td>
<td>0.007</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td>0.445</td>
</tr>
<tr>
<td>Smoker</td>
<td>9 (25)</td>
<td>31 (27)</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>16 (44)</td>
<td>44 (39)</td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>7 (19)</td>
<td>29 (25)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>4 (11)</td>
<td>10 (9)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean(s.d.). †χ² test, except ‡Student’s t test.

Table 7-1 Characteristics of patients undergoing CEA. Values in parentheses are percentages unless otherwise indicated.

Carotid Plaque Volume

CPV was significantly increased in those patients who had suffered a recent episode of cerebral ischaemia compared to those who had not (0.91(0.35)cm³ vs 0.72(0.31)cm³, p=0.012); this difference remained following adjustment for gender and a diagnosis of hypercholesterolaemia (p=0.002). CPV was also significantly greater in males (0.94(0.36)cm³ vs 0.72(0.23)cm³, p<0.001), those with a diagnosis of hypercholesterolaemia (0.91(0.37)cm³ vs 0.75(0.24)cm³, p=0.008) and those who
had suffered a previous episode of cerebral ischaemia (1.02(0.41)cm$^3$ vs 0.82(0.31)cm$^3$, p=0.009) (Table 7-2). CPV was significantly increased in patients whose plaques were classed as being histologically unstable compared to those that were classed as histologically stable (0.90(0.34)cm$^3$ vs 0.71(0.26)cm$^3$, p=0.004) (Figure 7-1) and this difference remained following adjustment for gender, diagnosis of hypercholesterolaemia, and previous episode of cerebral ischaemia (p=0.003). However there was no significant difference between CPV values in relation to degree of plaque inflammation as assessed histologically (0.84(0.33)cm$^3$ vs 0.87(0.31)cm$^3$, p=0.746), nor were CPV and Lp-PLA$_2$ values correlated (r=0.205, p=0.127) (Table 7-3).

![Figure 7-1 Mean(s.d.) carotid plaque volume (CPV) in patients classed as having a histologically stable and histologically unstable plaque. P=0.004 (Student's t-test)](image-url)
<table>
<thead>
<tr>
<th></th>
<th>CPV*</th>
<th>P-value</th>
<th>Lp-PLA₂*</th>
<th>P-value</th>
<th>Hs-CRP†</th>
<th>P-value</th>
<th>P-selectin*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td>&lt; 0.01</td>
<td></td>
<td>0.036</td>
<td>0.99</td>
<td></td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>0.94</td>
<td>(0.36)</td>
<td>139 (39)</td>
<td>2.6 (5.9)</td>
<td>44 (12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.71</td>
<td>(0.23)</td>
<td>115 (57)</td>
<td>2.7 (5.4)</td>
<td>43 (15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.89</td>
<td>(0.32)</td>
<td>125 (48)</td>
<td>1.7 (2.7)</td>
<td>44 (10)</td>
<td>0.92</td>
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<td></td>
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<tr>
<td>Hypertension</td>
<td>0.89</td>
<td>(0.36)</td>
<td>135 (44)</td>
<td>2.7 (5.6)</td>
<td>45 (13)</td>
<td>0.44</td>
<td></td>
<td></td>
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<tr>
<td>Hypercholesterol</td>
<td>0.91</td>
<td>(0.37)</td>
<td>139 (48)</td>
<td>2.6 (5.2)</td>
<td>45 (13)</td>
<td>0.39</td>
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<tr>
<td>IHD</td>
<td>0.88</td>
<td>(0.45)</td>
<td>145 (61)</td>
<td>2.4 (5.6)</td>
<td>45 (11)</td>
<td>0.82</td>
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<tr>
<td>Previous MI</td>
<td>0.93</td>
<td>(0.33)</td>
<td>139 (60)</td>
<td>2.3 (3.2)</td>
<td>40 (9)</td>
<td>0.28</td>
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<tr>
<td>Previous CVA/TIA</td>
<td>1.02</td>
<td>(0.41)</td>
<td>133 (40)</td>
<td>1.1 (2.1)</td>
<td>43 (9)</td>
<td>0.64</td>
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<tr>
<td>Smoking History</td>
<td>0.748</td>
<td></td>
<td>0.807</td>
<td>0.12</td>
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<td></td>
<td>0.688</td>
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<tr>
<td>Smoker</td>
<td>0.88</td>
<td>(0.28)</td>
<td>136 (47)</td>
<td>4.4 (5.8)</td>
<td>48 (14)</td>
<td></td>
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</tr>
<tr>
<td>Ex-smoker</td>
<td>0.89</td>
<td>(0.35)</td>
<td>143 (49)</td>
<td>2.5 (6.0)</td>
<td>44 (14)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Never smoked</td>
<td>0.85</td>
<td>(0.43)</td>
<td>127 (41)</td>
<td>4.4 (5.8)</td>
<td>43 (12)</td>
<td></td>
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</tr>
</tbody>
</table>

Table 7-2 Characteristics of patients undergoing CPV measurement and plasma testing of Lp-PLA₂, Hs-CRP and P-Selectin.*values are mean (sd) & students t-test applied. †values are median (IQR) & Mann-Whitney U test applied.
### Table 7-3

<table>
<thead>
<tr>
<th>Symptom Status</th>
<th>Symptomatic</th>
<th>Asymptomatic</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>CPV*</td>
<td>0.91 (0.35)</td>
<td>0.72 (0.31)</td>
<td>0.012</td>
</tr>
<tr>
<td>Lp-PLA₂*</td>
<td>128 (48)</td>
<td>139 (41)</td>
<td>0.406</td>
</tr>
<tr>
<td>Hs-CRP†</td>
<td>4.28 (6.11)</td>
<td>2.07 (2.05)</td>
<td>0.193</td>
</tr>
<tr>
<td>P –selectin*</td>
<td>43 (13)</td>
<td>48 (14)</td>
<td>0.194</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Plaque Stability</th>
<th>Unstable</th>
<th>Stable</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPV*</td>
<td>0.90 (0.34)</td>
<td>0.71 (0.26)</td>
<td>0.004</td>
</tr>
<tr>
<td>Lp-PLA₂*</td>
<td>140 (48)</td>
<td>127 (37)</td>
<td>0.288</td>
</tr>
<tr>
<td>Hs-CRP†</td>
<td>2.3 (5.97)</td>
<td>3.12 (4.81)</td>
<td>0.971</td>
</tr>
<tr>
<td>P –selectin*</td>
<td>47 (13)</td>
<td>42 (15)</td>
<td>0.225</td>
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<table>
<thead>
<tr>
<th>Plaque inflammation</th>
<th>Marked</th>
<th>Mild</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>CPV*</td>
<td>0.84 (0.33)</td>
<td>0.87 (0.31)</td>
<td>0.746</td>
</tr>
<tr>
<td>Lp-PLA₂*</td>
<td>141 (45)</td>
<td>105 (20)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hs-CRP†</td>
<td>2.76 (5.55)</td>
<td>1.08 (3.39)</td>
<td>0.516</td>
</tr>
<tr>
<td>P –selectin*</td>
<td>45 (14)</td>
<td>46 (11)</td>
<td>0.976</td>
</tr>
</tbody>
</table>

Table 7-3 A) Relationship between CPV; Lp-PLA₂, Hs-CRP; P-Selectin and symptom status. (B) Relationship between CPV; Lp-PLA₂, Hs-CRP; P-Selectin and plaque stability as assessed histologically. (C) Relationship between CPV; Lp-PLA₂, Hs-CRP; P-Selectin and plaque inflammation as assessed histologically. †values are median (IQR) & Mann-Whitney U test applied.

**Lp-PLA₂**

Lp-PLA₂ was found to be significantly increased in males (139(39) ng/ml vs 115(57) ng/ml, p=0.036) and those with a history of hypercholesterolaemia (139(48) ng/ml vs 116(39) ng/ml, p=0.045). Lp-PLA₂ was significantly increased in those patients with marked inflammation of the carotid plaque as assessed histologically (141(45)ng/ml vs 105(20)ng/ml, p=0.001) (Figure 7-2), and remained significant following adjustment for gender and hypercholesterolaemia (p=0.024). Lp-PLA₂ also trended towards an increase in those with unstable plaques although this finding did not reach statistical significance (140(48)ng/ml vs 127(37)ng/ml, p=0.288). Lp-
PLA₂ demonstrated a weak correlation with P-selectin ($r=0.233$, $p=0.043$). There was no relation to symptom status and was actually found to be increased in asymptomatic patients.

Figure 7-2 Mean(s.d.) Lp-PLA2 levels in patients whose plaques were graded as having marked and mild inflammation. $P=0.001$ (Student’s t-test)
High-Sensitive CRP

Whilst Hs-CRP was increased in symptomatic patients and those with marked inflammation it did not reach statistical significance (U=616.5, Z=1.301, p= 0.193 and U=230.5, Z=0.650, p=0.516, respectively). There was weak correlation between Hs-CRP and P-selectin (r_s=0.273, p=0.021), but no demonstrable difference in Hs-CRP between those with stable and unstable plaques.

Plaque Histology

A diagnosis of hypercholesterolaemia was significantly associated with a histologically unstable plaque (OR 2.75; [95% CI: 1.19,6.14], p=0.016). Hypertension was significantly associated with plaques classed as having marked inflammation (OR 1.35; [95% CI: 1.2,1.51], p=0.021).
7.5 Discussion

This to our knowledge is the first paper to report that CPV is significantly related to a histologically unstable plaque and adds further evidence to the view that cerebral ischaemia, secondary to carotid disease, may be related more closely to CPV than to the severity of stenosis per se. In addition to replicating the authors previous findings relating CPV to symptom status\(^8\) we also found CPV to be significantly increased in males, those diagnosed with hypercholesterolaemia, and those who had suffered a previous episode of cerebral ischaemia.

Lp-PLA\(_2\) was also found to be significantly increased in males and those diagnosed with hypercholesterolaemia. Hypercholesterolaemia was also significantly associated with plaque instability. Nearly all patients in this study were receiving statin therapy which would have been commenced as part of secondary CV prevention irrespective of their lipid levels. Therefore any association with lipid levels was not explored. Lp-PLA\(_2\) plays an active role in atherosclerosis through hydroxylation of Ox-LDL. It is possible that prolonged exposure to increased levels of LDL may be the stimulus for these more advanced atherosclerotic plaques, as evidenced by the significant association between Lp-PLA\(_2\) levels and a diagnosis of hypercholesterolaemia.

Lp-PLA\(_2\) was significantly increased in those patients with marked inflammation and increased in those with a histologically unstable plaque, likely reflecting the pathophysiological role of Lp-PLA\(_2\). This is consistent with work from Sarlon-Bartoli et al, who found no difference in Lp-PLA\(_2\) between degrees of symptom status, but found significantly increased levels of Lp-PLA\(_2\) in those with unstable plaques.\(^{34}\) Mannheim et al\(^{35}\) also found significantly increased levels of Lp-PLA\(_2\) expressed within the plaques of those regarded as unstable. As mentioned, lysoPC is the main end product of Ox-LDL hydroxylation and has both pro-inflammatory and pro-atherogenic properties by stimulating macrophage proliferation and expression of vascular adhesion molecules known to be integral in atherosclerosis.\(^{22}\) Nishi has reported that macrophage infiltration is a landmark factor for plaque instability, and subsequently demonstrated that the amount of plaque Ox-LDL was positively correlated with plaque instability and macrophage content.\(^{36, 37}\) This could be due
to the effects of Lp-PLA$_2$. Mannheim et al also reflected this finding, reporting lysoPC to be significantly increased in symptomatic patients and positively correlated with Lp-PLA$_2$. Ox-LDL and macrophage content were also significantly increased and collagen content significantly decreased in unstable plaques. They hypothesised that the instability was related to the apoptotic effects of lysoPC given its strong correlation with TUNEL+ cells, a marker of apoptosis.

We could not replicate findings reported in the literature between Hs-CRP and symptomatic patients.$^{(38, 39)}$ Many believe the instability in plaques is related to the level of inflammatory activity within such plaques and Hs-CRP levels reflect this.$^{(40-42)}$ Others have postulated that Hs-CRP has a role at a cellular level through an ability to bind macrophages, promoting endothelial cell activation and expression of adhesion molecules $^{(42-48)}$ as well as a pro-coagulant effect.$^{(39, 49-51)}$ Whilst we found Hs-CRP trended towards increase in patients in whom plaques were scored as having marked inflammation, it did not reach statistical significance, nor did we find any relation to plaque instability. Interestingly, when reporting the significance of Hs-CRP with symptomatic disease, Garcia et al$^{(39)}$ also noted that Hs-CRP increased with time from symptom onset, which questions how much of a role Hs-CRP plays in unstable plaques. When looking at the results in more detail, Hs-CRP levels were reduced in all patients with known CV risk factors and significantly reduced in those who had suffered a previous episode of cerebral ischaemia ($p=0.042$). Further work needs to be completed to either confirm or refute this finding with larger numbers and ascertain if there is any relation to medications.

The relevance of P-selectin to symptomatic CAD could not be found in this study. Despite it being increased in symptomatic patients and those with unstable plaques it did not reach statistical significance raising doubts about its significance in symptomatic CAD.

That we couldn’t replicate findings from other studies of Lp-PLA$_2$, Hs-CRP and P-selectin being associated with plaque instability could be explained by the fact we had a small number of asymptomatic patients who had advanced disease evidenced by 26 out of the 36 having a carotid stenosis $>$80% with 9 of the remaining 10 being greater than 70%. All lesions were classed as advanced atherosclerotic lesions on
histology meaning their circulating levels of Lp-PLA₂, Hs-CRP and P-selectin would be expected to be increased.

Lp-PLA₂ plays a part in advancing atherosclerotic lesions; however, based on results in this paper and the related literature, it cannot be used as an indicator for carotid intervention in patients with asymptomatic disease. This paper only measured plasma Lp-PLA₂ and further work could involve measuring the amount of Lp-PLA₂ expressed within carotid plaques to ascertain if this correlates with plasma levels. It may be that circulating Lp-PLA₂ is absorbed into the plaque as it becomes unstable, hence decreasing the circulating level, and could account for why we found Lp-PLA₂ levels to be lower in recently symptomatic patients.

The main limitation to this study is the relatively small numbers involved and especially the small number of asymptomatic patients. Only one third of patients underwent the full battery on investigations with regards to CPV calculation, histological analysis and plasma markers. This was due to the clinical nature of the study meaning that logistically it was not always possible due to time constraints and the increased recognition that CEA should be performed expediently in symptomatic patients. CEAs were performed out of hours meaning collection and processing of the plaque according to protocol was not always possible along with venepuncture.

The observation that CPV is related to plaque instability is of potential significance given our preliminary findings associating symptoms of cerebral ischaemia with significantly increased CPV. Based on preliminary findings already published it is possible to accurately measure CPV in-vivo using t-US. This paper further strengthens our view that CPV can be used as an indicator for carotid intervention, especially in those with asymptomatic disease. This now needs to be further studied in a longitudinal, prospective cohort study.
7.6 References


CHAPTER 8: ANTIPLATELET RESISTANCE IN THOSE PATIENTS UNDERGOING CAROTID ENDARTERECTOMY

S Ball, R Taylor, C McCollum

Contributions and role:

S Ball: Patient recruitment, data collection, venepuncture, data analysis, manuscript writing

R Taylor: Patient recruitment, data collection

Prof C McCollum: Conception, supervisor
8.1 Abstract

**Background:** Platelet inhibitory therapy is prescribed to reduce cardiovascular (CV) risk in patients with atherosclerotic disease. Although taken by millions of people, around 30% may be resistant to the treatment they are being prescribed.

**Objectives:** To determine whether symptoms of cerebral ischaemia or pre-operative cerebral emboli in patients admitted for a carotid endarterectomy (CEA) were associated with resistance to aspirin or clopidogrel.

**Methods:** Venous blood from 133 patients undergoing CEA was analysed for resistance to aspirin and clopidogrel by multiplate impedance aggregometry. The number of emboli entering the ipsilateral middle cerebral artery over one hour was counted by transcranial Doppler (TCD) on the day before surgery in 33 of these patients.

**Results:** Resistance to aspirin and clopidogrel was found in 21 (26.3%) of 100 patients taking aspirin and 14 (42%) of 33 taking clopidogrel. Mean (sd) residual platelet aggregation was significantly higher in patients who had suffered recent symptoms of cerebral ischaemia at 41.9(32) Au compared to 30.8(16) Au in asymptomatic patients (p=0.012). Residual platelet aggregation also correlated with the number of emboli/hour in the ipsilateral middle cerebral artery detected by TCD (r=0.45, p=0.009).

**Conclusion:** Antiplatelet resistance was associated with the number of cerebral emboli and recent symptoms of cerebral ischaemia in patients with carotid disease. Definitive clinical studies are needed to explore whether testing for antiplatelet resistance should be undertaken routinely in patients starting platelet inhibitory therapy for CV disease.
8.2 Introduction

Atherosclerotic arterial disease is the single most frequent cause of death worldwide causing 10 million deaths due to ischaemic heart disease and 5.5 million deaths due to stroke. (1) Atherosclerosis is a chronic inflammatory disease, with a latency period of many years, leading to atheroma and plaque development within the arterial wall.

Stroke is the leading cause of disability in the UK and third leading cause of death with > 150 000 new strokes/year, costing the UK economy £7 billion per year. (2, 3) Atherosclerotic emboli originating from CAD are thought to cause 30% of all ischaemic strokes. Antiplatelet therapy has become established as an essential treatment for all patients with carotid disease reducing annual stroke risk by 9% and preventing 20% of strokes in patients with recent symptoms of cerebral ischaemia. (4-7) However, in the U.S. alone, 185,000 recurrent strokes occur each year, with a third occurring in patients receiving antiplatelet therapy. (8) Aspirin and Clopidogrel impact on platelet activation and aggregation via their own distinct mechanisms but share the common endpoint of reducing thromboembolism from atherosclerotic disease. (9) True antiplatelet resistance occurs at a biochemical and genetic level through polymorphisms of the COX-1/2 genes and thromboxane synthase in aspirin resistance and the cytochrome P450 family in clopidogrel resistance. (8, 10-12)

Following several major clinical trials in the 1990s, statins have been widely adopted for all patients with symptomatic arterial disease. (13) Not only have they been shown to reduce LDL, (14) they have a plaque stabilising effect and may even lead to plaque regression. (15) Of the several beneficial effects of statins, their anti-inflammatory and anti-oxidant effects and potential to restore endothelial function are thought to be the most important. (16-20) It is well documented that inflammatory activity relates to the vulnerability of plaques to rupture; potentially this anti-inflammatory effect was thought to be an important benefit of Aspirin in the Physicians Health Study. (21) Nevertheless, the main benefit of platelet inhibition is almost certainly the prevention of thrombus formation on vulnerable atherosclerotic plaques. Following plaque rupture, collagen and vWF are exposed
causing platelet adherence and subsequent thrombus formation which then leads to MI or stroke due to arterial occlusion or distal thromboembolism.

The role of platelet-inhibitory therapy in CV disease has been confirmed by several major clinical trials with the largest recruiting patients with symptomatic arterial disease in any territory (cerebrovascular, coronary artery and peripheral artery). The Antithrombotic Trialists Collaboration meta-analysis including 135,000 patients with symptomatic arterial disease, reported a 25% reduction in CV events in patients taking a range of platelet inhibitory drugs, with Aspirin the most widely studied.

Antiplatelet resistance can be classified as ‘laboratory’ or ‘clinical’. "Laboratory” antiplatelet resistance is usually measured as continued platelet activity despite platelet inhibitory therapy. "Clinical” antiplatelet resistance is defined as treatment failure; CV events occurring despite antiplatelet therapy, however this may be an oversimplification as strokes can be caused by other pathologies that antiplatelet therapy will not affect such as embolic phenomena secondary to atrial fibrillation. In either event, failures of compliance can lead to “resistance” and true resistance demands confirmation that the relevant drug was taken.

Since aspirin resistance was first reported in the 1980s, resistance to antiplatelet therapy has been widely reported. In post stroke patients demonstrating aspirin resistance at baseline, 40% experienced a serious vascular event at two years compared with 4.4% of responders.

As part of a major study on the importance of CPV as a cause of cerebral ischaemia, we also explored of the role of resistance to aspirin and clopidogrel in patients admitted for CEA. The aim being to ascertain if resistance is associated with cerebral emboli measured pre-operatively or symptoms of cerebral ischaemia.

8.3 Methods

**Patients.** All patients undergoing CEA over an 18 month period at the University Hospital of South Manchester were invited to participate. These patients are a subgroup of the patients recruited in chapter 6. A detailed past medical history was
taken and exclusion criteria were; non-compliance with antiplatelet therapy, atrial fibrillation, concomitant use of non-steroidal anti-inflammatories (NSAIDs) within the last three months, unable to give informed consent and a diagnosis of or receiving treatment for cancer within the previous 12 months. Patients were classified as symptomatic or asymptomatic depending on whether they had suffered symptoms of cerebral ischaemia within 6 months of surgery.

Measurement of antiplatelet resistance- Antiplatelet resistance was measured using multiplate impedance aggregometry. (33, 34) The electrical impedance generated by platelet aggregation following the administration of a known platelet agonist was measured to evaluate residual platelet function. (35) Multiplate analysers are also widely used for the measurement of intraoperative platelet function during cardiac surgery. (34, 35)

Venous blood (3ml) was taken pre-operatively by atraumatic puncture using an 18 gauge needle into a double wall Hirudin blood tube and gently inverted three times to ensure adequate mixing with the anticoagulant. These samples were transported by hand to avoid excessive shaking, stored at room temperature and analysed 90 minutes post venepuncture. 300ul of blood was pipetted into each multiplate test cell and diluted 1:1 with 0.9% saline before being incubated for three minutes. 10ul of each agonist was then added to individual cells; Arachidonic Acid, ADP and Thrombin Receptor Activating Peptide (TRAP). Aggregation was recorded in aggregation units (Au) over 6 minutes producing an aggregation curve plotted against time. Patients were classed as resistant to Aspirin or Clopidogrel if the area under the curve was greater than 40Au or 47Au respectively. (36, 37)

Transcranial Doppler. Pre-operative transcranial Doppler (TCD) insonation of the ipsilateral MCA using a 2-MHz pulsed-wave Doppler probe (Acuson Multiprobe JH-6007 TCD) was used to count microemboli over one hour in a subgroup of 33 patients less than 24 hours before CEA. Microembolic signals, defined as transient, unidirectional signals occurring within the Doppler spectrum at least 3 dB higher than the background blood flow and lasting <300msec, were counted by two trained and blinded observers using the 1995 International Consensus Criteria. (38)
**Carotid Plaque Volume.** The volume of the endarterectomised plaque was measured immediately following carotid surgery using a validated water suspension technique, which has been shown to be accurate and reproducible. (39) The reliability of this method was also confirmed in our major study reporting the association between CPV and recent symptoms of cerebral ischaemia. (40)

**Statistics.** Bivariate associations between antiplatelet resistance and gender, diabetes, hypertension, hyperchoesterolaemia, rheumatoid arthritis, ischaemic heart disease, previous myocardial infarct and smoking status were assessed with the use of Chi-Squared and Fishers Exact test for categorical variables and Independent t-tests for continuous variables with a significance level of <0.05. To assess the relationship between continuous variables, Pearson Correlation Coefficient and Spearman’s rho was used for parametric and non-parametric data respectively. Histograms and normality plots were used to assess normality. All statistical analysis was performed using SPSS version 22.
8.4 Results

Of the 133 recruited to this study, 102 (76%) had suffered recent symptoms of cerebral ischaemia and 31 were asymptomatic. Thirty three of the 133 also underwent TCD insonation of the ipsilateral MCA to count cerebral emboli over one hour.

As our clinical policy was to stop dual platelet inhibitory therapy, 100 patients were taking Aspirin (75%) and 33 Clopidogrel (25%). The mean age (range) was 69 (47-85) with 94 men (91%). Resistance to platelet inhibitory therapy was found in 21 (21%) of the 100 patients taking aspirin and 14 (42%) of the 33 taking clopidogrel; an overall frequency of resistance of 35 (26.3%) in the 133 patients.

Mean (sd) residual platelet activity measured as aggregation units (Au) was significantly higher in 102 symptomatic patients at 41.9(32) Au compared with 30.8(16) Au in the 31 asymptomatic patients (p=0.012) (Figure 8-1). There was no significant difference in residual platelet function depending on the symptom of cerebral ischaemia with TIA, stroke, or amarousis fugax being associated with mean (sd) aggregation of 42.8(32.9)Au for the 53 TIA patients, 42.1(31.6)Au for the 35 stroke patients and 37.7(31.2)Au for the 13 amarousis fugax patients (p=0.870).
Of the 33 patients undergoing preoperative TCD to detect cerebral emboli, the 13 patients with antiplatelet resistance had significantly more frequent cerebral emboli of 5.19±2.93/hr compared with just 1.93±1.99/hr in the responders (p=0.002). There was a positive correlation between the frequency of cerebral emboli and residual platelet aggregation (r=0.450, p=0.009). (Figure 8-2)

Hypertension, but none of the other CV risk factors, was significantly associated with antiplatelet resistance (p=0.018). (Table 8-1) Despite this association between the diagnosis of hypertension and antiplatelet resistance, there was no correlation between the pre-operative systolic blood pressure and residual platelet aggregation. (r=-0.131, p=0.180) There was also no correlation between residual platelet aggregation and patient’s age, BMI or pre-operative blood markers.
<table>
<thead>
<tr>
<th></th>
<th>Resistant (n=35)</th>
<th>Responder (n=98)</th>
<th>p-value</th>
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<tr>
<td>Gender M:F</td>
<td>23:12</td>
<td>71:27</td>
<td>0.452*</td>
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<td>6 (17)</td>
<td>8 (8)</td>
<td>0.848*</td>
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<tr>
<td>Hypertension</td>
<td>27 (77)</td>
<td>43 (44)</td>
<td>0.018*</td>
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<td>26 (74)</td>
<td>48 (49)</td>
<td>0.140*</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>2 (6)</td>
<td>3 (3)</td>
<td>0.648†</td>
</tr>
<tr>
<td>IHD</td>
<td>11 (31)</td>
<td>11 (11)</td>
<td>0.248*</td>
</tr>
<tr>
<td>Previous MI</td>
<td>6 (17)</td>
<td>7 (7)</td>
<td>0.375†</td>
</tr>
<tr>
<td>Current smoker</td>
<td>10 (29)</td>
<td>14 (14)</td>
<td>0.773*</td>
</tr>
</tbody>
</table>

Table 8-1 Characteristics of patients undergoing impedance aggregometry testing. Values in parentheses are percentages unless otherwise stated. *Chi-square, † Fishers Exact

Mean (sd) CPV in patients resistant to antiplatelet therapy tended to be greater at 1.03(0.47) cm$^3$ compared with 0.95(0.46) cm$^3$ in responders but this small difference did not approach statistical significance (p=0.371). Nor did CPV correlate with residual platelet aggregation ($r=0.072$, P=0.438).
Figure 8-2 Scatterplot demonstrating correlation between residual platelet aggregation and number of cerebral emboli. ($p=0.009$)
8.5 Discussion

We found a clear link between resistance to platelet inhibition, the number of cerebral emboli detected by TCD before CEA and recent symptoms of cerebral ischaemia in patients with CAD. The association between resistance to antiplatelet therapy and microembolic signals in the middle cerebral artery has been reported previously.\(^{(41)}\) Microembolic signals on TCD of the ipsilateral middle cerebral artery have been reported in 40% of patients with symptomatic carotid disease and their detection is known to be associated with stroke risk.\(^{(42,43)}\)

The association between antiplatelet assistance and hypertension has also been reported previously suggesting platelet reactivity caused by increased arterial stiffness, shear stress and endothelial dysfunction in hypertensive patients.\(^{(44-47)}\) Nifedipine, verapamil and diltiazem have all been reported to have a platelet inhibitory effect, but the number of patients on each antihypertensive medication in our study were too small to explore this possibility. We could not confirm a previously reported association between resistance to antiplatelet therapy and systolic blood pressure.\(^{(45)}\)

The finding of an overall prevalence of antiplatelet resistance of 26.3% is in line with published literature, although the published prevalence varies widely at between 5.5%-61% for aspirin and 4-30% for clopidogrel.\(^{(8,48)}\) This may be due to the number of different platelet function assays being used. Harrison et al explored antiplatelet resistance in TIA and ischaemic stroke patients using three methods and reported aspirin resistance in 17% of patients using the Verify Now-Aspirin Assay, 22% using the PFA-100 assay and 5% using optical aggregometry.\(^{(35,49)}\) Multiplate impedance aggregometry has been reported to produce some of the most robust and consistently reproducible data.\(^{(33-35)}\)

Clinical drug resistance can be due to poor patient compliance, inadequate dosage, drug interactions or increased platelet turnover.\(^{(8,12,50)}\) Non-compliance is thought to be frequent and possibly the most frequent cause of recurrent CV events; up to 40% of patients with CV disease do not comply with their aspirin therapy.\(^{(8)}\)
Patients who admitted non-compliance to their antiplatelet therapy were excluded from this study.

The main limitation to this study was the small proportion of our patients undergoing TCD studies for cerebral emboli. This was due to the length of time required for TCD to be performed and a change in unit policy to perform CEAs first on the list. We were also reliant on the patient being accurate regards their compliance to medication. Also, as was discussed in the introduction, the wide range of reported antiplatelet resistance is secondary to the number of assays available to test platelet function. While impedance aggregometry is thought to be the most robust method, it is still not wholly reproducible. Hence, given the poor reproducibility, it cannot be fully implemented into clinical practice and maybe genetic testing to detect cellular defects should be sought. Our study would also have been strengthened if we had taken fasting blood levels to explore whether resistance to platelet inhibitory therapy was associated with fasting lipids or HbA1C.

This study shows for the first time that resistance to platelet inhibitory therapy is associated with recent symptoms of cerebral ischaemia in patients with CAD. It also confirms previous studies i) showing that cerebral emboli are more frequent in patients with antiplatelet resistance and ii) reporting that platelet inhibitory therapy inhibits the number of cerebral emboli in patients with carotid disease.\(^{51}\) As microemboli are recognised to be risk factors for both TIA and future stroke, these results tend to confirm that resistance to platelet inhibitory therapy may be a risk factor for stroke in patients with carotid disease. These results have important implications to the over seven million people with symptomatic atherosclerotic disease in the UK alone raising the possibility that around two million of these are taking medication that may not be fully effective. These studies emphasise the need to prioritise research on whether all patients with CV disease should be tested for resistance to the antiplatelet therapy being prescribed.
8.6 References


SECTION 4: OVERALL DISCUSSION
9.1 Overall Discussion

It is clear from the evidence in the literature and this thesis that severity of stenosis is not the best predictor of stroke risk in patients with asymptomatic CAD and stenosis alone is not sufficient to justify carotid intervention in such patients. Best medical therapy suffices for the majority of patients given the reported annual risk of stroke being near 1%.\(^{(17, 69, 191-196)}\) However, some of these patients have plaques that will become high risk and ultimately unstable, causing symptoms of cerebral ischaemia. It is because of the huge health impact a stroke has on a person’s life that surgeons generally feel nervous not offering intervention for patients with a more severe stenosis i.e. over 80%, as evidenced in chapter 7 where 72% of the patients undergoing CEA for asymptomatic CAD had a stenosis greater than 80%. Based on this and the fact that CEA confers a 2-3% peri-operative risk of stroke, it is important that a marker of impending plaque instability is found that can be easily measured. Therefore patients who would benefit most from carotid intervention can be identified.

The results presented in chapter 5 demonstrate the insufficiency of using the degree of carotid stenosis in determining who should undergo intervention and when. Only 15% demonstrated progression from a moderate to a severe stenosis and disease progression was not associated with symptom development. The fact out of the 19 who underwent a prophylactic CEA, only 7 had evidence of progression from a moderate to severe stenosis points to the uncertain nature of the decision making surrounding asymptomatic carotid disease and highlights the need for a better discriminator. 4 of the 19 had a moderate stenosis and 8 had a severe stenosis that was identified on entry to the surveillance programme. If patients with a severe stenosis are to be operated on then why wait? Patient factors need to be taken into account as well as morphological features noted on duplex that were not explored in this study due to its retrospective nature. This also prevented discussion with the surgeon about what factors were involved in their decision to operate. However, whilst the economic impact of such a programme
was outside the scope of this research it could be inferred that the cost to benefit ratio would be high given that none of the patients who developed symptoms had any evidence of disease progression during surveillance.

As mentioned in chapter 2.3, there is much in the literature regarding what contributes to an unstable plaque and many of these features are detectable on either CT or MR imaging. It is not feasible to perform such investigations on all patients with asymptomatic CAD due to the cost involved and the exposure of patients to radiation and nephrotoxic contrast. Therefore a simpler, quicker and non-invasive way of determining high risk plaques is needed.

This makes our most important finding of CPV being significantly increased in patients with recent symptoms of cerebral ischaemia more promising especially given the early results suggesting it can be reliably measured in vivo by 3D t-US. The need for a marker of a high risk plaque is clearly evident as is the inferiority of using stenosis severity as a marker of risk. Perhaps the most important finding from chapter 6 is the fact the mean CPV from patients undergoing CEA at 8 weeks post symptoms was close to that of asymptomatic patients. We know the benefit of CEA in preventing further ischaemic events is maximal in the first two weeks, with more recent studies suggesting it should take place within 48 hours,\(^\text{[209-211]}\) and that by 3 months the benefit is negligible.\(^\text{[212, 213]}\) It is then fair to assume that CPV must play a role given it regresses at a similar rate and may even suggest that the benefits may even be shorter. Given CPV correlated with the number of cerebral emboli detected by TCD, albeit weak, and the evidence to support emboli as a risk of infarction, this strengthens our belief that CPV has a role to play. CPV having no relation to the degree of stenosis also demonstrates the unreliability of stenosis severity. Further work needs to be done regarding the regression of CPV over time and should be conducted in the cohort of patients who evidently have symptomatic CAD but do not undergo carotid intervention either due to their fitness for anaesthesia or the severity of the stroke. Ideally the CPV and number of cerebral emboli should be measured in these patients over a period of time to evaluate the rate of regression and confirm/refute the relation to cerebral emboli.
The results in chapter 7 regarding the histological analysis adds further weight to our belief of CPV being an important marker given those with an unstable plaque had significantly increased mean CPVs. This paper also found significantly increased mean CPVs in males, those diagnosed with hypercholesterolaemia and those who had suffered a previous episode of cerebral ischaemia. However these results must be interpreted with caution as they did not fit with the results in chapter 6 where only a significant association with male gender was found. This paper contained a much larger number of patients and while the mean CPV for those diagnosed with hypercholesterolaemia was increased it did not reach statistical significance.

However, a diagnosis of hypercholesterolaemia must play an active role in the atherosclerotic process given the significant association between the diagnosis and unstable plaques along with significantly increased Lp-PLA2 levels. The relationship with cholesterol levels was not explored in this study as the vast majority of patients were receiving statin as part of secondary CV prevention, irrespective of their cholesterol levels. We therefore didn’t feel it would be a true representation. Patients were classed as hypercholesterolaemic if they had been commenced on statin secondary to elevated lipid levels. I have previously mentioned that a period of untreated hypercholesterolaemia may play a role in explaining the associations in this study. A number of genetic studies have shown that the level of LDL cholesterol may not be the most important factor but more the length of time to which the body is exposed to LDL. Several studies have shown that patients with genetic mutations causing decreased concentrations of LDL have a greater impact on reducing CV risk than those achieving the same LDL concentrations through statin use. They proposed this was due to statin therapy being commenced later in life hence longer exposure to increased LDL.\(^{(214-219)}\) This has also been shown through epidemiological studies finding that earlier lowering and prolongation of LDL concentrations yield better results on reducing CV risk.\(^{(220, 221)}\) This fits in with our hypothesis of how a diagnosis of hypercholesterolaemia may be related. Other studies have suggested that consideration must be given to the number of circulating LDL particles in patients with normal LDL levels as a large number of small LDL particles confer greater risk.\(^{(222, 223)}\) This could be due to the increased
amount of free cholesterol that is subsequently transported and the increased susceptibility of the small LDL particles to oxidisation.\textsuperscript{(224, 225)}

These above theories may go some way to explain why cholesterol levels per se have never been directly linked to stroke risk.\textsuperscript{(226)} While it is acknowledged that there has been a reduction in the number of CV events since statin therapy became prevalent, lifestyle factors have also contributed a significant part.\textsuperscript{(227)}

Lp-PLA\textsubscript{2} plays a role in atherosclerosis from the evidence presented in the literature and the finding of Lp-PLA\textsubscript{2} being significantly increased in those plaques with marked inflammation. The fact those with a diagnosis of hypercholesterolaemia also had significantly raised mean values points to the role it must have in initiating and maintaining atherosclerosis. It may be that this period of untreated hypercholesterolaemia is enough to stimulate the start of the atherosclerotic process and then the inflammatory reaction/pathway takes over of which Lp-PLA\textsubscript{2} seems to play an integral part. It is evident that inflammation has role in atherosclerosis and it is this perpetuation and magnification of the inflammatory process at the time of plaque fissuring that causes the fibrous cap to rupture leading to thrombus formation. It would seem logical that plaque fissuring is caused by the thinning of the cap secondary to the decreasing amounts of collagen due to the proteolytic properties of the inflammatory mediators released. Is it this sudden increase in inflammatory activity and subsequent IPH that contributes to an increased CPV secondary to the increasing amounts of IPH, necrotic material and free lipids? This may explain how CPV is significantly increased in plaques classed as unstable. It may also explain why CPV was found to be significantly increased in males. It is known that the incidence of CV events and stroke is increased in males before the age of 75 \textsuperscript{(228, 229)} with the protective effects of pre-menopausal hormones on atherosclerosis assumed to be the reasoning.\textsuperscript{(229, 230)} Recent studies have demonstrated, both histologically and on MRI, that carotid plaques removed from males have a greater prevalence of IPH.\textsuperscript{(231, 232)} Given that IPH is a recognised marker of plaque instability could CPV be a surrogate marker of IPH? Figure 9-1 demonstrates the histological features of an unstable, inflamed plaque compared
to the features of a stable, mildly inflamed plaque in figure 9-2, with the main
difference being the intact fibrous cap and amount of IPH.
Figure 9-1 Histological sections of an unstable, inflamed carotid plaque
Top left represents the whole plaque stained with H&E. (A) represents IPH with (B) fibrous cap rupture. (C) is a magnified view demonstrating the typical appearances of foam cells. (D) is a section stained with CD 68 demonstrating an abundance of macrophages (brown dots) and (E) is a section stained with CD 3 demonstrating T-Cells (blue dots)
Figure 9-2 Histological sections of a stable, mildly inflamed plaque

Top left demonstrates the whole plaque stained with H&E. A magnified view demonstrates a thick, intact fibrous cap (A) and the typical appearances of a lipid rich necrotic core (B). (C) demonstrates few macrophages (brown dots) within the actual plaque but more contained to the fibrous cap and (D) demonstrates very few T-Cells (blue dots) as stained by CD 3.

When comparing sections from the plaques above (figures 9-1 and 9-2) it is evident how much more activity is taking place within the unstable plaque compared to the stable plaque. Therefore it would seem logical that CPV could be related to the amount of IPH present.
In terms of identifying a biomarker for symptomatic CAD I believe this would be very difficult given the atherosclerotic process is the same throughout the arterial tree and it would be fair to assume that atherosclerotic CAD is unlikely to occur in isolation. The only slight difference regards CAD is in terms of the initiation of the atherosclerotic process with the turbulent nature of the blood flow at the bifurcation playing a role. The same processes that cause a femoral artery or coronary artery plaque to rupture and thrombose will be the same as in CAD and hence while we may find biomarkers that generally reflect the level of inflammatory activity and atherosclerotic process it will be very difficult to pinpoint exactly where in the arterial tree this is. Inflammatory biomarkers would more likely be of benefit in the quiescent phase of atherosclerosis in terms of monitoring the level of atherosclerotic activity and its progression hence being able to titrate treatment. It is evident that administering statin therapy and measuring cholesterol levels alone is not sufficient as a proportion of patients will have a CV event while on medical therapy and cholesterol levels have never been specifically linked to the risk of stroke. Perhaps we should move away from using cholesterol levels and age as a guide to starting medical therapy in primary prevention and look to identify biomarkers that reflect the level of atherosclerotic activity, such as Lp-PLA₂, so that more aggressive treatment measures can be employed for those with increased activity. It could be argued, given the evidence regarding the length of time of exposure to LDL, that LDL lowering therapy should be commenced earlier in life.

In terms of best medical therapy for CAD it is evident from chapter 8 that platelet aggregation is related to embolic phenomena and this is of great importance given the prominent role of antiplatelet therapy in secondary CV prevention. Whether this increased aggregation is due to true resistance at the cellular level or other factors need to be addressed. However, regardless of what the cause is, the fact increased aggregation is associated with emboli, which we know are markers of increased stroke risk, and the relatively high prevalence of antiplatelet resistance means a number of patients are not receiving the perceived benefits of antiplatelet therapy. It is likely the majority can be explained by patient non-compliance despite patients claiming to take all their medications. Further work needs to be done on this subject matter to ascertain the following;
1) Is it a true resistance at the cellular level due to genetic factors? And if genetic, is there any potential for genetic testing to tailor antiplatelet therapy for the individual’s need.

2) Do patients have varying tolerance to the dose i.e. does a patient demonstrating resistance at 75mg also demonstrate the same at 150mg?

3) Does resistance become more prevalent the longer the patient is taking the medication?

4) What is the impact of medication interaction?

All these factors need to be addressed in a large multicentre longitudinal study before any recommendations can be made. However, if the prevalence reported in the literature is a true reflection, around 20%, then routine antiplatelet testing could be argued for those patients suffering a recurrent CV event while on antiplatelet therapy. If it transpires it is largely related to non-compliance this is also of equal importance and re-educating the patient on the importance of such therapy needs to be stressed at primary care level.

9.2 Limitations to the Study

While the numbers of patients recruited to the study were relatively large the numbers that underwent 3D t-US, TCD, plasma sampling, and histological analysis were relatively small. This is in part due to only being able to perform TCD at one site and to ensure uniformity in sample collection only patients recruited from the UIHSM site had their plasma and plaque analysed. There has also been a change in clinical practice with the recognition that the sooner CEA takes place the greater the benefit. This led to more CEAs being performed out of hours hence while the plaque was stored in the theatre fridge it could only be used for calculation of CPV as the degradation process would have affected the histological analysis. Also, patients tended to be admitted on the day of surgery with CEA performed first on the list whenever possible meaning little time for TCD, blood sampling and 3D t-US.
9.3 Future Work

Future work that could stem from this research can be split into two arms – clinically based studies focusing on how to incorporate CPV into clinical practice and basic science related to focusing on the suggestions in the discussion regarding what contributes to an increased CPV and the implications of the TGS discovered.

Clinical Studies

The accuracy and validation of 3D t-US measurements of CPV is currently underway in a larger cohort of patients with the additional use of Sonovue contrast to ascertain if this has any bearing on accuracy having secured a Horizon 20:20 grant. Computer algorithms to automatically generate the CPV are also being studied. Ethics has been granted for a study evaluating the rate of regression of CPV in those patients with symptomatic CAD who do not undergo a CEA. The CPV will be measured over a 12 week period to test our hypothesis that CPV is dynamic and decreases with time from symptom at the same rate as the benefit from CEA for symptomatic disease does.

The relationship of fibrous cap thickness to CPV also needs to be explored as it is evident from the literature that the thinning of the cap is a precursor to impending rupture. Thus the question to be asked is does cap thickness decrease as CPV increases?

If 3D t-US is shown to be able to measure CPV accurately then a 5 year multi-centre cohort study measuring CPV in asymptomatic patients over a minimum of two years is needed to ascertain whether CPV can be used as a screening tool. To detect a doubling of stroke risk from 2% to 4% in patients with asymptomatic CAD and progression of CPV, power calculations reveal 3700 patients will need to be recruited.

Basic Science

At a cellular level the cause of an increased CPV needs to be addressed. If this finding can be replicated and further understood then alternative treatment
strategies could be sought that target alternative pathways contributing to atherosclerosis.

9.4 Final Conclusion

Based on the results, stenosis is not the best indicator for carotid intervention in patients with asymptomatic CAD and the use of CT or MR imaging to identify high risk plaques is both too costly and carries risk to the patient. I believe CPV has the potential to solve this problem and be used as a predictor of risk in patients with asymptomatic CAD. The early results from 3D t-US are encouraging and if the accuracy is proven with ongoing studies then using CPV as an indicator for carotid intervention in asymptomatic patients become realistic. That CPV is not only increased in patients with recent symptoms of cerebral ischaemia and decreases at a rate akin to that of the beneficial effects of CEA for symptomatic disease but the fact it is also related to unstable plaques graded histologically adds weight to the notion that CPV, hence plaque burden, is potentially more important than stenosis. The identification of a biomarker specific to high risk carotid plaques is unlikely to be achievable given the systemic, widespread nature of atherosclerosis but there may be biomarkers that could be used to assess the response of patients to best medical therapy. The testing of antiplatelet resistance in patients with CAD should be considered, especially those that are high risk and have suffered a previous CV event.
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