The use of microparticles and inflammatory cytokines as potential biomarkers for plaque instability in patients with carotid disease

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CONTENTS

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Abbreviations

ACAS	Asymptomatic Carotid Atherosclerosis Trial
ACST	Asymptomatic Atherosclerosis Trial
AHA	American Heart Association
BSA	Bovine Serum Albumin
CCA	Common carotid artery
CCL2	Chemokine ligand -2
CD	Cluster of differentiation
CEA	Carotid endarterectomy
c-MET (HGFR)	Receptor tyrosine kinase(Hepatocyte Growth Factor receptor)
CTA	Computed tomographic angiography
CTACK	Cutaneous T-Cell Attracting Chemokine
CXCR	Chemokine receptor
DAB	Diaminobenzidine solution
EC	Endothelial cell
ECA	External carotid artery
eGFT	Glomerular Filtration Rate
ELISA	Enzyme linked Immunoassay
EMP	Endothelial microparticle
eNOS	Endothelial Nitric oxide Synthase
GM-CSF	Ganulocyte- Macrophage Colony stimulating Factor
GRO-α	Growth regulated oncogene alpha
H&E	Haematoxylin and Eosin
HGF	Human Growth Factor
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells

ICA	Internal carotid artery
ICAM	Intercellular cell adhesion molecule
IL	Interleukin
INF-Y	Interferon Gamma
IPH	Intra-Plaque Haemorrhage
LDL	Low density lipoprotein
LIF	Leukocyte Inhibitory Factor
LIF	Leucocyte Inhibitory Factor
MCP	Monocyte chemoattractant protein
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage Colony stimulating Factor
M-CSF	Monocyte Colony Stimulating Factor
MIF (CXCL9)	Macrophage migration inhibitory factor
MIG	Monokine induced gamma interferon
MMP	Metalloproteinase
MP	Microparticle
NASET	North American Symptomatic Endarterectomy Trial
NGF	Nerve Growth Factor
NHS	National Health Service
NO	Nitric oxide
OC	Osteocalcin
OPG	Osteoprotegrin
OPN	Osteopontin
OxHb	Oxidised Haemoglobin
OxLDL	Oxidised Low Density Lipoprotein
PBS	Phosphate-Buffered Saline
PC	Phosphatidylcholine

PDGF	Platelet Derived Growth Factor
PE	Phosphatidylethanolamine
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule
PS	Phosphatidylethanolamine
PSV	Peak systolic velocity
PTFE	Polytetrafluoroethylene
PTX3	Pentraxin-related protein
RANKL	Nuclear Factor Κβ Ligand
ROCK	Rho-associated kinase
SCF	Stem Cell Factor
SCGF-β	Stem cell growth factor beta
SDF1	Stromal Cell-derived Factor
SM	Sphingomyelin
SMC	Smooth muscle cell
SOST	Sclerositin
TF	Tissue factor
Th	T-helper
TIA	Transient Ischaemic Attack
TNF-α	Tumour necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
VCAM	Vascular cell adhesion molecule
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand Factor

Abstract

Aim: Endothelial microparticles (EMPs) are released from dysfunctional endothelial cells. We hypothesised that patients with unstable carotid plaque have higher levels of circulating microparticles compared to patients with stable plaques, and this may correlate with serum markers of plaque instability and inflammation.

Method: Circulating EMPs and inflammatory markers were measured in twenty healthy controls and seventy patients undergoing carotid endarterectomy. EMPs were quantified using flow cytometry. Bioplex assays profiled systemic inflammatory and bone-related proteins. Immunohistological analysis detailed the contribution of differentially-regulated systemic markers to plaque pathology. Alizarin red staining showed calcification.

Results: EMPs were significantly higher in patients with carotid stenosis (≥70%) compared to controls, with no differences between asymptomatic *vs* symptomatic patients. Asymptomatic patients with unstable plaques exhibited higher levels of EMPs compared to those with stable plaques, with a similar trend observed in symptomatic patients. CXCL9 and SCGF-β were significantly elevated in asymptomatic patients with unstable plaques, with IL-16 and macrophage inhibitory factor significantly elevated in the stable plaque group. CXCL9, CTACK and SCGF-β were detected within all plaques, suggesting a contribution to both localised and systemic inflammation. Osteopontin and osteoprotegerin were significantly elevated in the symptomatic *vs* asymptomatic group, while osteocalcin was higher in asymptomatic patients with stable plaque. All plaques exhibited calcification, which was significantly greater in asymptomatic patients. This may impact on plaque stability.

Conclusions: Circulatory EMP, CXCL9 and SCGF-β levels are raised in asymptomatic patients with unstable plaques, which could be important in identifying patients at most benefit from intervention.

Declaration

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Publications

1. **Endothelial microparticles as conveyors of information in atherosclerotic disease.** Schiro, A., Wilkinson, F. L., Weston, R., Smyth, J. V., Serracino-Inglott, F. &Alexander, M. Y. (2014). *Atherosclerosis* **234,** 295-302. See Appendix 6

2. Elevated levels of endothelial derived microparticles and serum CXCL9 and SCGF-β are associated with unstable asymptomatic carotid plaques. Submitted to Atherosclerosis, Thrombosis and Vascular Biology (ATVB), pending review June 2015. See Appendix 7

Presentations

1. Vascular Society of Great Britain and Ireland (VSGBI)-Nov 2013- Manchester
Could microparticles act as potential biomarkers of plaque instability in patients with carotid disease? (Eposter presentation)

2. British Society for Cardiovascular research (BSCR) - May 2014- Manchester
The role of Microparticles in Carotid disease (Poster presentation)

3. Audit meeting, Vascular Unit Manchester Royal Infirmary- Oct 2014- Manchester
Markers of Atherosclerotic plaque activity: Have we discovered anything new? (Oral presentation)

4. European Society for Vascular Surgery (ESVS) - May 2015- Frankfurt
Elevated EMP's in asymptomatic patients: Markers of unstable plaque? (Poster presentation)

5. M60 Vascular group meeting- June 2015- Manchester
Could blood tests predict atherosclerotic plaque instability? (Oral presentation)

CHAPTER 1

Clinical importance of carotid disease

1.0 Clinical importance of carotid disease

More than one million people are living with the consequences of stroke in the UK. (BHF, 2010) Every year about 110,000 patients have a stroke which accounts to the third largest cause of death in Britain. More than half of these patients become dependent on their family and society. (Stineman *et al.*, 1997) In the UK, stroke is estimated to cost the economy around £7 billion per year.(BHF, 2010)

In 2010, the Interstroke study was published. This study showed that 90% of strokes are attributable to ten main risk factors: hypertension, smoking, abdominal obesity, poor diet, lack of physical activity, diabetes, alcohol, stress, cardiovascular disease and hypercholesterolemia.(O'Donnell *et al.*, 2010) Primary prevention of atherosclerosis is aimed at reducing stroke in asymptomatic individuals by reducing risk factors. In developed countries, better patient care has led to a substantial decrease in mortality of patients following stroke.(Carandang *et al.*, 2006)

1.1 Types of stroke

Ischaemic brain injuries together with intracerebral haemorrhage are the two major mechanisms that cause a stroke. Around 80% of all strokes are ischaemic and result from thrombotic and embolic events within diseased arteries supplying the brain resulting in a diminished supply of blood to the brain tissue that leads to a stroke. (Rosamond *et al.*, 2008)

Atherosclerosis is a pathological process whereby lipid, calcium and cellular debris are deposited within the intima of the artery wall. With time, atherosclerotic plaques increase in size and encroach into the vessel lumen. Susceptible atherosclerotic plaques may ulcerate or rupture, exposing thrombogenic atherosclerotic plaque contents to the blood stream. During this process, platelets ad-

here to the damaged endothelium initiating vessel repair but as a consequence they initiate a coagulation response resulting in thrombus formation. Apart from restricting blood flow to the brain, thrombus within the carotid artery may embolise to the brain resulting in a stroke.(Fisher *et al.*, 2005)

The remaining 20% of all strokes are caused by non-traumatic intracerebral haemorrhage. Intracerebral haemorrhage results from a leak or rupture of a blood vessel within the brain, commonly caused by hypertension, arteriovenous malformations or intracerebral aneurysms. Blood collects within the brain forming a haematoma resulting in a pressure effect on the surrounding brain tissue causing ischaemia and eventually results in a stroke. (Keep *et al.*, 2012) This thesis will focus on ischaemic strokes.

1.2 Stroke risk in symptomatic and asymptomatic carotid disease

The carotid arteries are the two main vessels supplying the brain. Carotid atherosclerosis accounts for 15-20% of all strokes. (Bamford *et al.*, 1991) Symptomatic carotid disease can manifest as a transient ischaemic attack (TIA) or a hemispheric stroke. A TIA is defined as a “clinical syndrome characterised by an acute loss of focal cerebral or monocular function, with symptoms lasting less than twenty four hours and which are thought to be due to inadequate cerebral or ocular blood supply, as a result of low blood flow, thrombosis or embolism associated with disease of the arteries, heart and blood”. (Hankey *et al.*, 1991) A hemispheric stroke is a medical emergency that occurs when blood flow to the brain acutely interrupted. It is characterised by a focal neurological defect lasting for more than twenty four hours. The effect of the resulting stroke depends on the region of the brain affected. Symptoms include paralysis, speech defect, memory loss and death. Early recognition of stroke symptoms and prompt medical intervention improves survival and limits neurological damage in stroke survivors.(Heros, 1994)

Patients, who survive a stroke, are more likely to suffer from another stroke in the first two weeks following the first event, accounting for a substantial increase in morbidity to the patient. (Albers, 2000) Studies have shown that the risk of recurrent stroke varies from 1.7% to 4% in the first 30 days. In the first year, the risk of a second stroke is 6% to 13%, 5% at 2 years and 8% at 5 years. The cumulative risk of recurrent stroke, at 5 years, is between 19% and 42%, (Rundek & Sacco, 2004) therefore improving our understanding of the mechanisms underpinning stroke and reducing the incidence of stroke is vital to reduce the burden on patients and the NHS.

Patients who have a stroke are referred to as 'symptomatic' patients i.e. those with symptomatic internal carotid disease. These patients are offered an operation called carotid endarterectomy (CEA) to prevent further strokes. (See section 1.4.1) This operation involves clearing out of carotid plaque within the carotid artery, aimed at preventing a second stroke. (Touzé, 2012) The effectiveness of CEA was confirmed by the North American Symptomatic Endarterectomy Trial (NASET) in the 1980s. This trial compared medical treatment versus CEA in symptomatic patients and it showed clear evidence in benefit of CEA. The study followed patients who were either treated medically or surgically. Over a two year period, patients in the medical arm had a stroke risk of 26% but this risk was reduced to 9% in patients with high grade stenosis (70-99%) who were operated. This study showed an absolute stroke risk reduction of 17% (Ferguson *et al.*, 1999)

The second group of patients are those patients with asymptomatic carotid disease who are diagnosed by chance. Most commonly, these patients present to the ophthalmologist with loss of vision or for a routine check-up and are referred to the vascular surgeons to rule out carotid disease as a cause of their visual symptoms. Other patients are diagnosed in the vascular or cardiac surgical

out-patients where they undergo carotid scans as part of pre-operative assessment to dismiss carotid disease prior to major surgery. Using duplex ultrasound, radiologists are able to evaluate and quantify the extent of carotid disease. The annual risk of stroke in patients with asymptomatic carotid stenosis of ≤ 75% is around 1.5% or less and 3.3% in those with a stenosis of >75%.(Mineva *et al.*, 2002; Norris *et al.*, 1991) Based on this information, assessment of the patients' health in general and life expectancy vascular surgeons are able offer surgery accordingly since the benefit of surgery on patients with asymptomatic disease is of long term rather than an immediate benefit. (Halliday *et al.*, 2010) Although ultrasound is an important tool in identifying carotid stenosis and identifying irregular friable and potentially unstable plaque, duplex alone can't predict which plaques are likely to become unstable and manifest in a stroke.

Following the introduction of carotid surgery in 1953(DeBakey, 1975), for the prevention of stroke, controversy deepened amongst surgeons and neurologists as to whether it was appropriate to offer surgery to asymptomatic patients. This controversy lasted until two randomised controlled trial results were published. The two randomised controlled trials provided evidence showing that carotid endarterectomy was beneficial for selected patients with asymptomatic internal carotid stenosis of 60 to 99 %. (Halliday *et al.*, 2004; Hobson *et al.*, 1993) The Asymptomatic Carotid AtherosclerosiS trial published in 1995, (USA) (ACAS) (1995) studied patients with a greater than 60% internal carotid artery stenosis and were randomised to surgery or best medical therapy. The results showed that, at 5 years, the risk of stroke for patients treated with best medical therapy was 11.8%, which dropped by half; 6.4% in those treated surgically. This trial concluded that, CEA for asymptomatic patients of <75 years of age, significantly reduces their risk of stroke over a 10 year period, compared to those treated with best medical therapy. The results of the second and larger study called the

Asymptomatic Carotid Atherosclerosis Trial were published in 2004(UK) (ACST). (Halliday *et al.*, 1994) This trial also demonstrated that CEA conferred a 50% relative risk reduction in a 5-year risk of stroke in patients with carotid stenosis of ≥70% from 12% to 6%. ACST showed that immediate CEA conferred a 4.6% absolute risk reduction in stroke compared to best medical treatment. This equates to 46 strokes prevented per 1000 operations over a 10 year period. Approximately 95% of CEAs were being performed unnecessarily. Statistically, these studies showed that for every 19 carotid endarterectomies performed, one stroke was prevented over a 5 year period. Clearly many patients were being treated unnecessarily and subjected to post-operative complications of surgery which include a 1-2% risk of peri-operative stroke and cranial nerve damage. (Seiden, 1995)

Currently we have no means of isolating those patients who are more likely to stroke because we cannot accurately identify patients with unstable asymptomatic plaques. The lack of diagnostic tools to identify asymptomatic patients at risk of stroke provided the incentive for this research.

1.3 AHA guidelines on the treatment of patients with asymptomatic carotid disease

Despite the results of two randomised controls studies – ACAS and ACST published 20 and 10 years ago respectively, both trials remain the cornerstones of current guidelines. The American Heart Association (AHA) updated its published guidelines in 2011 regarding the treatment of patients with asymptomatic disease. The AHA guidelines recommend that patients with asymptomatic disease should be screened for treatable risk factors and the adoption of life style changes should be implemented together with the institution of best medical therapy. They recommend CEA for patients with a reasonable life expectancy and co-morbidities who have an internal carotid artery stenosis of 70-99% pro-

vided that the procedure is performed in a centre with a procedural risk of <3%.(Goldstein *et al.*, 2011)

1.4 Carotid surgery

In 1953, DeBakey performed the first successful CEA.(DeBakey, 1975) Over the years, carotid surgery became standardised and adopted worldwide. Pre-operative imaging is critical prior to performing CEA. Standard first-line imaging includes duplex ultrasound which is operator dependant. Computed tomographic angiography (CTA) and magnetic resonance imaging (MRI) are second-line imaging investigations which are of use when ultrasound is inconclusive. The choice of the diagnostic test boils down to the clinicians' preference and the availability of the services provided within the hospital setting.

1.4.1 Carotid endarterectomy

1.4.2 Incision

CEA is performed under general or local anaesthesia. An incision is made parallel to the anterior border of the sternocleidomastoid over the carotid bifurcation and extended proximally or distally as appropriate. The following are intra-operative images taken during CEA surgery at the Manchester Royal Infirmary (Patient consent was obtained pre-operatively). See Figure1. The upper end of the incision is angulated towards the earlobe to avoid the parotid gland. The incision is deepened passing through the platysma whilst the sternocleidomastoid is retracted laterally using a self- retaining retractor.

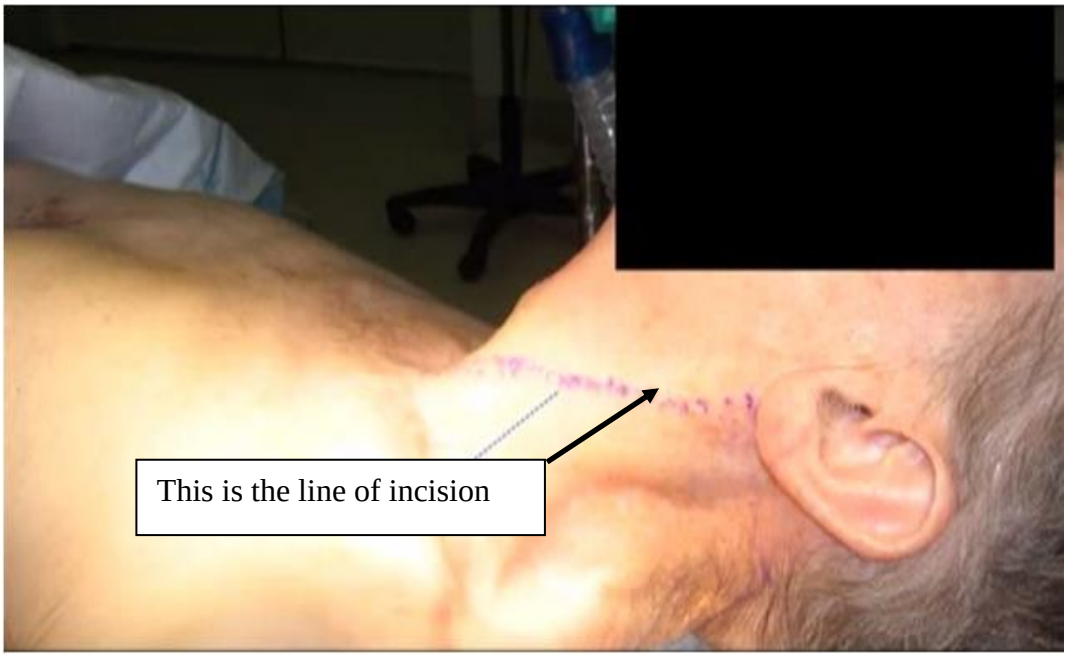

Figure 1. Incision for CEA

1.4.3 Exposure of the carotid artery

The internal jugular vein is identified and the carotid sheath is opened. The internal jugular vein is then retracted laterally whilst the facial vein is ligated to allow the carotid artery to be exposed. See Figure 2. Meticulous dissection is carried out anterior to the common carotid artery (CCA) to avoid injury to the vagus nerve as well as the glossopharyngeal, accessory and hypoglossal nerves.

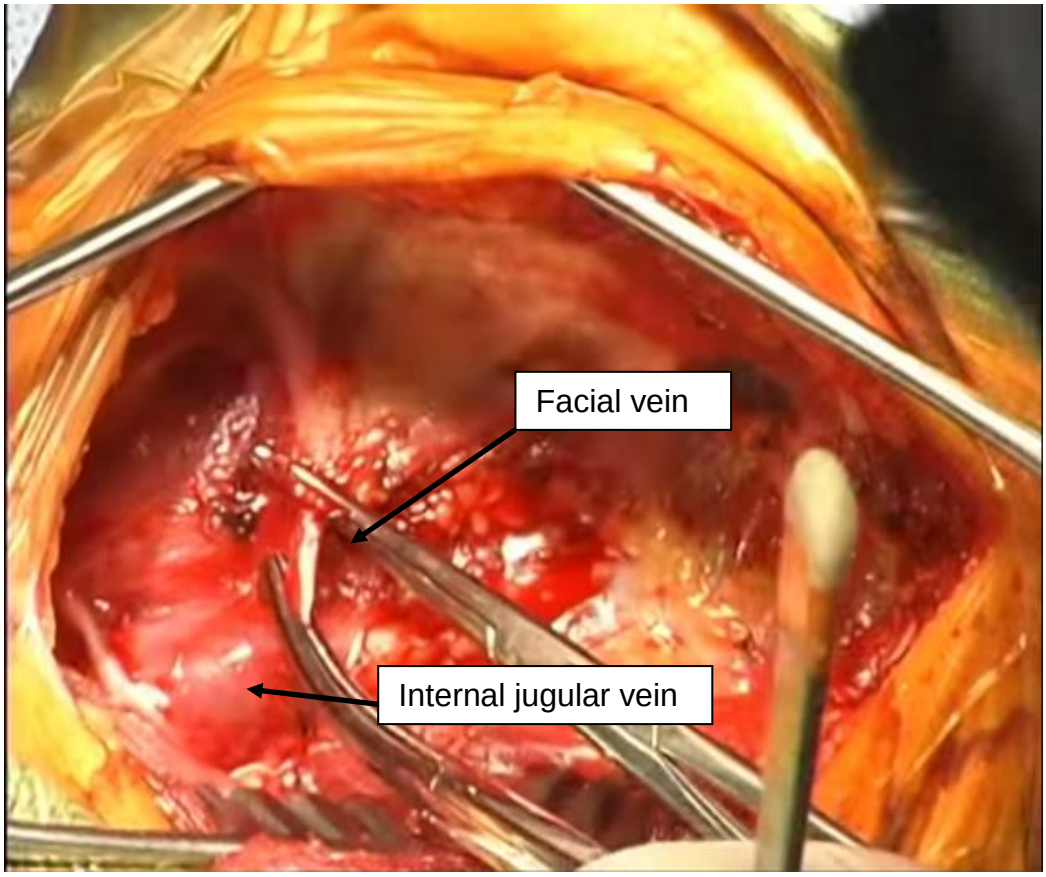

Figure 2. Ligation of the facial vein

The CCA is the first to be dissected and slung to achieve proximal control followed by the external carotid artery (ECA) and its first branch, the superior thyroid artery. The internal carotid artery (ICA) is then mobilised up to a point where it is normal taking special care not to damage the hypoglossal nerve in the process. See Figure 3.

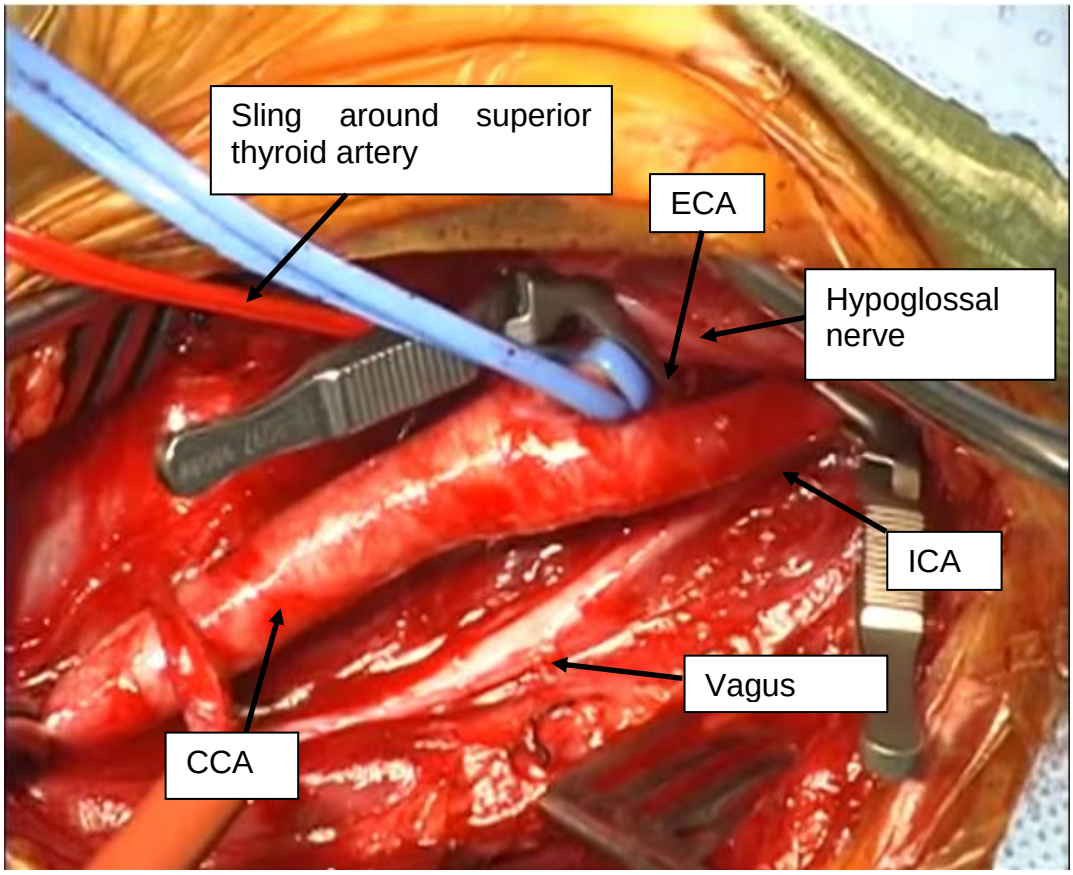

Figure 3. Exposure of the carotid bifurcation

Extreme care is taken throughout the dissection to minimise manipulation of the vessels to reduce the risk of embolisation and as well as damage to the surrounding nerves mainly the vagus and hypoglossal nerves.

1.4.4 Arteriotomy

Prior to clamping the vessels, unfractionated heparin is administered (3000-5000U). The ICA is first to be clamped followed by the CCA and ECA. An arteriotomy is made with a size 11 blade and extended into the ICA with Potts scissors. The arteriotomy is extended distal to the plaque to a point where the ICA is relatively normal. See Figure 4. When surgery is performed under general anaesthetic without cerebral monitoring or when neurological changes are noted during monitoring the ICA is shunted.

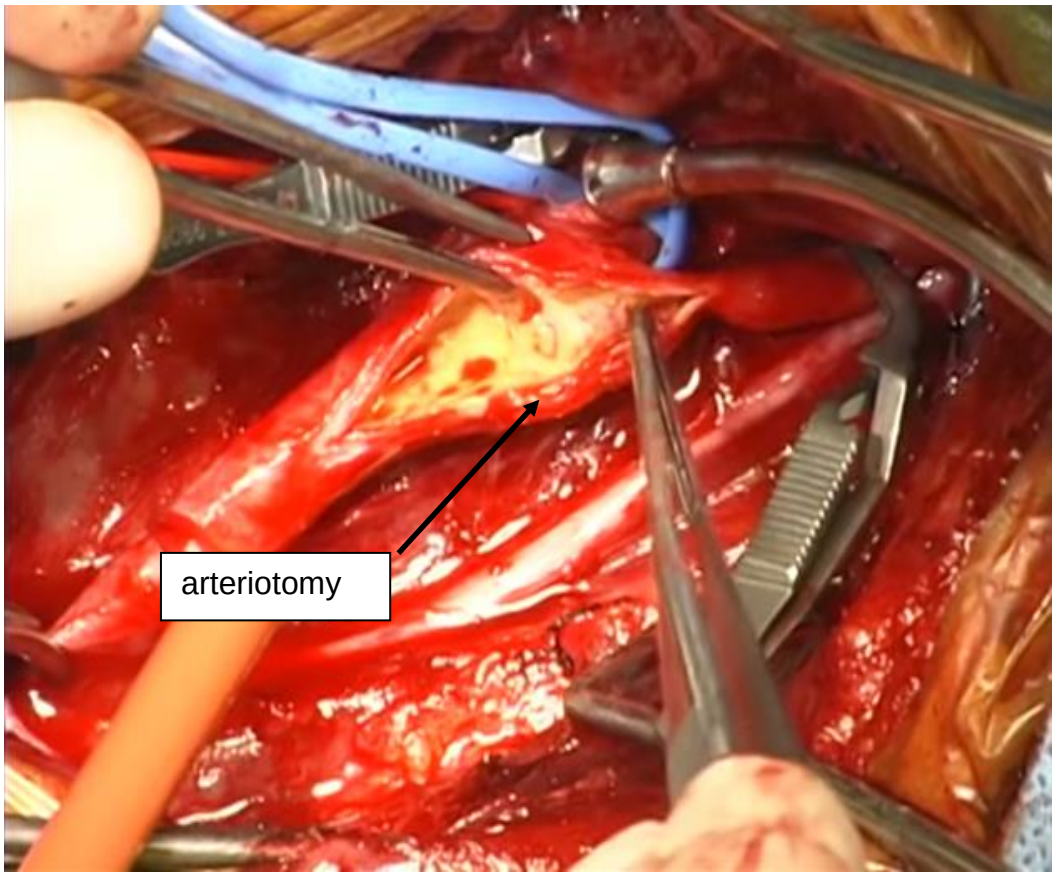

Figure 4. Arteriotomy

1.4.5 Plaque removal

Care is taken to identify the optimal endartectomy plane between the inner and outer medial layers using a Watson-cheyne dissector. At the levels of the CCA, the plaque is carefully dissected and lifted from the CCA and incised using Potts scissors. The plaque is then elevated under vision and the endarterectomy is continued into the carotid bulb and ECA. At the level of the ICA care is taken to achieve an adequate endpoint. Once the plaque is removed an eversion endarterectomy is performed on the ECA. The distal edge of the plaque in the ICA is tacked down with interrupted 7'0 non-absorbable monofilament sutures to secure the endpoint. See Figure 5.

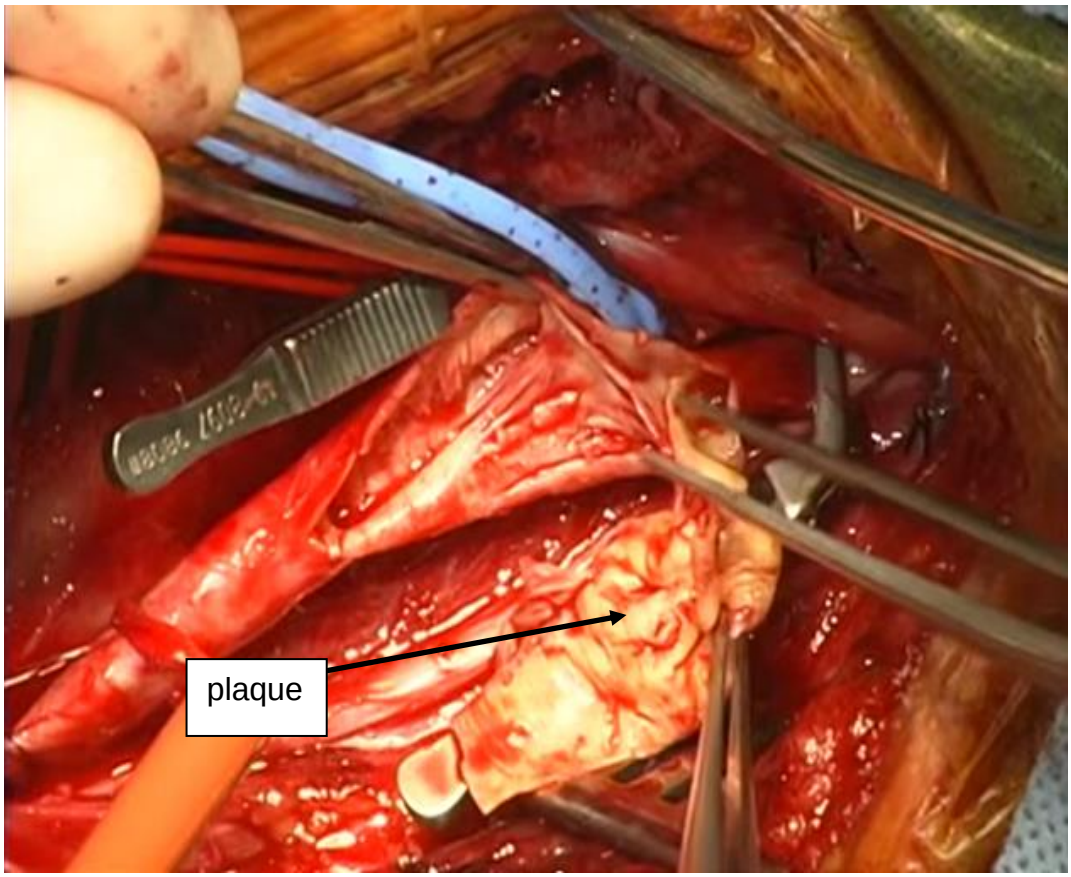

Figure 5. Endarterectomy

After the endarterectomy is complete all residual debris and loose medial fibres are excised. The endarterectomised surface is then irrigated with heparinised saline to clear of all debris.

1.4.6 Patch Closure

Patch angioplasty closure of the arteriotomy is carried out with a 6'0 non-absorbable monofilament suture; this ensures that the carotid artery is not narrowed in the process. Various types of patches can be used and these include: bovine pericardium, Dacron, polytetrafluoroethylene (PTFE) and autologous saphenous vein. Before closure of the arteriotomy the ICA, ECA and CCA are back bled and flushed with heparinised saline.

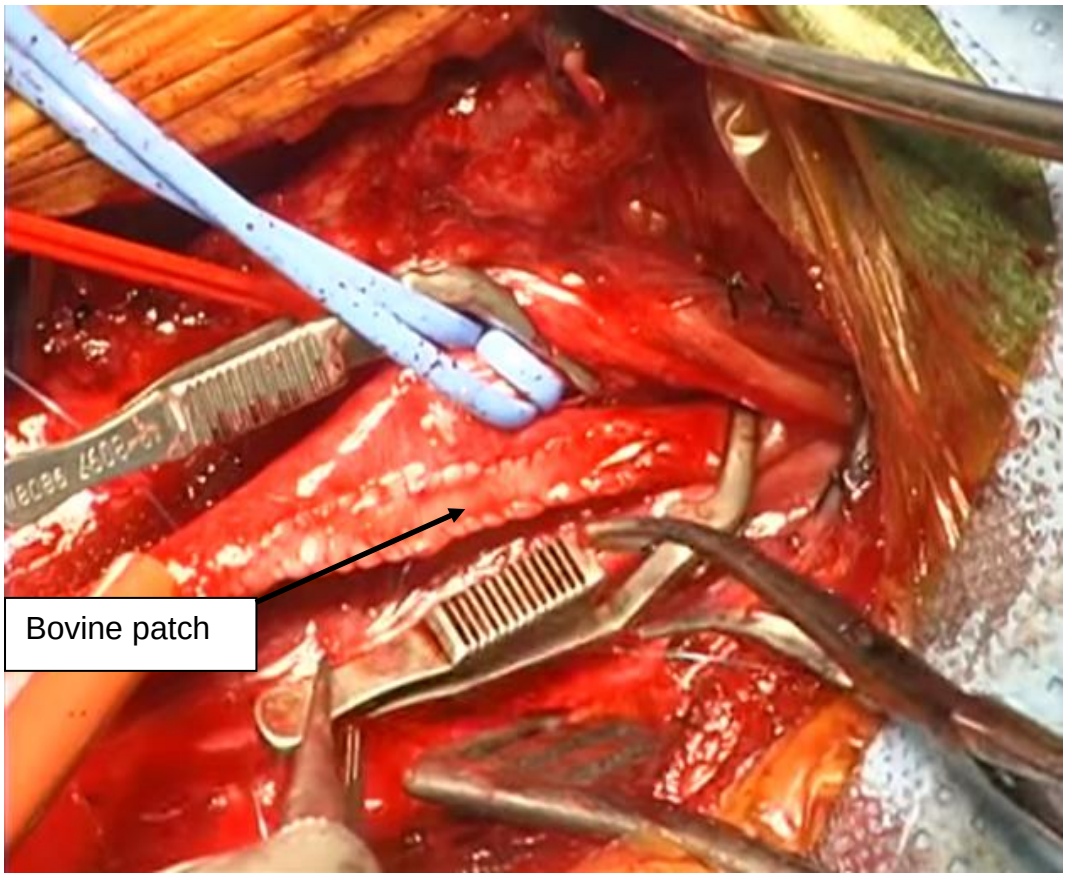

Figure 6. Patch angioplasty with bovine pericardium

The wound is subsequently closed in layers and a vacuum drain is left in the wound for 24 hours. Postoperatively the patients are fully awakened. In recovery the patients' neurological status, blood pressure and wound are observed continuously.

1.5 Endothelial microparticles and their significance in atherosclerosis

Over the years, extensive research has been carried out on carotid plaque morphology with a view to identifying vulnerable plaques. Researchers have studied the complex composition of carotid plaques and correlated their constituents and the presence of inflammatory molecules such as cytokines to plaque stability (Tedgui & Mallat, 2006). Recent studies have taken interest in the role of microparticles (MPs) in this process and their potential value as novel biomarkers of cardiovascular disease. (Leroyer *et al.*, 2007)·(Chalela, 2009)

Microparticles (MP) are anucleoid submicron sized fragments (50 nm-1µm diameter) of plasma membranes. They are made up of oxidised phospholipids and specific proteins that represent the cells from which they originate. MPs are released from virtually all eukaryotic cells (platelets, erythrocytes and leucocytes) following stimulation or apoptosis (cell death). (VanWijk *et al.*, 2003)

MPs, are released from budding of plasma membranes following an increase in intracellular calcium which disrupts the lipid membrane asymmetry and cytoskeleton proteins.(Zwaal & Schroit, 1997) Plasma membrane remodelling, results from an imbalance between the cytoplasmic and exoplasmic aminophospholipids. Cytoplasmic aminophospholipids include phosphatidylserine (PS) and phosphatidylethanolamine (PE) whilst phosphatidylcholine (PC) and sphingomyelin (SM) constitute the outer part of the plasma membrane. Following cell stimulation, PS translocates into the exoplasmic membrane and results in MP release. See Figure 7

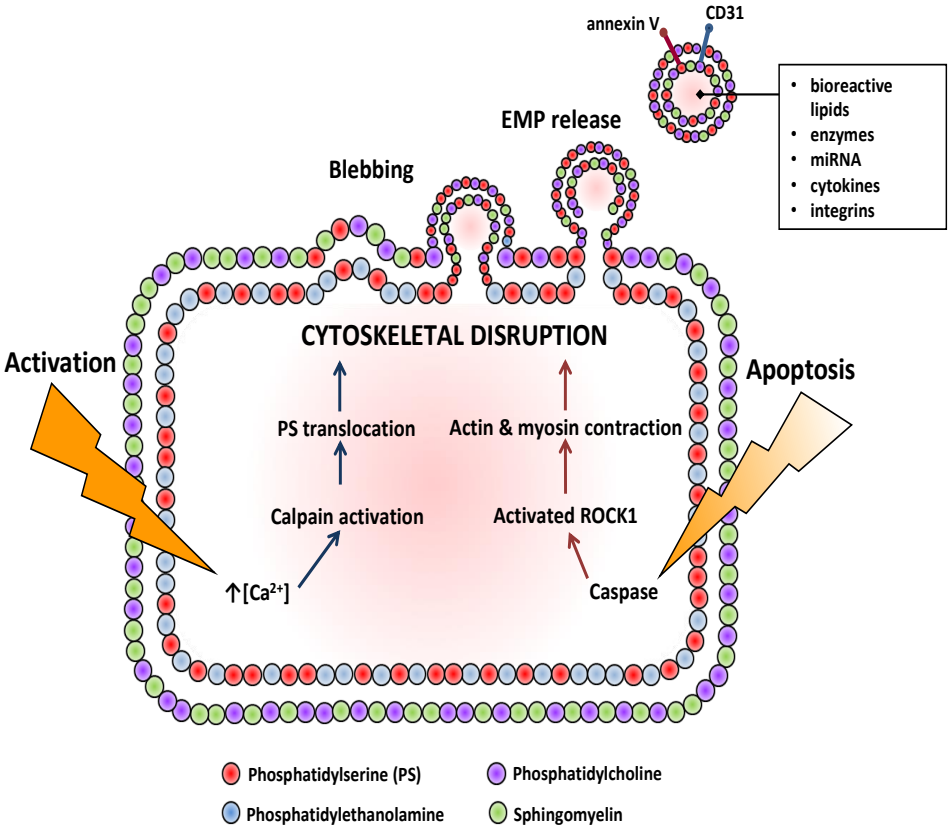

Figure 7. Cell activation and the release of microparticles. Loss of membrane asymmetry results in the translocation of phosphatidylserine (PS) to the outer surface membrane resulting in the release of microparticles.
Abb- Rho-associated kinase (ROCK I)(Schiro *et al.*, 2014)

An increase in intracellular calcium results in the inhibition of flippase (a lipid transporter enzyme which aids the translocation of membrane phospholipids), the activation of floppase (ATP-dependent enzyme involved in the transport of aminophospholipids and cholinephospholipids in the plasma membrane) and scramblase (involved in the translocation of phospholipids between the two monolayers of a cells plasma membrane). Calpain (a calcium dependent cytosolic cystenine protease) is subsequently activated leading to cleavage of long actin filaments (Comfurius *et al.*, 1990) as well as gelsolin (an actin binding protein

necessary for filament assembly and disassembly). When actin capping proteins are cleaved, this leads to cytoskeleton disruption. Rho-associated kinase (ROCK I) is also involved in the process of membrane blebbing. (ROCK is mainly involved in regulating the shape and movement of cells by acting on the cytoskeleton). ROCK I is cleaved and activated by caspases. (Cysteine-aspartic proteases essential in apoptosis) (Coleman *et al.*, 2001). Activated ROCK I induces actin-myosin contraction resulting in disruption of the cell membrane, resulting in loss of plasma membrane asymmetry, leading to MP formation. During this process, DNA fragments may become incorporated in MPs. (Coleman *et al.*, 2001) The protein constituents in the outer plasma membranes of MPs are dependent on the type of stimulus cells are submitted to. This was revealed after proteinomic analysis (sophisticated analysis of membrane proteins) of MPs released *in vitro* from cultured cells.(Miguet *et al.*, 2006)

In our lab, endothelial cells have been stimulated with the inflammatory cytokine TNFα (Tumour necrosis factor alpha) *in vitro* and this process successfully produced endothelial microparticles (EMPs). Proteinomic analysis, performed on these EMPs, showed raised PTX3 (Pentraxin-related protein), also known as tumour necrosis factor-inducible gene 14 protein, which has been proposed as a biomarker for atherosclerosis and ischemic stroke (Rolph *et al.*, 2002; Smalley *et al.*, 2007) (See section 1.6.2.9) Currently our lab is working on a protocol to extract EMPs from serum form patients with carotid disease to compare their EMP protein constituents.

1.5.1 Endothelial microparticle composition and structure

Endothelial microparticles (EMPs) are complex vesicular structures that contain several characteristic protein molecules in their outer membrane. Their constituents vary depending on the stimulus that promoted their release. The

EMP structure consists of an outer and inner phospholipid membrane with membrane proteins and receptors. (Jimenez *et al.*, 2003; Miguet *et al.*, 2006) Recently, it has been shown that microparticles also contain mRNA and micro RNA which may indicate that they may have a regulatory role. (Chen *et al.*, 2010)

See Figure 8.

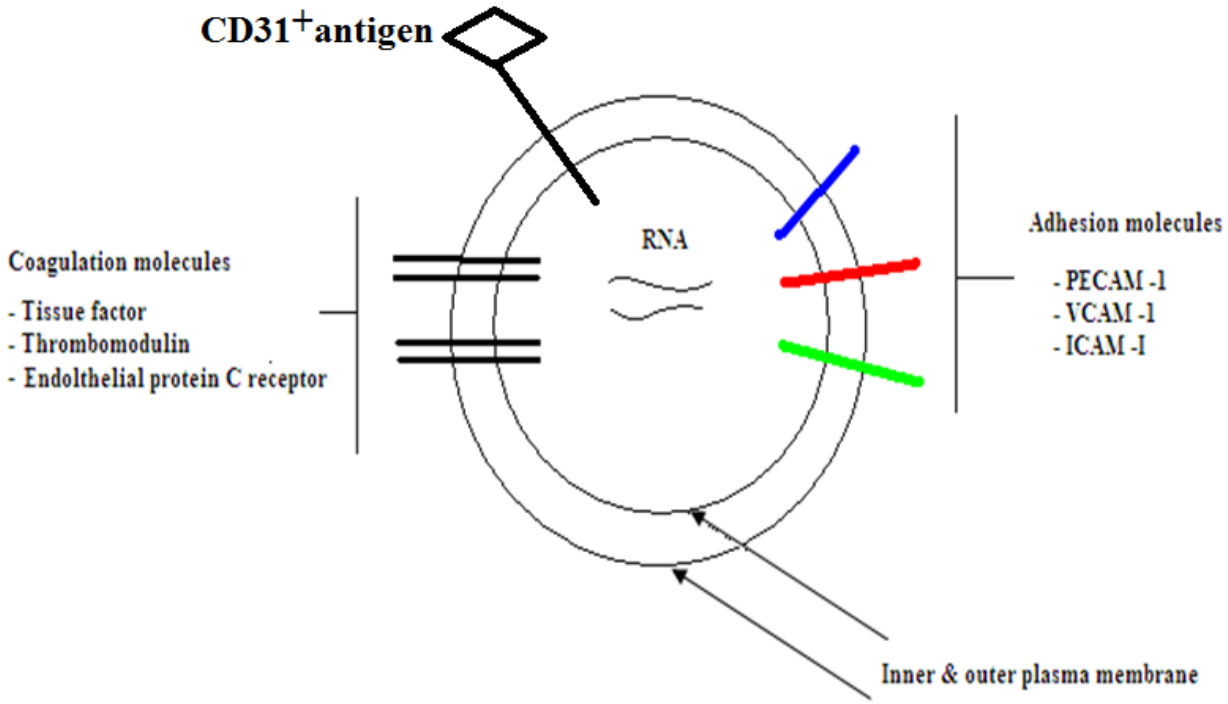

Figure 8. Endothelial microparticle. EMPs harbour a panel of receptors, enzymes and molecules which confer them a role in coagulation, inflammation, angiogenesis and immune function. TF, tissue factor; TM, thrombomodulin; EPCR, endothelial protein C receptor; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule.

1.5.2 The role of microparticles and cytokines in atherosclerotic plaque development

1.5.3 Atherosclerotic lesion initiation

Arteries are lined with a single layer of endothelial cells known as the endothelium. The endothelium forms a highly selective non-thrombogenic selective barrier that synthesises and releases protective vasoactive substances such as nitric oxide (NO).(Lüscher & Noll, 1996) Endothelial cells produce NO from the amino acid L-arginine via the enzyme endothelial NO synthase (eNOS) and release it following shear stress in vessels. NO acts as a i) vasodilator, ii) inhibitor of leukocyte migration into the vessel wall, iii) inhibitor of platelet aggregation and iv) inhibitor of smooth muscle cell (SMC) proliferation all of which are involved in the early and late stages of the atherosclerotic process. (Lüscher & Noll, 1996). However EMPs could be released in the process of endothelial cell damage.

EMPs have been shown to act as independent predictors of vascular disease since they are released into the circulation following damage to endothelial cells. (Amabile *et al.*, 2005; Suades *et al.*, 2012) EMPs bear membrane proteins such as $CD31^+$ antigens that are also present on endothelial cells and thus can be detected on this basis. However, since the $CD31^+$ antigens are also found on myeloid cells which also harbour $CD42^-$ antigens, EMPs are selected against MPs of myeloid origin by excluding MPs with $CD42^-$ antigens during analysis.(Sinning *et al.*, 2011) Thus EMPs can act as potential bio-markers of cardiovascular disease processes since they are released following endothelial damage. In this context, EMPs have a potential of being used as a marker of damaged / vulnerable plaques in patients with asymptomatic disease in the clinical setting. (Chalela, 2009) This is the main purpose of this thesis.

Several studies have shown that circulating microparticles can also promote atherosclerotic plaque development. This concept was demonstrated *in vitro* whereby microparticles were generated from activated white blood cells and

platelets. These MPs were shown to stimulate the expression of adhesion molecules on endothelial cells and induce the formation of inflammatory molecules in cultured endothelial cells. These adhesion molecules favoured leucocytes adhesion to damaged endothelium which is a classical mechanism of atherosclerotic plaque initiation.(Barry *et al.*, 1997; Mesri & Altieri, 1998; Mesri & Altieri, 1999) MPs have also been shown to impair the NO pathway, by stimulating the formation of free radicals, and/or decrease Ser1179- endothelial nitric oxide synthase phosphorylation (Amabile *et al.*, 2005; Densmore *et al.*, 2006), thus leading to vasoconstriction of vessel walls (Boulanger *et al.*, 2001). Interestingly, elevated MPs have been observed in smokers and in healthy individuals subjected to physical inactivity, and this may be explain why atherosclerosis is prevalent in smokers since MP may be kick starting the atherosclerotic process (Gordon *et al.*, 2011; Navasiolava *et al.*, 2010) *In vitro* and *in vivo* experiments have also demonstrated that shear stress and turbulent flow within vessel walls stimulate endothelial cell apoptosis favouring EMP release. (Tricot *et al.*, 2000)

Atherosclerotic plaques tend to occur where hemodynamic forces are greatest and these include the inner curvature of vessels and arterial branches such as the carotid, femoral and aortic bifurcations. (Ku *et al.*, 1985) See Figure 9. The dragging forces of blood flow within the arterial wall, creates 'wall shear stress' contributing to the formation of a dysfunctional endothelium. (Gimbrone, 1999) Interestingly endothelial permeability is increased when endothelial cells are exposed to microparticles *in vitro* implicating their involvement in disrupting the endothelial barrier integrity. (Densmore *et al.*, 2006) Arterial bifurcations are known to house dysfunctional endothelial cells. When analysed these cells are polygonal in shape and not aligned to the direction of flow, making them highly permeable to macromolecules such as Low density lipoprotein (LDL), a critical component in the atherosclerotic process. (Lusis, 2000)

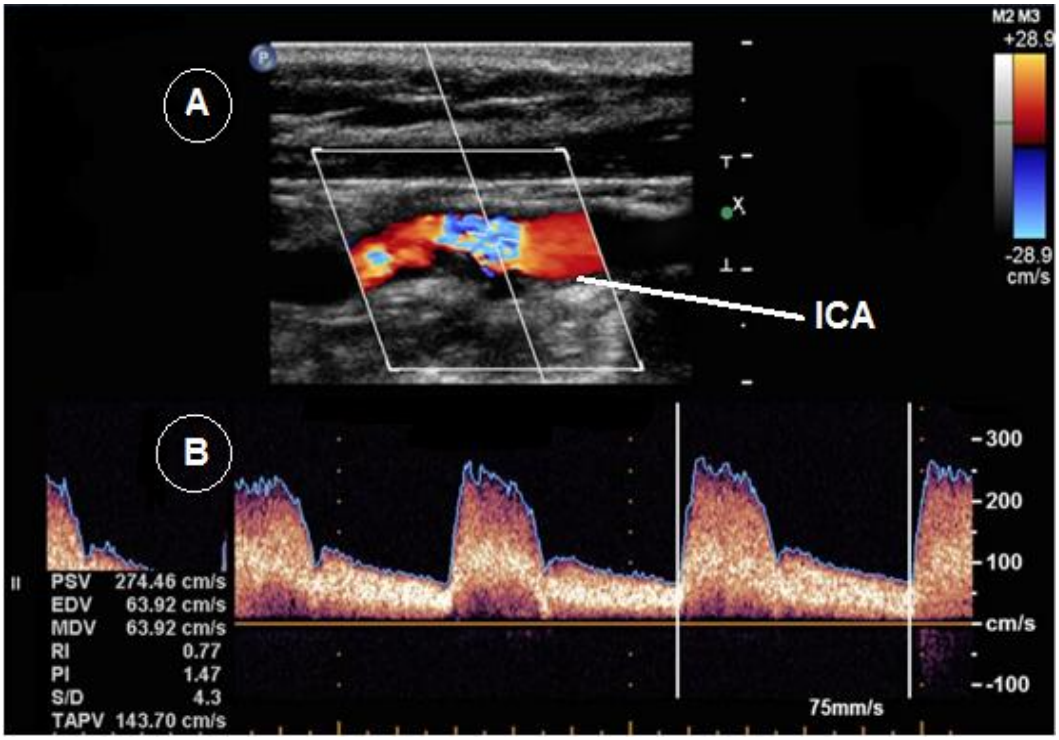

Figure 9. Doppler ultrasound image from a patient with symptomatic internal carotid disease who was scanned in the vascular lab at the Manchester Royal Infirmary. A) The ultrasound image shows an area of internal carotid artery (ICA) stenosis (blue). B) Shows a Doppler trace of the turbulent flow within the ICA with a high peak systolic velocity PSV (normal range < 125 cm/s)

The passage of LDL into the vessel wall results in LDL oxidation, lipolysis, proteolysis and aggregation. Oxidised LDL (oxLDL), a powerful inflammatory agent, promotes the expression of adhesion molecules on endothelial cells. (Kim *et al.*, 1994) In turn, OxLDL acts as a potent chemoattractant for monocytes promoting their differentiation into macrophages and inhibits their mobility. (Goldstein *et al.*, 1979)

As LDL accumulates within the arterial wall, it further attracts more monocytes and lymphocytes to the affected area resulting in the secretion of large amounts of cytokines activating smooth muscle cells (SMC) migration into the area of damage. These events result in a unending, self-sustained, inflammatory process which eventually becomes independent of oxidised LDL.(Meyer *et al.*, 2014) See Figure 10.

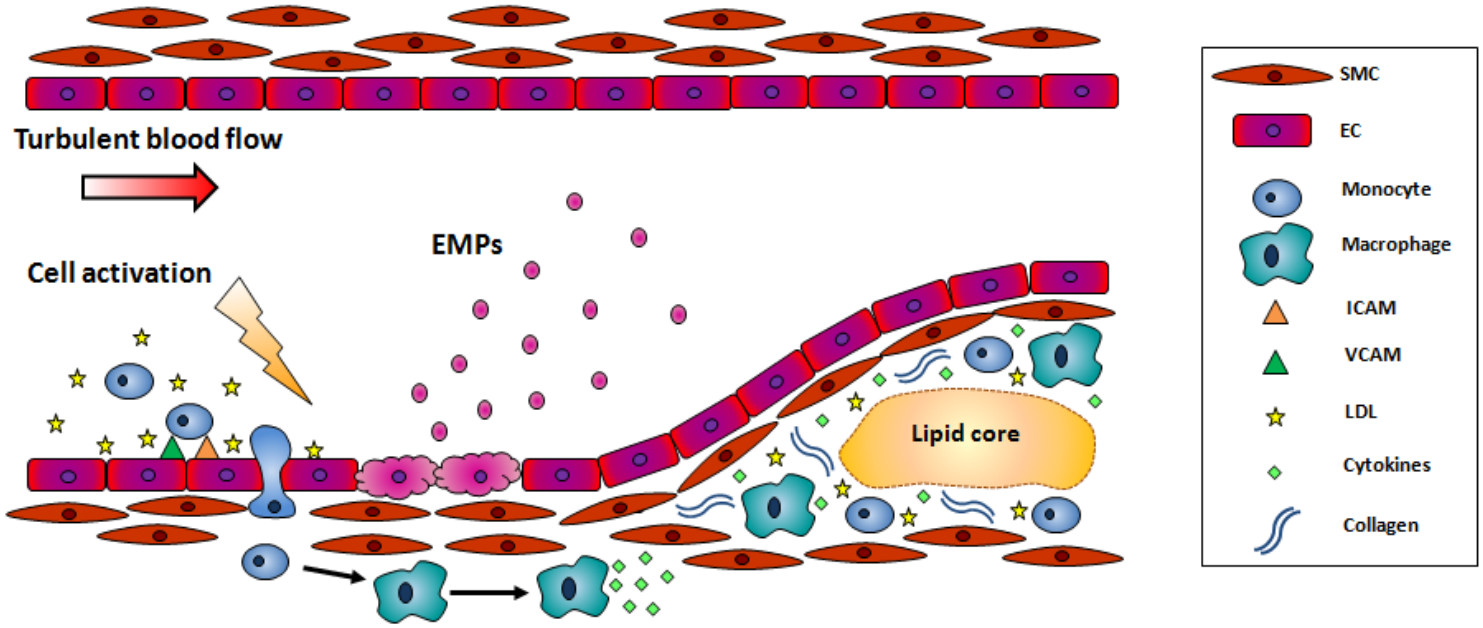

Figure 10. Stages in atherosclerotic plaque development. (Schiro *et al.*, 2014)

1.5.4 Plaque Progression – Endothelial damage and recruitment of inflammatory cells.

Histological analysis of atherosclerotic plaques have shown that monocytes are recruited in the early stages of atherosclerotic process and throughout all the stages of plaque formation.(Libby *et al.*, 2009) Endothelial cell (EC) damage results in the expression of adhesion molecules a well as the production of cytokines such as macrophage migration inhibitory factor (MIF) that facilitate the ingress of monocytes into the intima. Monocytes attach to activated endothelial cells by binding to ICAM-1 (intercellular adhesion molecule), VCAM-1 (vascular adhesion molecule), P and E selectin. (Tedgui & Mallat, 2006) See figure 10 and 11.

In vitro MPs, originating from different cells, have been shown to promote leucocytes and endothelial cells to synthesise and release the cytokines IL-6 and IL-8 promoting further leukocyte recruitment to the site of endothelial injury. (Barry *et al.*, 1998; Mesri & Altieri, 1998; Scanu *et al.*, 2008) MPs were also shown to be responsible for stimulating endothelial cells to express ICAM-1 and monocytes to express the counter adhesion receptors CD11a and CD11b leading to further leukocyte adhesion and transmigration into the endothelium. (Barry *et al.*, 1998; Huber *et al.*, 2002) A study on advanced carotid endarterectomy plaques by Mallat *et al* has shown a significant number of leukocyte microparticles within the atherosclerotic plaque that may support this hypothesis. (Mallat *et al.*, 1999) High levels of plaque MPs harbouring ICAM-1 molecules have been shown to transfer adhesion molecules to endothelial cells *in vitro* promoting macrophage diapedesis into plaques. (Rautou *et al.*, 2011) See Figure 11. Once monocytes invade the intima, they differentiate into macrophages by taking up oxidised lipids, and are transformed into “foam cells” which constitute the lipid core forming the ‘fatty streak’. (Goldstein *et al.*, 1979)

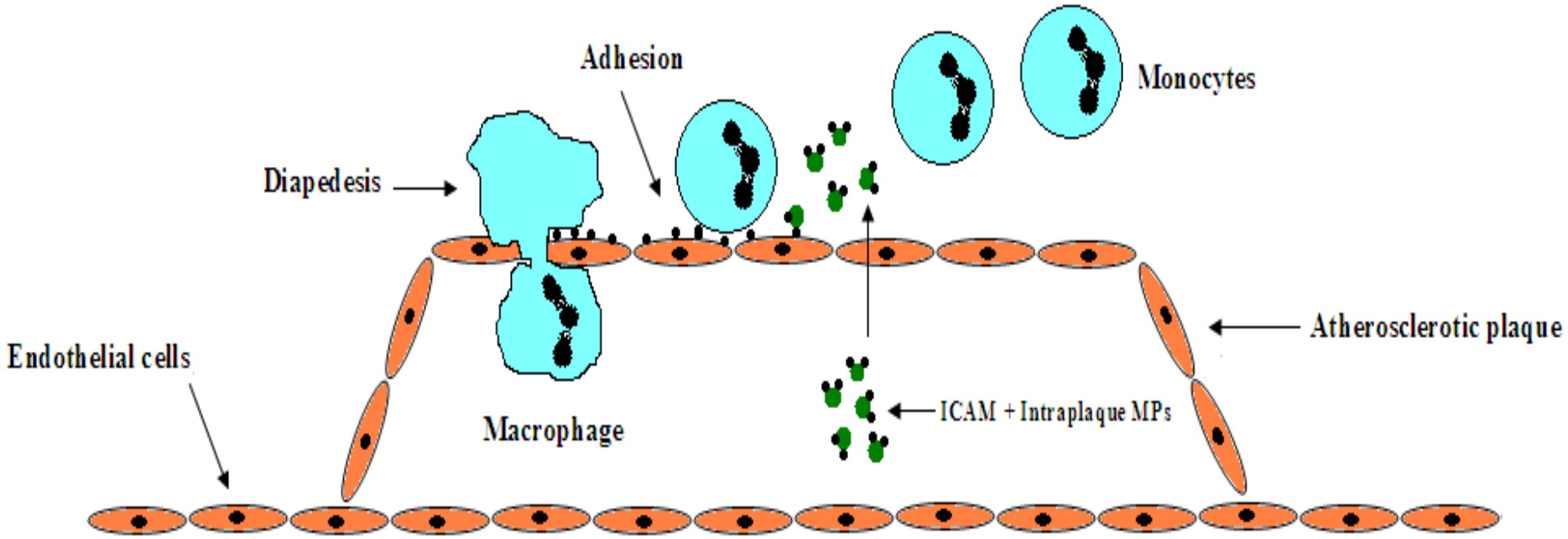

Figure 11. Schematic drawing of MP interaction with the endothelial layer.
Intra-plaque MPs (represented in green circles) transfer intracellular adhesion molecules (ICAM) to endothelial cells. ICAM-1 molecules facilitate diapedesis of leukocytes within the plaque, which eventually results in plaque progression.

1.6 Inflammatory cytokines

Cytokines are low molecular weight protein molecules (~ 5-20 kDa) released by cells that have a specific effect on cell to cell interaction and communication. See table 1. Inflammatory cytokines play a major role in the atherosclerotic inflammatory process. Cytokines are able to modulate various aspects of vascular inflammation by influencing the differentiation and proliferation of vascular and inflammatory cells. See Table 1. These regulatory mediators have been shown to modulate plaque morphology and stability.(Nilsson *et al.*, 2009)

Group	Cytokine
Pro-inflammatory	IL-1 IL-2 IL-12 IL-18 MIF TNF-α INF-γ M-CSF TNF-β
Variable	IL-4 IL-6 GM-CSF
Anti- Inflammatory	IL-5 IL-10 IL-13

Table 1. Summary of cytokines

1.6.1 Cytokine classification

There are six different types of cytokines which are classified according to their structure, function and their pro or anti-atherogenic effects. These include i) interleukins, ii) interferons, iii) tumour necrosis factors, iv) chemokines, v) growth factors and vi) colony-stimulating factors.

There are more than hundred known cytokines involved in regulating the complex inflammatory and immune responses in atherosclerosis. Analysis of cytokines associated with vascular inflammation reveals that there exists a balance between pro and anti-inflammatory cytokines involved in the development of atheroma.(Robertson & Hansson, 2006)

When T-lymphocytes are activated, they differentiate into T-helper (Th) 1 and Th2 cells. Within atherosclerotic plaques, Th1 macrophages are most prevalent and are responsible for secreting inflammatory cytokines involved in the inflammatory process.(Frostegård *et al.*, 1999) Th1 cells secrete the pro-inflammatory cytokines IL-2&12, IFN- γ, TNF-α and TNF-β whilst the Th2 cells secrete the athero-protective cytokines IL-4, 5 ,10 and 13.(Calcagni & Elenkov, 2006; Robertson & Hansson, 2006; Walch *et al.*, 2006) See table 1. In the next section we will review several of the important cytokines involved in the atherosclerotic process some of which will be investigated in this research project and used to identify unstable plaques.

1.6.2 Proatherogenic Cytokines

The following is a description of several important cytokines involved in atherosclerosis.

1.6.2.1 Interferon gamma (IFN- γ)

IFN- γ is a Th1 cytokine and is expressed by the majority of cell types within atheroma. These include T cells, macrophages and SMCs. ECs as well as macrophages and SMCs synthesise and release IFN-γ in response to inflammation resulting in further recruitment of Th1 cells, leading to a propagation of the inflammatory response. IFN-γ induces the expression of ICAM and VCAM-1 on ECs which in turn facilitate the entry of T cells into the atheroma.(Tedgui & Mallat, 2006)

1.6.2.2 Tumour necrosis factor (TNF-α)

TNF-α is a member of the TNF family and is primarily secreted by monocytes and macrophages. TNF-α plays a role in the early and late stages of atherosclerosis from the initial stages of intimal thickening to vessel occlusion(Barath *et al.*, 1990). Like IFN- γ, TNF-α stimulates the expression of adhesion molecules in ECs and SMCs creating a pro-inflammatory/ thrombotic environment, where leucocytes and endothelial cells interact.(Young *et al.*, 2002) Together with IFN-γ, TNF-α is involved in the early stages of atherosclerosis in that it alters endothelial cell function. TNF-α and IFN- γ are able to disrupt the intercellular adhesion molecules between endothelial cells (Kleinbongard *et al.*, 2010). TNF-α favours an influx of calcium within ECs resulting Rho A and myosin chain activation. This process results in a loss of barrier function of the endothelial cells leading to increased porosity facilitating the transmigration of leukocytes within atherosclerotic plaques. (Komarova & Malik, 2010) Serum TNF-α levels have been shown

to correlate to the degree of carotid stenosis and inflammation within atherosclerotic plaques, indicating to its possible use as a biomarker of atherosclerosis (Skoog *et al.*, 2002) Furthermore studies have also shown that TNF-α *in vitro* enhances the expression of metalloproteinases in SMCs, which are proteinases capable of digesting extracellular matrix within arterial walls, implicating their role in plaque remodelling and rupture. (Galis *et al.*, 1995) More importantly, TNF-α induces the expression of tissue factor (TF) a thrombogenic protein, in ECs, SMCs and macrophages, which can be detected in plaque and patients' serum. Elevated TF levels have been observed in patients with cardiovascular risk factors such as hypercholesterolemia, diabetes, hypertension and smoking and may be considered in future therapeutic strategies to combat cardiovascular disease. (Steffel *et al.*, 2006)

1.6.2.3 Interleukin 1 beta (IL-1β)

The IL-1 cytokine family are Th1 cytokines. The family is composed of IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18. IL-1 is pro-inflammatory and has been one of the first cytokines implicated in the early stages of vessel wall inflammation in the atherosclerotic process. (Dinarello, 2000) IL-1 exerts its effect through the IL-1R receptor on ECs which induces the generation and secretion of other cytokines, chemokines, matrix- degrading enzymes and adhesion molecules which promote further recruitment of inflammatory cells to the site of inflammation. (Libby *et al.*, 1995) Raised IL-1 levels have been shown to be associated with a higher chance of plaque rupture by maintaining an ongoing local inflammatory process. (Libby *et al.*, 1995)

1.6.2.4 Interleukin 16 (IL-16)

Il-16 is released by a variety of cells including lymphocytes and epithelial cells. It functions as a chemo-attractant and modulator of immune cells that express the CD4 surface molecule on their outer membranes such as T-cells.(Cruikshank *et al.*, 2000) Il-16 has been recently used in risk prediction models in patients with coronary artery disease (Cross *et al.*, 2012)

1.6.2.5 Interleukin 3 (IL-3)

IL-3 is a haemopoetic cytokine predominately released by CD4 T lymphocytes infiltrating atherosclerotic plaque. IL-3 is able to induce angiogenesis resulting in neo-vessel formation as well as promote EC proliferation (Brizzi *et al.*, 1993; Dentelli *et al.*, 1999). IL-3 has been shown to induce the expression of E and P selectin adhesion molecules on ECs which further promote T cells adherence to the endothelium.(Khew-Goodall *et al.*, 1996) The presence of IL-3 in early and advanced human atheroma indicates its role in the initial stages of inflammation.(Brizzi *et al.*, 2001)

1.6.2.6 Interleukin 18 (IL-18)

IL-18 is a pro-atherogenic cytokine related to the IL-1 cytokine. IL-18 has been identified in human carotid atherosclerotic plaques and has been proven to have an inflammatory effect within the plaques themselves. (Mallat *et al.*, 2001; Uyemura *et al.*, 1996) IL-18 is produced by macrophages and acts synergistically with IL-12 to induce T cells, macrophages and SMCs to produce and release pro-atherogenic cytokines such as IFN-α. (Gerdes *et al.*, 2002) (Puren *et al.*, 1998) The effect of IFN-α effect on the atherosclerotic process has been proven in *apoe* $^{-/-}$ mice where it has been shown to have a potent inflammatory effect, favouring

atherosclerotic plaque formation.(Whitman *et al.*, 2002) Inhibition of IL-18 may have a future role clinically in reversing atherosclerosis. (Mallat *et al.*, 2001)

1.6.2.7 Macrophage inhibitory factor (MIF) and Monokine induced by gamma interferon (MIG aka CXCL9)

MIF is a key pro-inflammatory cytokine involved in atherosclerosis. (Calandra & Roger, 2003) (See Figure 3) MIF is strongly involved in triggering the atherosclerotic process following endothelial injury, and is thought to contribute to lesion instability.(Schrans-Stassen *et al.*, 2005) Pro-atherogenic factors such as oxLDL induce MIF expression in endothelial cells, SMC and intimal macrophages within plaques.(Burger-Kentischer *et al.*, 2002) MIF induces endothelial cells to upregulate adhesion molecules promoting monocyte adhesion to the areas of damage. Furthermore, MIF induces endothelial cells to produce CCL2 (MCP1) which is responsible for the recruitment of monocytes and T cells to the site of inflammation and the expression of chemokine receptors 2 and 4 (CXCR2 / CXCR4) on these cells. When MIF binds to these receptors, this translates into a rapid increase in intracellular calcium that results in monocyte and T cell transmigration, resulting in plaque growth and instability. (Bernhagen *et al.*, 2007)

MIG is another cytokine secreted by macrophages, endothelial cells as well as fibroblasts. Similar to MIF, MIG also acts as a T cell attractant and is induced by IFN-α secreted by macrophages.(Müller *et al.*, 2010) MIG attracts leukocytes to the site of inflammation by binding to the CXCR3 receptor present on activated T-lymphocytes making it an important mediator of T-lymphocyte migration. MIG plays a pivotal role in mediating cell recruitment and activating necessary processes involved in the repair of inflamed tissue. (Müller *et al.*, 2010)

1.6.2.8 Hepatocyte growth factor (HGF)

HGF is an important growth factor involved in inflammation and plaque development. Together with its receptor c-Met, it has been localised in human atherosclerotic plaques, and systemic levels of HGF are often raised in patients with cardiovascular disease. (Liu *et al.*, 2007) HGF acts as a potent angiogenic factor on endothelial cells and has been shown to have chemotactic effects on pericytes *in vivo* and *in vitro* inducing its effect via the c-Met receptor on endothelial cells. Pericytes are contractile cells present around endothelial cells and are thought to act in stabilising mature blood vessels. HGF may also be involved in plaque neovascularisation resulting in the formation of intra-plaque microvessels which are immature vessels that are more prone to rupture. Whether HGF contributes to plaque development or is up-regulated indirectly as a result of other factors remains to be elucidated. (Liu *et al.*, 2007)

1.6.2.9 Pentraxin-3 (PTX 3)

PTX3 is an protein that is produced by several cell types which include macrophages, fibroblasts and well as endothelial cells in response to inflammation. PTX3 behaves as an acute phase protein which increases dramatically in serum in response to inflammation. Peripheral leucocytes release PTX3 in response to the inflammatory cytokines IL-1 and TNF-α (Bottazzi *et al.*, 2009)

1.6.3 Anti-atherogenic cytokines

The majority of cytokines present within atherosclerotic plaques mainly originate from TH1 macrophages which are pro-atherogenic. There are several studies which characterised and studied their activity to the expense of giving little importance to the anti-atherogenic cytokines which may hold greater therapeutic potential.

1.6.3.1 Interleukin 10 (IL-10)

IL-10 cytokine is produced by Th2 macrophages as well as B cells and monocytes. IL-10, which has been detected in human atherosclerotic plaques, has potent anti-inflammatory properties on macrophages limiting their inflammatory response in vessel walls as well as decreasing apoptosis in lipid cores. IL-10 is also responsible for inhibiting Th1 lymphocyte cytokine production and T-cell proliferation.(Tedgui & Mallat, 2006)

1.6.3.2 Interleukin 33 (IL-33)

IL-33 is expressed by Th2 macrophages and is responsible for an increase in expression of the anti-atherogenic cytokines IL-4, 5 and 13 and down regulation of IFN-γ. (Miller *et al.*, 2008) Miller *et al* have demonstrated that mice treated with IL-33 expressed IL-4 and 5 as well as anti-bodies to ox-LDL which in turn resulted in plaque regression. Modulation of the IL-33 may be of therapeutic value in the future. (Miller *et al.*, 2008)

1.6.3.3 Transforming growth factor (TGF β)

TGFβ is a potent anti-inflammatory cytokine produced by Th2 macrophages, T lymphocytes, endothelial cells as well as vascular smooth muscle cells (VSMC). Low levels of TGF-β were found in patients with severe atherosclerotic disease.(Grainger *et al.*, 1995) The anti-atherogenic properties and immune-modulatory effects of TGF-β on T-cell differentiation within plaques have been extensively studied. TGF-β is responsible for collagen deposition to the plaque matrix adding strength to the atherosclerotic plaque structure. Studies in mice showed that TGF β depleted atherosclerotic plaques were more prone to intra-plaque haemorrhage and plaque rupture.(Gojova *et al.*, 2003)

1.6.3.4 Stem cell growth factor beta (SCGF-β)

Little is known about the effect of SCGF-β on the cardiovascular system although raised levels were found in patients with heart failure; the mechanism responsible is still unknown. (Wang *et al.*, 2013) SCGF-β is a haemopoetic growth factor that is involved in the early stages of haematopoesis. It is expressed in bone marrow and skeletal tissues stimulating the proliferation of erythrocytes, macrophages and lymphocytes. (Da Riva *et al.*, 2011)

1.6.3.5 Platelet derived growth factor (PDGF)

Following intimal injury, SMCs are attracted to the surface of the plaque to aid in the healing process. This results in a fibro-proliferative response below the fibrous cap lining atherosclerotic plaques. PDGF synthesised by platelets, ECs and activated macrophages is responsible for this process as well as its mitogenic effects on SMCs. (Sano *et al.*, 2001)

1.7 Vascular calcification

1.7.1 Serum human bone biomarkers

Vascular calcification was once thought to result from passive infiltration of calcium within vessel walls and was until recently thought to be a non-modifiable disorder of aging. (Rifkin *et al.*, 1979) Arterial calcification is now a recognised independent predictor of cardiovascular morbidity and mortality. Vascular calcification is a regulated process which resembles that of bone formation resulting in the deposition of organised extracellular matrix within vessel walls. (Hruska *et al.*, 2005)Calcium spicules are mainly deposited within the intima and media of vessels walls in the final stages of plaque development, although calcification has been noted in the early stage of atherosclerosis. (Amann, 2008) Intimal calcification occurs in areas associated with macrophages and mast cells, whilst medial calcification occurs in the elastic fibres around SMCs. (Jeziorska *et al.*, 1998) Vessel wall calcification reduces arterial elasticity weakening vasomotor responses. Studies looking at patients with coronary disease have correlated unstable angina to the presence of plaques with speckled calcification patterns however patients with stable angina had large calcified deposits.(Ehara *et al.*, 2004) Atherosclerotic plaque calcification seems to promote plaque stability which in turn decrease the risk of clinical events. (Abedin *et al.*, 2004) (Abedin *et al.*, 2004) (Abedin *et al.*, 2004) (Abedin *et al.*, 2004) (Abedin *et al.*, 2004) (Abedin *et al.*, 2004) (Abedin *et al.*, 2004) A growing number of studies suggest that there are numerous stimulatory and inhibitory molecules that play a part in vascular calcification process which will be assessed in this thesis.

Several proteins involved in bone metabolism have been detected within atherosclerotic plaques. These include osteocalcin (OC), osteopontin (OPN), osteoprotegrin (OPG) receptor activator of nuclear factor kappa-β ligand (RANKL) and sclerostin. (Dhore *et al.*, 2001)

1.7.2 Promoter of bone formation- Osteocalcin

OC is a protein synthesised by osteoblasts and is released into circulation. It acts like a hormone promoting bone formation. In the Asian population, high levels of OC were associated with a lower risk of developing heart disease. OC was also shown to have a negative correlation with carotid intima-media thickness in diabetic Asian patients(Kanazawa *et al.*, 2009) (Zhang *et al.*, 2010) Although the role of OC in atherosclerosis hasn't been fully understood, current data is promising of its use as a potential biomarker of plaque stability.

1.7.3 Inhibitors of bone formation- OPN, OPG & sclerositin

1.7.3.1 Osteopontin (OPN)

Osteopontin is a potent inhibitor of mineralisation which is expressed in chronic and inflammatory diseases. OC is released by a variety of cell types; osteocytes, osteoblasts, fibroblasts, macrophages and smooth muscle cells. (Scatena *et al.*, 2007) OPN aids in the recruitment of macrophages to sites of inflammation as well as regulating cytokine release and preventing ectopic calcification.(O'Brien *et al.*, 1994b) High plasma levels of OPN have been shown to correlate with severe coronary artery disease/calcification. (Ohmori *et al.*, 2003)

1.7.3.2 Bone resorption inhibitor Osteoprotegrin (OPG) and Receptor activator for nuclear factor κβ ligand (RANKL)

OPG is a decoy protein receptor protein that makes part of the TNF family. OPG is synthesised by osteoblasts and bone marrow cells. OPG is a competitive inhibitor of receptor activator for the nuclear factor κβ ligand (RANKL) preventing its activation resulting in the inhibition of osteoclastogenesis and hence jamming the process of bone resorption.(Ndip *et al.*, 2011) See Figure 12. Clinical studies

have shown that raised levels of OPG correlated to cardiovascular mortality.(Jono *et al.*, 2002)

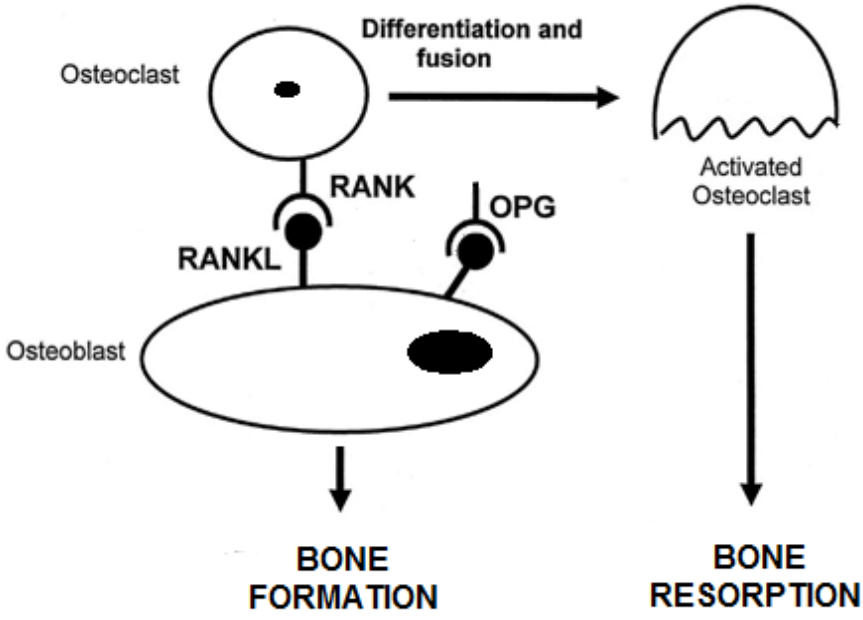

Figure 12 Interaction of the RANKL-RANK/OPG biomolecular complex

1.7.3.3 Sclerostin

Sclerostin is a glycorprotein produced by osteocytes which has a potent inhibitory effect on bone formation.(Balemans *et al.*, 2001) When the function of sclerositin was studied in bone, sclerostin was shown to be down regulated when bone was subjected to strain favouring bone formation.(Robling *et al.*, 2008) Similarly, sclerostin was found to be negatively correlated to carotid plaque calcification.(Register *et al.*, 2014)

1.8 Role of cytokines and MPs in fibrous cap weakening and neovessel formation leading to intra-plaque haemorrhage

1.8.1 Fibrous cap weakening

Atherosclerotic plaques consist of a central lipid core which is enclosed by a fibrous cap. (Davies *et al.*, 1993) Stable plaques are characterised by a thick fibrous cap (layer of cells lining the atherosclerotic plaque) composed of a matrix rich in type I and III collagen. The fibrous cap provides structural integrity to the lipid core making it less prone to thrombotic complications.(Lendon *et al.*, 1991) In the case of unstable plaques, the fibrous cap is thinner and encloses a larger lipid core which is more prone to rupture and subsequent risk of thrombus formation when exposed to high blood flow in stenosed vessels. (Virmani *et al.*, 2000) Figure 13 shows the sequence of events that lead to an acute plaque rupture, thrombosis and remodelling. Kolodgie *et al*, have shown that ruptured coronary plaques contain a high concentration of apoptotic macrophages within the fibrous cap in the area where plaque rupture is most likely to occur. (Kolodgie *et al.*, 2000) Monocytes also promote smooth muscle cell growth and atherosclerotic plaque formation. (Mesri & Altieri, 1998) Smooth muscle cells (SMC) migrate from the media to the intima and contribute to the development of atherosclerotic plaques. (Doran *et al.*, 2008) Later in the course of plaque development, SMC assume more of a synthetic role contributing to the production of extracellular matrix, collagen, cytokines and proteases rather than their usual contractile function, resulting in weakening of the atherosclerotic plaque. (Raines & Ferri, 2005)

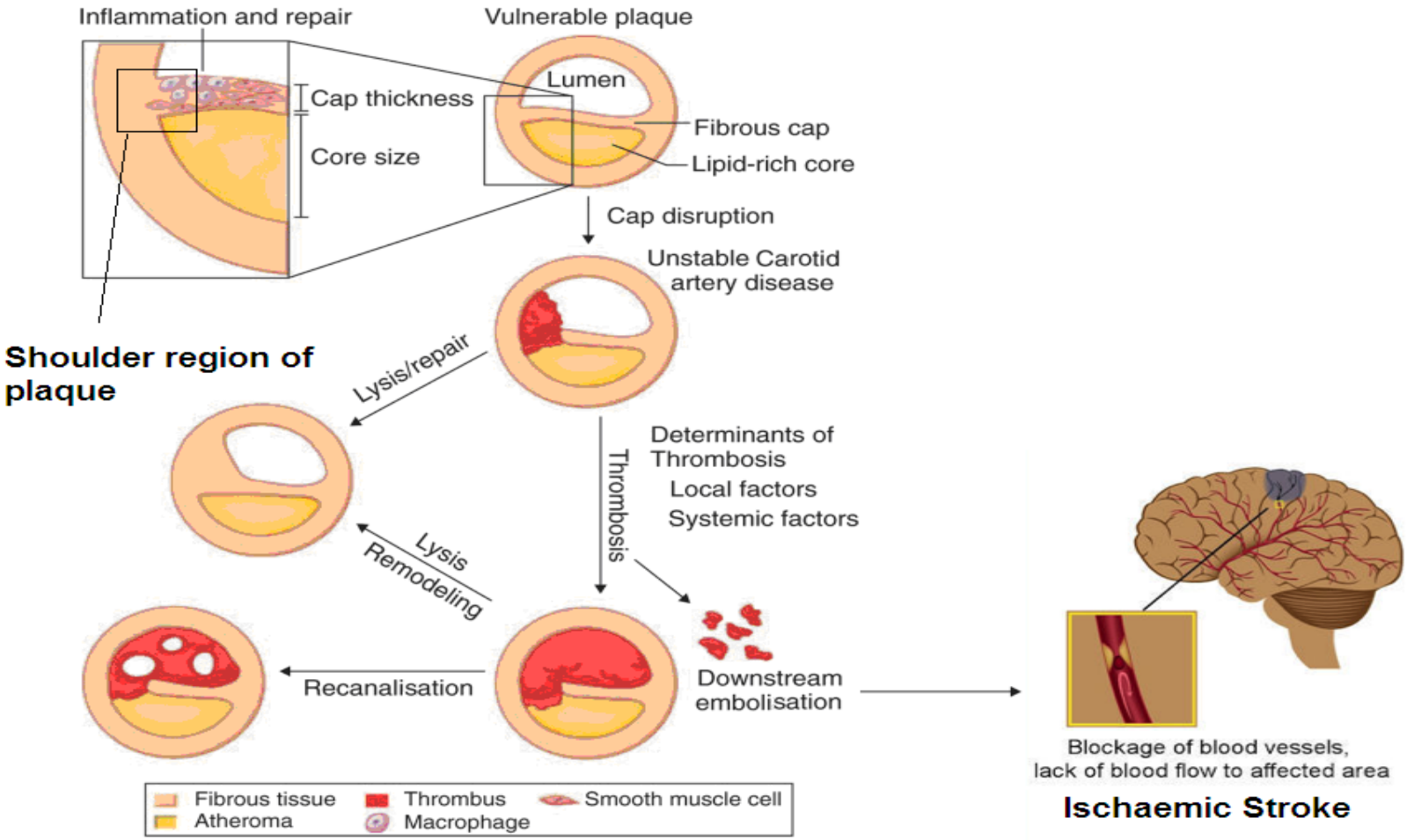

Figure 13. Plaque vulnerability, disruption and thrombosis. Changes leading to acute atherosclerotic plaque rupture, remodelling or stroke. (Modified from - Théroux P) (Théroux & Fuster, 1998)

Weakening and subsequent rupture of the fibrous cap results in thrombosis and is responsible for the most dreaded complications of atherosclerosis. There are several mechanisms and factors that act both at the cellular and molecular levels for this process to occur. In most cases, thrombotic events are dependent on the degree of plaque inflammation and endothelial damage rather than the degree of plaque stenosis. (Libby, 2001) Unstable plaques contain monocytes, macrophages and a large number of CD4+T-cells which are responsible for orchestrating the inflammatory environment within the plaque.(Hansson & Hermansson, 2011)

Histological analysis of complex advanced atherosclerotic plaques exhibited a paucity of SMCs at sites of rupture and show an abundance of macrophages within the fibrous cap, implicating their roles in plaque instability.(Ross *et al.*, 1984) Activated T-lymphocytes secrete i) IFN-γ, which is responsible for inhibiting collagen production by SMCs, interfering with the integrity of the interstitial collagen present within the fibrous cap(Amento *et al.*, 1991) ii) CD40 ligand and IL-1, are responsible for inducing macrophages to release interstitial collagenases, namely matrix metalloproteinases - MMP-1,8 and 13, with MMP-9 being more prominent in the 'shoulder region' of plaques accounting for weakening of the collagen structure in this region,(Gough *et al.*, 2006; Sukhova *et al.*, 1999) and iii) MIF which modulates migration, proliferation and synthetic function of SMCs and macrophages, resulting in the production and expression of MMP 1,2,9 &12. (Verschuren *et al.*, 2005) These processes are responsible for collagen degradation resulting in weakening of the atherosclerotic plaque, hence causing plaque destabilisation and rupture.

1.8.2 Neovessel formation & intra-plaque haemorrhage

Normal healthy arteries possess a vascular network in the adventitia and outer media called vasa vasorum whose main function is to nourish the vessel wall with oxygen and essential nutrients.(Virmani *et al.*, 2005) With the progression of atherosclerotic disease, there is an increase in intimal thickness and a tendency for increased neovessel formation within the atherosclerotic plaques to counter act tissue hypoxia and inflammation. (Moreno *et al.*, 2006; O'Brien *et al.*, 1994a; Virmani *et al.*, 2005). As atherosclerotic vasa vasora proliferate, they form a network of leaky vessels within the atherosclerotic plaque, allowing erythrocytes and leucocytes to leak into atherosclerotic lesions. (Hellings *et al.*, 2010) As the lipid core increases in size, there is greater tissue hypoxia within the atheroma which further simulates neovessel formation, leading to a greater chance of intra-plaque haemorrhage and eventually plaque rupture. (Kolodgie *et al.*, 2003) Released red cells are broken down within the plaque favouring the release of cholesterol rich cell membranes that accumulate within the plaque constituting the necrotic lipid core. (Kolodgie *et al.*, 2003)

MPs may also be responsible for this process of neovessel formation. Since MPs are involved in the production of metalloproteinases, this results in the degradation of interstitial matrix that favours endothelial cell invasion into the surrounding matrix promoting angiogenesis by providing more room for neo-vessel formation. (Dashevsky *et al.*, 2009; Distler *et al.*, 2005) Evidence for this process was shown *in vitro* using human umbilical vein endothelial cells (HUVECs). MPs isolated from carotid endarterectomy specimens were found to increase endothelial cell proliferation and promote capillary tube formation in HUVEC. The presence of CD40 ligands on the surface of plaque microparticles and their interaction with CD40 receptors on endothelial cells promoted vascular endothelial growth factor release that was responsible for this effect(Leroyer *et al.*, 2008), Interest-

ingly, MPs extracted from symptomatic plaques had significantly more CD40L+ve MPs (Leroyer *et al.*, 2008).

In summary, there are three mechanisms which may contribute to plaque rupture. The first mechanism involves shear stress on the thin fibrous cap from the physical forces of blood flow. This can result in superficial erosions or desquamation of endothelial cells. The commonest site of rupture is at the ‘shoulder region‘(see Figure 3) of the fibrous cap and the vessel wall which is the most vulnerable point. (Kaartinen *et al.*, 1994)

The second involves cellular mechanisms within the fibrous cap and the plaque itself. These mechanisms involve active proteases capable of degrading extracellular matrix. MPs *in vivo* and *in vitro* have been shown to possess proteases that are responsible for weakening the fibrous. (Canault *et al.*, 2007)

The third mechanism involves intra-plaque haemorrhage that results from rupture of neovessels within the plaque itself.

1.9 Role of platelets, cytokines, MPs in plaque rupture and activation of coagulation pathways

In normal physiological situations, platelets travel at the periphery of blood vessels close to the endothelium. Platelets are negatively charged so are the glycoproteins expressed on endothelial cells. Together, these negatively charged surfaces repel one another preventing contact between these two surfaces.(Massberg *et al.*, 2002)

Cardiovascular risk factors such hypercholesterolemia, hypertension, diabetes and smoking promote the generation of reactive oxygen species that are pro-thrombotic and inflammatory resulting in damage to the endothelial cells lining the endothelium. Endothelial damage promotes platelet adhesion to the vascular wall.(Massberg *et al.*, 2002) When endothelial cells are damaged they express adhesion molecules which include P-selectin, VCAM-1, (platelet endothelial cell adhesion molecule) PECAM-1, and von Willebrand factor (vWf) which attract platelets to the site of injury. (Bröijersén *et al.*, 1998; Manduteanu *et al.*, 1992; Massberg *et al.*, 2002; Nityanand *et al.*, 1993; Nowak *et al.*, 1987) vWF is also released from endothelial cells following stimuli such as hypoxia, inflammatory cytokines, histamine, endotoxin and thrombin. vWF produced by endothelial cells is released into the circulation whilst a significant amount is also deposited into the sub-endothelial space. This mechanism initiates the initial platelet response at the site of injury. (Paleolog *et al.*, 1990).

When the endothelial surface enclosing atheroma is damaged, this results in changes in plaque geometry and exposure of vascular matrix components to blood. Matrix components include collagen, laminin, fibulin and tissue factor that are a very thrombogenic substrates to which platelets readily adhere to. Platelet adhesion to the damaged vessel wall results in the activation of the coagulation cascade leading to plaque thrombosis. The resulting thrombus leads to a reduction in blood flow to the target organ which in the case of carotid disease is the

brain. Thrombi may also dislodge and embolise leading to a stroke. This process is the result of a sudden transformation of a stable plaque into an unstable plaque. (Falk *et al.*, 1995; Ruggeri & Mendolicchio, 2007)

Plaque stability is dependent on the ratio of extracellular matrix to lipid content. Histologically there is a marked difference between stable and unstable atherosclerotic plaques. Early lesions harbour an abundance of SMCs which account for about 90-95% of plaque contents but this decreases to half the amount in advanced plaques .(Badimón *et al.*, 2009) Vulnerable plaques were also found to have larger lipid cores with increased neovascularisation and contained smaller amounts of collagen and SMCs. As plaques progress, the fibrous cap becomes thinner making them more prone to rupture (Badimón *et al.*, 2009).

The inflammatory microenvironment within the core of the atherosclerotic plaques, consists of cytokines e.g. tumour necrosis factor (TNF-α) and interleukins that stimulate tissue factor (TF) expression in monocytes, VSMC and EC. Typically plaques that harbour large amounts of TF, are more vulnerable to rupture and thrombosis. (Steffel *et al.*, 2006)

Tissue factor is a transmembrane glycoprotein responsible for initiating the coagulation cascade playing a major role in endothelial repair after injury as part of a normal haemostatic mechanism. (Wilcox *et al.*, 1989) The majority of TF activity within atherosclerotic plaques is derived from perishing foam cells and macrophages. (Wilcox *et al.*, 1989) Interestingly TF-rich MPs were found to be increased in patients with diabetes and coronary artery disease but were not actually associated with coagulation but it is postulated that they may be associated with transcellular signalling or angiogenesis.(Diamant *et al.*, 2002) TF has also shown to be responsible for SMC migration and proliferation resulting in atherosclerotic plaque remodelling.(Cirillo *et al.*, 2004) TF is also involved in angiogenesis *in vitro* so it may also be involved in plaque microvessel formation favouring

plaque rupture.(Carmeliet *et al.*, 1996) This evidence supports the theory of MP involvement in plaque thrombosis, since MPs harbouring tissue factor favours plaque rupture, resulting in subsequent extravasation of procoagulant atherosclerotic plaque contents into the blood stream. The thrombogenic potential of the exposed plaque is a key determinant in vascular thrombosis and luminal narrowing.(Davies, 1996)

1.9.1 Anti-inflammatory effect of MPs

Certain MPs possess an anti-inflammatory role and may be involved in the resolution phase after the inflammatory process commences. Two studies have shown that EMPs may play a role in anti-coagulant activity. In the first study, Perez-Casal *et al* have shown that when HUVECs are exposed to activated protein C (APC) (protein C reduces the production of i) thrombin by inactivating factors Va & VIII, ii) tissue factor and iii) cytokines IL-1, IL-6 and TNF-α) they release endothelial microparticles with APC protein in the outer membrane. (Pérez-Casal *et al.*, 2009) Microparticles with APC proteins were shown to bind to the endothelial protein C receptor (EPCR) on endothelial cells forming and EPCR-APC complex that has an anti-coagulant effect.

In the second study, MPs released from polymorphonuclear neutrophils (PMN) were found to block the inflammatory response of macrophages to lipopolysaccharide (LPS) (a major component of the outer membrane of Gram negative bacteria) and zymosan (a polymerised chain of glucose molecules found in yeast cell walls). (Gasser & Schifferli, 2004) These microparticles expressed PS, which attached to the PS receptor on the cell surface membrane of macrophages that ingest apoptotic cells. These MPs induced the release of TGFβ1 in macrophages (transforming growth factor beta 1), which has an anti-inflammatory effect and suppressed the release of the pro-inflammatory mediator cytokines IL-8 and

IL-10. (Fadok *et al.*, 1998; Huynh *et al.*, 2002) This suggests that EMPs may also be involved in the fine tuning of the anti-coagulant / coagulation equilibrium.

1.10 Hypothesis

We hypothesised that unstable carotid plaques are associated with increased levels of EMPs and have a specific cytokine profile. This concept could potentially be adapted in clinical practice to detect patients with unstable plaques.

1.11 Aim

The aim of this study is to formulate a metric for diagnosing 'immune health' by making serological, cellular and molecular analyses of the clinical samples from patients with carotid disease. This aim will be achieved in the following objectives.

1.12 Objectives

To establish whether plasma concentrations of EMPs and cytokines levels differ in patients with:

1) symptomatic and asymptomatic disease,
2) symptomatic and asymptomatic disease compared to healthy controls,
3) stable vs unstable plaques and
4) to determine whether a correlation exists between serum and plasma level of these markers with symptomatology and carotid plaque morphology.

CHAPTER 2

Materials and methods

2.0 Materials and methods

2.1 Ethical approval

Ethical approval for these studies was obtained from the Ethics committee in Leeds - REC reference 12/YH/0107. All patients who gave written consent for investigation of their blood and carotid samples were included in the study.

2.2 Patients

Seventy patients who underwent a carotid endarterectomy at the Manchester Royal Infirmary between April 2012 and April 2013 were studied prospectively. Indications for surgery included symptomatic carotid artery stenosis of ≥50% ICA stenosis or high grade asymptomatic ICA stenosis of ≥70%. Healthy age matched controls with no evidence of carotid disease on ultrasound were included. Control patients recruited from the gastroenterology and orthopaedic clinics.

Patient demographics and risk factors were reviewed and noted. Risk factors included age, sex, hypertension, hyperlipidaemia, diabetes, ischaemic heart disease and renal disease. Symptomatic patients were those who experienced an acute non-disabling stroke, TIA, or retinal embolic disease, whilst asymptomatic patients were the ones with no symptoms. Nineteen asymptomatic patients, 51 symptomatic patients and 20 controls were recruited into the study. Of the symptomatic patients, 13 patients had an acute stroke, 31 patients had a TIA, whilst 7 patients had retinal embolic disease on the ipsilateral side of their carotid stenosis.

2.3 Preoperative carotid disease evaluation

All patients underwent a duplex scan of their carotid arteries in the vascular lab pre-operatively to assess the degree of internal carotid stenosis; a standard procedure carried out on all patients with suspected carotid disease. Using information obtained from the carotid duplex assessment, surgery was planned accordingly. Occasionally, when duplex scans were inconclusive as a result of abnormal carotid artery anatomy or heavy plaque calcification, a CT angiogram scan of the carotid arteries was performed to give a clear picture of the extent of carotid disease prior to surgery. In total twenty eight patients (40%) had a CT angiogram.

2.4 Carotid plaque processing

Fresh carotid plaques were obtained following carotid endarterectomy. Procedures were performed according to standard protocol, by consultant vascular surgeons, with minimal disruption of the carotid plaque. Plaques were photographed using a Canon 650 D camera mounted on a tripod prior to histological evaluation. Plaques were fixed for 12-16 hours in 4% formaldehyde at 4ºC. The carotid specimens were transected transversely into 3 sections: I (proximal), II (middle) and III (distal) parts. Each section was appropriately labelled and placed in a tissue cassette. All sections were processed in an automatic processor for wax embedding, which consisted of several containers holding increasing concentrations of ethanol – 3 containers of 70%, 90% and absolute ethanol, 3 containers of xylene and 2 paraffin wax baths. The total process lasted for 16 hours where the specimens were allowed to soak into the individual solutions for 2 hours each.

Following this process, all tissue cassettes were placed into a paraffin wax bath for 15 minutes at 58ºC to melt away any excess wax. Tissue cassettes

were opened to view and orientate the dehydrated tissues which were then placed into moulds corresponding to tissue size. Using a heated forceps, the carotid tissue was placed into a mould in molten wax, which was then transferred to a cold plate to harden the wax and fix the tissue in place. Moulds were allowed to cool on a cold plate for 30 minutes until solid and then stored in a cold room at 4ºC.

2.4.1 Tissue sectioning

Paraffin wax blocks were placed face down on ice packs for 10 minutes to harden and ease sectioning. Blocks were cut in 7µm sections using a microtome and suspended in a water bath at 35-37ºC. Two consecutive sections were then mounted on appropriately labelled negatively charged slides (Superfrost ®, Germany). Slides were kept in an oven at 65ºC overnight allowing the wax to bond to the glass slides. Sections were then stored at 20^0C .

2.4.2 Preparation of samples prior to staining

Paraffin sections of carotid plaques were de-waxed 3 times in xylene for 10 minutes. Sections were rehydrated in decreasing concentrations of ethanol followed by water and kept moist throughout the immunohistochemical procedure.

2.5 Tissue staining and immunohistochemisty

2.5.1 Haematoxylin and Eosin staining

H&E staining was used to stain nuclei blue and cytoplasmic structures pink. Slides were placed in haematoxylin stain for 2 minutes and washed well in tap water. Slides were examined under the microscope to assess the stain quality. If the staining was inadequate this process was repeated until the desired level of stain was achieved. Excess staining was decolourised with 0.5-1% hydrochloric acid in 70% alcohol. After washing off the excess haematoxylin stain with tap water, slides were submerged in Eosin stain for 1 to 5 minutes. Optimal staining was assessed microscopically.

2.5.2 Alizarin stain

In order to visualise calcium deposition within the carotid plaques, alizarin red was used. Slides were dewaxed and rehydrated as in section 2.4.2. Sections were stained for 5 minutes with 2% Alizarin red at a pH of 4.2 (pH was adjusted with ammonium hydroxide). The excess dye was washed off with deionised water. Slides were dehydrated with acetone for 30 seconds followed by acetone- xylene (1:1) solution for 15 seconds and shaken off. This was then followed by xylene.

2.5.3 Immunohistochemistry

Slides each containing two sections of tissue, were dewaxed and rehydrated as in section 2.4.2. A wax pen was used to draw a circle around sections of tissue thus preventing the spread of stain into neighbouring tissue. Antigen retrieval was performed by adding 100-150µl of 1% SDS (sodium dodecyl sulphate) to the tissue sections. After allowing the sections to incubate for 5 minutes, SDS was washed off with PBS solution. Endogenous H_2O_2 was blocked with 100µl of 3% H_2O_2 in water for 5 minutes at 20^0C followed by a wash in PBS solution. Non-

specific binding sites were blocked using 10% blocking buffer solution consisting of 10% rabbit or goat serum in 1% BSA/PBS solution. Following an incubation period of 1 hour, sections were washed with PBS and exposed to the i) primary antibody ii) isotype matched IgG control overnight in a cold room at 4ºC. See Figure 14.

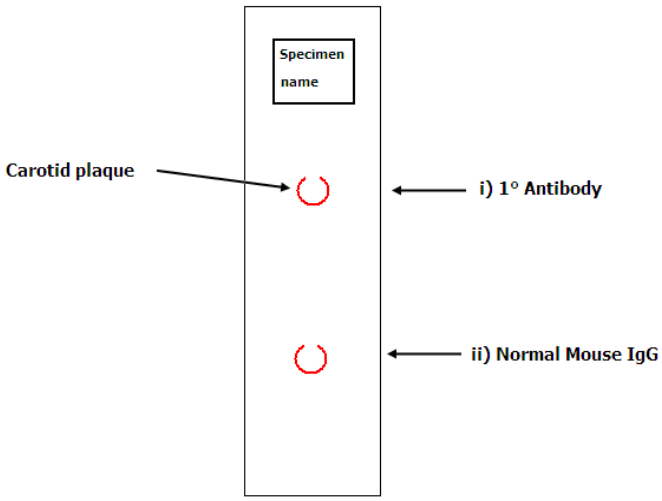

Figure 14. Slide showing carotid plaque sections.

This was followed by exposure to the secondary antibody which was diluted in blocking solution according to manufacturer recommendations shown in table 1. Slides were incubated for 1 hour at 20^0C . Sections were subsequently incubated with 100µl of 3,3- diaminobenzidine solution (0.7 $mg.ml^{-1}$ DAB, 1.g $mg.ml^{-1}$ Urea hydrogen peroxide) for 5 minutes at 20^0C which allowed the antibody binding to be visualised as a brown stain. Sections were washed in deionised water and counterstained with Mayers haematoxylin for 2 minutes before dehydrating the specimens in increasing concentrations of ethanol and xylene.

Following the staining procedures slides were mounted with a cover slip. This was carried out by adding a drop of xylene based mounting medium to the section and gently allowed to spread along the slide preventing any air bubbles from

getting trapped beneath the coverslip. The following table shows the different immunohistological stains used to stain the carotid plaques. See Table 2

Protein	1° Antibody	Dilution	2°Antibody	Dilution
Osteopontin	Mouse anti-human (R&D systems, MAB1433)	1:10	Rabbit anti-mouse HRP	1:50
CD68	Mouse anti-human (DAKO M0718)	1:10	Rabbit anti-mouse HRP	1:100
Smooth Muscle actin	Mouse anti-human (SIGMA A2547)	1:600	Goat anti-mouse	1:500
Stem cell growth factor(SCGF)	Mouse anti-human (AbD Serotec) MCA 2155 T	1:600	Goat anti-mouse	1:500
CXCL9 (MIF)	Mouse anti-human (Abcam®) Ab7207	1:200	Goat anti-mouse	1:500

Table 2. Summary of the primary antibody concentrations used for immunohistochemistry

2.6 Plaque classification

Atherosclerotic lesions were characterised histologically using the American heart association (AHA)(Virmani *et al.*, 2000) classification as well as the newer 'Oxford classification of carotid plaques' published in 2008.(Redgrave *et al.*, 2006)

Conventional AHA classification	
Type I	Initial lesion with foam cells
Type II	Fatty steak with multiple foam cell layers
Type III	Pre-atheroma with extracellular lipid pools
Type IV	Atheroma with a confluent extracellular lipid core
Type V	Fibroatheroma
Type VI	Complex plaque with possible surface defect, haemorrhage, or thrombus
Type VII	Calcified plaque
Type VIII	Fibrotic plaque without lipid core

Table 3 Classification of Atherosclerotic Plaques according to the AHA classification.(Károlyi *et al.*, 2013; Virmani *et al.*, 2000)

The Oxford plaque classification was used to classify plaques into stable or unstable groups according to plaque histological characteristics. See Table 4. Plaques were stained with haematoxylin/eosin (H&E), Alizarin Red (calcium stain), anti-smooth muscle actin antibody (Dako), anti-CD68 antibody (Dako) anti-TNFα antibody (Santa Cruz), anti-MIF antibody, MIG, CXCL9(Abcam), anti-OPN, anti-MIF, anti-SCGF, Cathepsin K and CTACK (R&D Systems) and anti-PTX3 (Abnova). The plaque composition, cellularity, severity of inflammation, athero-ma-associated macrophages and foam cells were analysed.

Inflammation score	*CD 68 (macrophages)*
0	No CD 68 stained cells
+	Occasional scattered cells or 1 group of >50 cells
++ (Marked inflammation)	Several groups (<5) of >50 cells
+++ (Marked inflammation)	Many groups (>5) of >50 cells or 1 group of >500 cells
Overall Plaque instability	
-Stable	Predominantly fibrous plaque with thick, intact cap
-Predominantly stable	Features of instability e.g. inflammation but thick intact cap
-Unstable with intact cap	Thin cap, large lipid core but no definite rupture or surface thrombus, intra-plaque haemorrhage
-Unstable with ruptured cap	Intra-plaque haemorrhage, Rupture or thrombus present

Table 4 Scales used to assess the presence of inflammatory infiltrates and overall instability in the plaques on histology(Redgrave *et al.*, 2006)

2.7 Blood samples

After an overnight fast, venous blood samples were collected pre-operatively on the day of surgery using a 21 G needle (BD Vacutainer ® systems). After discarding the first 5 mls of blood, blood for microparticle analysis was the first to be collected in Blue cap, citrate bottles (Lot 2118290, Ref 357691, BD Vacutainer®). The rest of the blood was collected in Yellow cap, rapid serum tubes (Lot 211896, Ref 367954, SST™ II Advance).

Bloods samples were centrifuged within 1 hour of blood collection. Citrate tubes were centrifuged at 1700g for 10 minutes at 4°C in a Sigma 3-16K centrifuge using a 121131 rotor. This was followed by harvesting of the plasma layer. The plasma layer was ultra-centrifuged again at 20,000g for 10 minutes at 4°C using an 11180 rotor to obtain platelet-poor plasma (PPP). After discarding the platelet pellet, the PPP was stored in aliquots of 200µl and stored at -80°C. Blood in the Rapid serum tubes were centrifuged at 4°C at 1700g for 15 minutes, to obtain serum, which was stored in aliquots of 200µl at -80°C for future Bio-Plex® cytokine analysis.

2.8 FLOW CYTOMETERY

2.8.1 Flow cytometry overview

Flow cytometry is used to analyse the physical and chemical characteristics of particles within a fluid as it passes through laser lights. Fluorescently labelled antigens emit light at different wavelengths as they traverse laser light. The emitted light called fluorescence is then measured to determine the properties of particles. (Khan *et al.*, 2005)

A flow cytometer is made up of three systems; i) the fluidic system which is responsible for transporting particles in a stream to the laser beam, ii) the optic system which is made up of lasers that illuminate particles as they pass through and the iii) electronic system which translates fluorescent light signals into electronic pulses which are processed by a computer. The data obtained from the computer analysis is then presented in the form of a plot and is referred to as a cytogram.(Khan *et al.*, 2005; Saxena & Anand, 2008)

2.8.2 Plasma preparation and MP labelling

To quantify the levels of EMPs within samples, 50µl of PPP and 50µl of 10µm diameter flow counting beads (Flow-count™ flourospheres; Cat No #7547053 Beackman Coulter, UK), were added to 900µl of calcium-rich buffer (eFluor®, e450 annexinV marker kit, Cat No # 48800669 eBioscience, UK). Analysis of the EMP population was carried out using specific flourochrome-labelled antibodies. These antibodies included 5µl of phycoerythrin (PE) - conjugated antihuman CD31 (endothelial marker) (Cat No # 555446 BD Bioscience, UK), 5µl of allophycocyanin (APC) - conjugated antihuman CD42b (platelet marker) (Cat No# 551061, BD Bioscience, UK) and 10µl eflour450 annexin-V marker (MP marker) (Cat No#4800669, eBioscience, UK), all of which were allowed to incubate for 10

minutes in the dark. Prepared samples were analysed in batches of 10 specimens using a Cyan flow cytometer (BD Biosciences) according to a predetermined protocol using specific gates to exclude artefact. EMPs were analysed according to their size and fluorescence using a logFS-logSS plot. On a PE count vs Annexin log plot events $>10^1$ were counted. When 1000 beads were counted by the flow cytometer, analysis was stopped.

2.8.3 Gating protocol for EMP measurement

Figure 15 shows a representative flow cytometric density plot demonstrating the gating protocol used to identify EMP in platelet free plasma. Gate R1 represents the bead population in each sample. R1 is an important 'quality control' measure that ensures that the flow cytometer was actually measuring beads within the samples. Gate R2 and R4 show particles which are only bound to PE and Annexin respectively. Gate R3 represents cellular debris of unidentified origin which is too small to be evaluated by the flow cytometer. Gate R5 is the main area of interest. This shows particles that are only positive to PE (endothelial marker) and Annexin V (microparticle marker) i.e. the EMPs in the sample. This is achieved by eliminating the particles that are $CD42b^+$ (platelet marker). EMP levels were calculated automatically by the flowcytometer.

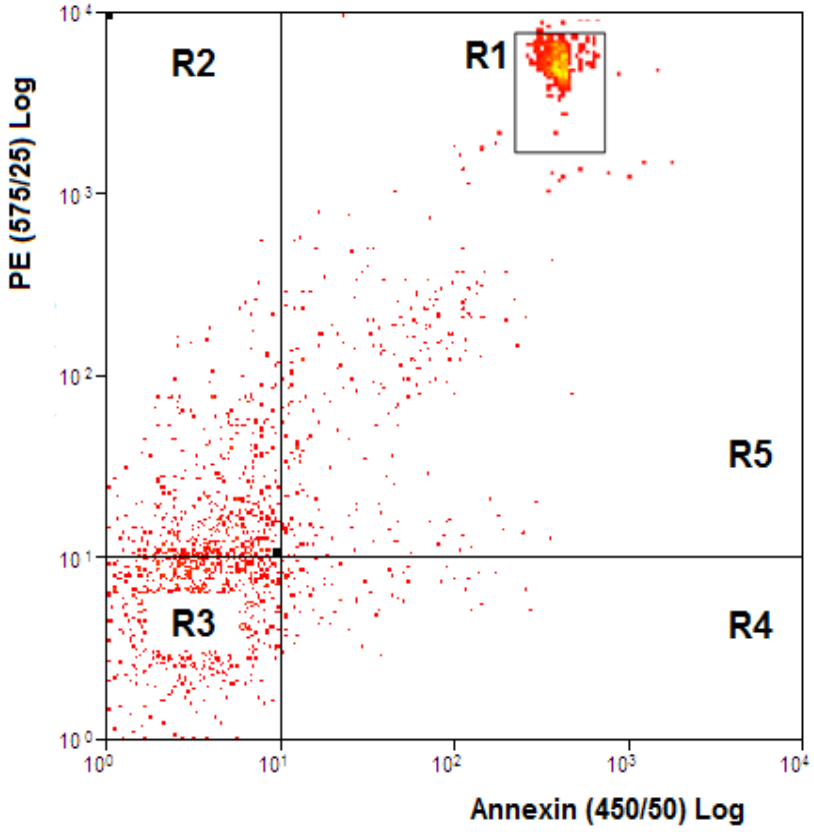

Figure 15. Density plot of phycoerythrin (PE) log against an Annexin log of a control patient

2.9 Bio-Plex® inflammatory cytokine suspension array

To quantify the level of inflammatory cytokines in the serum samples Bio-Plex® suspension arrays were used. Bio-plex® is a multiplate ELISA (Enzyme linked Immunosorbent assay) that permits the quantitative analysis of several biomolecules at the same time, in this case 21 molecules. Similar in principle to ELISA, capture antibodies directed against the biomarkers in question, are coupled to beads. Coupled beads react with the sample containing cytokines. After the sample is washed to remove any unbound protein a biotinylated detection antibody is added. Following the addition of sreptavidin-phycoerythrin (SA-PE) conjugate, a detection complex is formed which is detected using flow cytometry. See Figure 16.

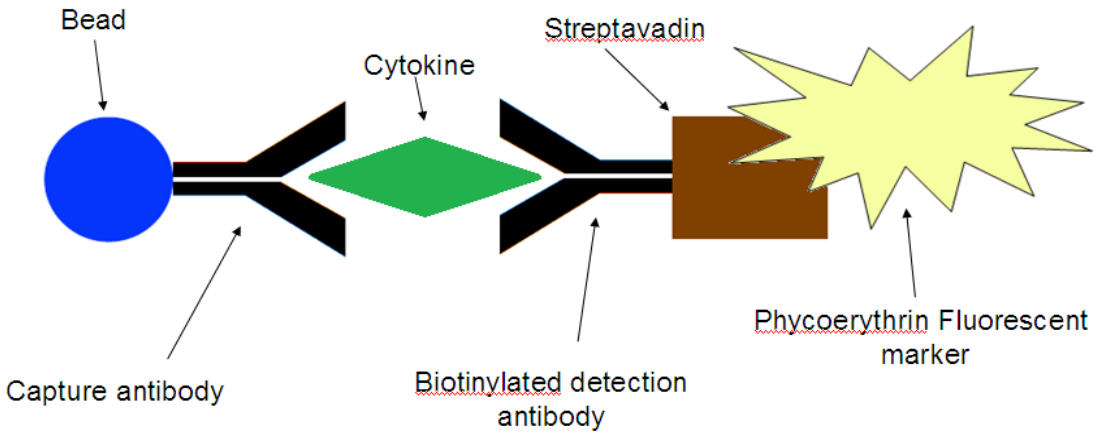

Figure 16. Bio-plex® immunoassay

2.9.1Serum preparation for cytokine analysis (see section 2.7)
2.9.2 Cytokines analysed using Bio-plex®.

The concentrations of 21 inflammatory cytokines in patients' serum were analysed using the Bio-plex Pro™ Human Cytokine 21-Plex immunoassay (Bio-Rad Laboratories) based on Luminex xMAP™ technology, according to the manufacturer's protocol.

These included:

interleukin-1a (IL-1a),

interleukin-2 receptor-alpha (IL-2Ra),

interleukin-3 (IL-3),

interleukin-12p40 (IL-12p40),

interleukin-16 (IL 16),

interleukin-18 (IL18),

cutanoeus T-cell-attracting chemokine (CTACK),

growth regulated oncogene-alpha (GROα),

hepatocyte growth factor (HGF),

interferon alpha-2 (IFN-α2),

leukocyte inhibitory factor (LIF),

monocyte chemoattractant protein-3 (MCP-3),

monocyte colony stimulating factor (M-CSF),

macrophage migration inhibitory factor (MIF),

monokine induced by gamma interferon (MIG),

nerve growth factor-beta (b-NGF),

stem cell factor (SCF),

stem cell growth factor-b (SCGF-b),

stromal cell-derived factor-1a (SDF1a),

tumour necrosis factor-beta (TNFβ) and

tumor necrosis factor-alpha (TNF-α).

Samples were analysed in duplicate and data were collected and analysed using **Bio-plex®** Manager Software (Bio-rad Laboratories) version 6.0.

2.9.2.1 Bio-plex® analysis

2.9.2.2 Instrument preparation and plate layout

Bio-Plex® manager software was used to calibrate the Bio-Plex® system before the assay was set up. A calibration validation kit (Lot # 503785) was run to standardise the fluorescent signal in the flow cytometer to ensure that the fluidic and optic systems were running smoothly. A 96 well plate was laid out so that the standards and samples were run in duplicate.

2.9.2.3. Standard and sample preparation

The standards were reconstituted with 500 µl of standard diluent, vortexed for 1-3 sec and incubated on ice for 30 minutes. Subsequently nine 1.5ml Eppendorf's were labelled S1 through S8 and blank. 72µL of standard diluent were added to S1 and 150µl to the rest. 128 µl of the reconstituted standard was then added to S1 from which 50 µls was pipetted out and transferred to S2. This process was repeated through the remaining Eppendorf's creating an eight-point standard curve with a fourfold dilution. The serum samples were diluted 1:4 by adding 1 volume of sample (50µl) to 3 volumes of sample diluent (150 µl).

2.9.2.4. Preparation of coupled magnetic beads

5472 µls of assay buffer and 288 µls of stock coupled beads (according to manufacturer instructions) were added to a 15 ml polypropylene tube creating a

1x concentration of beads. This tube was then vortexed, covered with aluminium foil and allowed to equilibrate at 20^0C for 20 minutes.

2.9.2.5 Assay

50µl of 1x magnetic coupled beads were added to each well which was then placed on a magnetic plate to prevent loss of beads during the washing process. Complete removal of wash buffer was ensured by inverting the plate and patting it dry on a clean paper towel. Following this process, 50µl of standards, blanks, controls and samples were added to the wells. The plate was allowed to incubate on a shaker at 300rpm in the dark for 30 minutes.

2.9.2.6 Assay antibodies

During the time the assay was incubating 1x detection antibodies solution was prepared by adding 150µl detection antibodies to 2850µl of detection antibody diluent (according to the manufacturer's instructions). After 30 minutes of incubation the plate was washed for 3 times with 100µl of wash buffer and 25µl of detection antibodies were added to each well and allowed to incubate in the dark on a shaker at 300 rpm for 30 minutes.

2.9.2.7 Streptavidin –PE (phycoerythrin fluorescent reporter) and plate reading

While the detection antibodies were incubating 60µl of streptavidin-PE was added to 5940µl assay buffer (according to the manufacturer's instructions). After the assay antibodies had incubated, the plate was washed with 100 µl of wash buffer for 3 times and 50 µl of streptavidin-PE was added to each well and allowed to incubate for 10 minutes. This was again followed by a wash with 100 µl of wash buffer for 3 times followed by the addition of 125 µl of assay buffer.

The plate was then covered with sealing tape and placed on a shaker at 1100rpm for 30 seconds. After this process was complete the sealing tape was removed and the plate was placed in the plate reader. The output data were analysed with Bio-plex Manager ™ software version 6.0

2.10 PDGF (Platelet-Derived Growth Factor) ELISA

A PDGF ELISA (Quantitative ® Human PDGF-BB ELISA) manufactured by R&D Systems® (Catalogue No. DBB00) was performed on all the samples as this cytokine was unavailable in the Bioplex® kit.

2.10.1 Standard preparation

All reagents and platelet poor plasma samples were brought to 20^0C. The PDGF-BB Standard was reconstituted with 1.0mL of deionised water producing a stock solution of 20,000pg/mL which was placed on a shaker for 15 mins at 20^0C. A seven point standard curve of PDGF-BB Standards using two fold serial dilutions was created by adding 900µL of Calibrator diluent into the first of seven 1.5mL Eppendorfs and labelled. Thereafter 500µL of Calibration diluent was added to the remaining tubes. 100µL of standard stock solution was added to the first tube which was then thoroughly mixed. This was followed by pipetting 500 µL from tube one into tube two and mixed. The same process was repeated for the remaining tubes.

2.10.2 Assay procedure

Two 96 well plates were used for the analysis. 100 µLs of Assay diluent (RD1X), standard, samples and controls were added to each well in duplicate except for four control samples which were run in single in view of limited space on the plate. The plates were then covered with adhesive strips and wrapped in foil leaving them to incubate for two hours. After the incubation period each well was aspirated and washed four times with 400 µL of wash buffer ensuring that all the liquid in each well was removed by inverting the plate and blotting it against a clean paper towel. 200 µL of PDGF-BB Conjugate was then added to each well and both plates were covered in adhesive strips and foil allowing them to incu-

bate for 2 hours at 20^0C. After incubation, the plates were then washed as described above. 200µL of Substrate solution (Colour reagent A (H_2O_2) mixed with Colour reagent B (stabilized chromagen) 50:50) was added to each well and allowed to incubate in the dark for 30 minutes. 50µL of Stop solution (2N H_2SO_4) was then added to each well. The colour of each well was seen to change from blue to yellow. The plates were then read using a microplate reader, setting the wavelength on the flow cytometer to 450nm. A standard curve was then generated using Microsoft Office Excel.

2.11 PTX3 ELISA (Human Pentraxin 3 Immunoassay)

A PTX3 ELISA manufactured by R&D Systems® (Catalogue No. DPTX30) was performed on all the samples as this cytokine was unavailable in the Bio-plex® kit.

2.11.1 Standard preparation

All samples, standards and controls were treated before being added to the microplate. 900µL of calibrator diluent RDS-24 was added to a polypropylene tube containing 100µL of Pentraxin 3 standard this produced a 20ng/mL high standard. 100µL of Pre-treatment D was then added to the 20 ng/mL polypropylene tube. Following this 10µL of Pre-treatment D was added to 100µL of each sample. All samples were then vortexed and incubated at 20^0C for 30 mins.

Six polypropylene tubes were labelled starting from 10ng/mL, 5ng/mL, 2.5 ng/mL, 1.25ng/mL, 0.625ng/mL and 0.313ng/mL. 500µL of the pre-treated 20ng/ml standard was mixed to each of the above mentioned tubes creating a dilution series.

2.11.2 Assay procedure

All reagents and samples were brought to 20^0C . 100µL of Assay Diluent RD1-56 was added to each well. This was followed by the addition of 20µL of pre-treated Standard, control and samples in their assigned wells. All samples were analysed in duplicate. The plate was then covered with the supplied adhesive strip and allowed to incubate for 2 hours at 20^0C on a horizontal plate shaker.

After incubation each well was aspirated and washed three times by filling each well with 400µL of Wash Buffer using a squirt bottle. The plate was then blotted down on clean paper towels. 200µL of Pentraxin 3 Conjugate was then added to each well and covered with an adhesive strip allowing it to incubate for

a further 2 hours at 20^0C on the shaker. The plate was then washed. 200µL of Substrate Solution was added to each well. Afterwards the plate was covered in tin foil and incubated for 30 mins at 20^0C. 50µL of Stop Solution was added to each well. This induced a colour change in the plate from yellow to blue. The plates were then read using a microplate reader, setting the wavelength on the flow cytometer to 450nm. A standard curve was then generated using Microsoft Office Excel.

2.12 RANKL (Receptor activator of nuclear factor kappa B ligand) ELISA

An sRANK ELISA, manufactured by Biomedical® (Catalogue No. BI-20462) was performed on all the samples as this cytokine was unavailable in the Bio-plex® inflammatory cytokine kit.

2.12.1 Standard preparation

All serum samples and reagents were brought to 20^0C before using the assay. 700µL of distilled water was added to each standard and left at 20^0C for 15 mins. This was followed by the preparation of the wash buffer. Wash buffer was diluted to 1:20; 50ml of wash buffer was added to 950 ml of distilled water and left at 20^0C until required.

2.12.2 Assay procedure

After washing the assay wells with 300µL of diluted wash buffer for 5 times, the plate was tapped against a clean paper towel after the last wash. 50µL of assay buffer was then added to each well and 150µL into the blank wells. Following this 150µL of each sample and control was added in duplicate to their respective wells. After covering the assay plate with supplied plastic film it was allowed to incubate at 20^0C for 2 hours.

When the incubation period was complete each well was aspirated, washed with 300µL of wash buffer for 5 times and the plate tapped dry against a clean paper towel. 200µL of Biotinylated anti- RANKL antibody was added to each well except the blanks wells to which 200µL of assay buffer was added. The plate was gently swirled, covered with plastic film and allowed to incubate at 4°C overnight.

The following morning all wells were aspirated and washed with 300µL of diluted wash buffer for 5 times and the plate tapped dry against a clean paper towel. After 200µLof streptavidin was then added to each well the assay plate was covered in aluminium foil and allowed to incubate in the dark at 20^0C for 30 minutes. After 30 minutes 50 µL of stop solution was added to each well. The plates were then read using a microplate reader, setting the wavelength on the flow cytometer to 450nm. A standard curve was then generated using Microsoft Office Excel.

2.13 Human Bone magnetic bead panel- Milliplex ® Suspension array

Following the successful results obtained from the inflammatory cytokine Bio-plex® we opted to run a human bone magnetic bead panel (Cat.# HBNMAG-51K using Milliplex ® map) to assess the role of bone metabolism biomarkers in vascular calcification.

The concentrations of 7 bone biomarkers were analysed. These included

Interleukin-6 (IL-6)

TNF-α (Tumour necrosis factor)

OPG (Osteoprotegrin)

OC (Osteocalcin)

OPN (Osteopontin)

SOST (Sclerostin)

2.13.1 Serum sample preparation

Serum samples were allowed to thaw completely at 20^0C and vortexed. Samples were diluted 1:2 in assay buffer

2.13.2 Preparation of reagents for immunoassay

A. PREPARATION OF ANTIBODY-IMMOBILISED BEADS

Each and every vial of antibody-bead was vortexed for 1 minute. 150µl form each antibody-bead vial was added to a mixing bottle and made up to 3.0ml with Bead diluent.

B. PREPARATION OF QUALITY CONTROLS

The supplied vials named Quality control 1 and 2 were reconstituted with 250µLdeionised water and allowed to sit for 10 minutes at 20^0C .

C PREPARATION OF WASH BUFFER.

60mls of 10X wash buffer at 20^0C was diluted with 540ml deionised water to make up the wash buffer solution.

D. PREPARATION OF SERUM MATRIX

To the supplied bottle containing lyophilsed serum matrix 1.0ml of deionised water and 1.0ml assay buffer was added and vorted. The reconstituted mixture was allowed to sit at 20^0C.

E. PREPARATION OF HUMAN BONE STANDARD

The supplied Human Bone Standard was reconstituted with 250 µL of deionised water to create a stock solution and labelled Standard 7 (reconstituted standard). Six polypropylene microfuge tubes were labelled Standard 1-6 to which 150µL of assay buffer was added. Serial dilutions were then prepared by adding 50µL of Standard 7 (reconstituted standard) to Standard 6 mixed and 50µL transferred to Standard 5. The rest of the Standards were created in this way.

F.IMMUNOASSAY PROCEDURE

1. 200 µL of Assay buffer was added to each well of the plate , sealed with the supplied plate sealer tape and placed on a shaker at 20^0C for 10 minutes
2. Assay buffer was then removed by tapping the plate on clean paper towels. 25µL of each Standard and Control was added to the appropriate wells followed by 25µL of Assay buffer.

3. 25µL of the appropriate matrix solution was then added to the wells followed by 25µL of the Samples.

4. After 25µL of Premixed beads were added to each well the plate was sealed with a plate sealer, covered with foil and placed on a plate shaker at 20^0C for 2 hours.

5. Following incubation the plate contents was gently tapped against a clean paper towel and washed with 200µL of Wash buffer for 3 times.

6. After adding 50µL of detection antibodies to each well the plate was sealed, covered in foil and incubated on a shaker for 30 minutes at 20^0C .

7. Following the incubation period the plate was uncovered and 50 µL of Streptavidin-Phycoerythrin and 50µL of Detection antibodies were added to each well.

8. The plate was again sealed and covered in foil and allowed to incubate on a plate shaker for 30 mins at 20^0C .

9. Following the incubation period the plate was washed with 200 µL of wash buffer for 3 times

10. 100 µL of Sheath fluid was added to each well and the plate was placed on a plate shaker for another 5 minutes.

11. The plate was run on a Luminex 200™ platform.

2.14 Statistical Analysis

Statistical analyses of data were carried out using SPSS version 19.0 (SPSS, Inc, Chicago, IL) using either Kruskal Wallis, Mann-Whitney U or Spearman's correlation coefficient non-parametric tests, taking $P<0.05$ as statistically significant. Chi-squared test was used for categorical data. All data analysis was discussed with a statistician at the University of Manchester.

CHAPTER 3

Results

3.0 Results

3.1 Patient details and clinical demographics

Blood samples from 70 patients undergoing a carotid endarterectomy as well as 20 healthy age matched controls were analysed. Controls patients had no past history of vascular disease or evidence of carotid disease on duplex ultrasound. Study patients included 19 asymptomatic patients who were diagnosed with carotid disease as part of their work up for cardiac or vascular surgery or incidentally diagnosed part of a routine vascular or diabetic examination. The 51 symptomatic patients were those patients who have suffered a stroke. These patients were divided into three groups i) patients with **retinal embolic disease**: patients who had focal or complete loss of vision for more than 24 hours, ii) patients with a **transient ischaemic attack** (TIA): patients who had symptoms lasting less than 24 hours. This group included patients with amaurosis fugax, upper or lower limb weakness or hemiplegia and iii) patients with **acute stroke**: this group included patients who had neurological symptoms lasting more than 24 hours. The demographic data and patient's current medication have been included in Table 5. Table 6 shows the duplex assessment results of the patient's scans prior to surgery.

	Asymptomatic (n=19)	***Symptomatic (n=51)***	**p value**
Age (average years) (Range)	67 (48-87)	70 (54-83)	0.05
Male	13 (68%)	26 (51%)	0.23
Diabetic	6 (32%)	10 (20%)	0.28
Hypertension	16 (84%)	46 (90%)	0.50
Hypercholesterolemia	15 (79%)	43 (84%)	0.56
Coronary artery disease [a]	4 (21%)	23 (45%)	0.07
Renal Disease [b]	3 (16%)	8 (16%)	0.99
Anti-platelet agent	17 (89%)	49 (96%)	0.30
Anti-hypertensive	16 (84%)	46 (90%)	0.48
Statin	19 (100%)	48 (94%)	0.28
Smoking	13 (68%)	22 (43%)	0.06

Table 5. Patient Demographics

[a] Coronary disease defined as having a history of any of the following: arrhythmia, myocardial infarction, congestive heart failure, previous coronary revascularization (percutaneous or surgical) and angina.

[b] Renal disease defined as patients with an eGFR GFR <60 mL/min/1.73 m^2.

% ICA stenosis	50-70%	70-99%
Asymptomatic	0	19
Symptomatic	33	18

Table 6 Duplex categories. The table above shows the results of duplex scan assessments of ICA stenosis in asymptomatic and symptomatic patients classified into categories of percentage stenosis. **Abb-** ICA (Internal carotid artery)

3.2 Gross plaque morphology

Representative samples of the different plaques studied are illustrated in Figure17. These plaques show a marked difference in morphology between the asymptomatic and symptomatic patient groups. In Figure 17, (A) shows an asymptomatic plaque of a patient with 80% stenosis of the ICA. This plaque had a smooth surface and no ulceration with a pronounced, non-ruptured atherosclerotic plaque with a thick fibrous cap which was a common occurrence amongst asymptomatic plaques. In Figure 17 (B), (C) and (D) we have symptomatic plaques which ruptured resulting in symptoms to a varying degree. Figure 18 shows representative plaques from patients with stable and unstable asymptomatic disease.

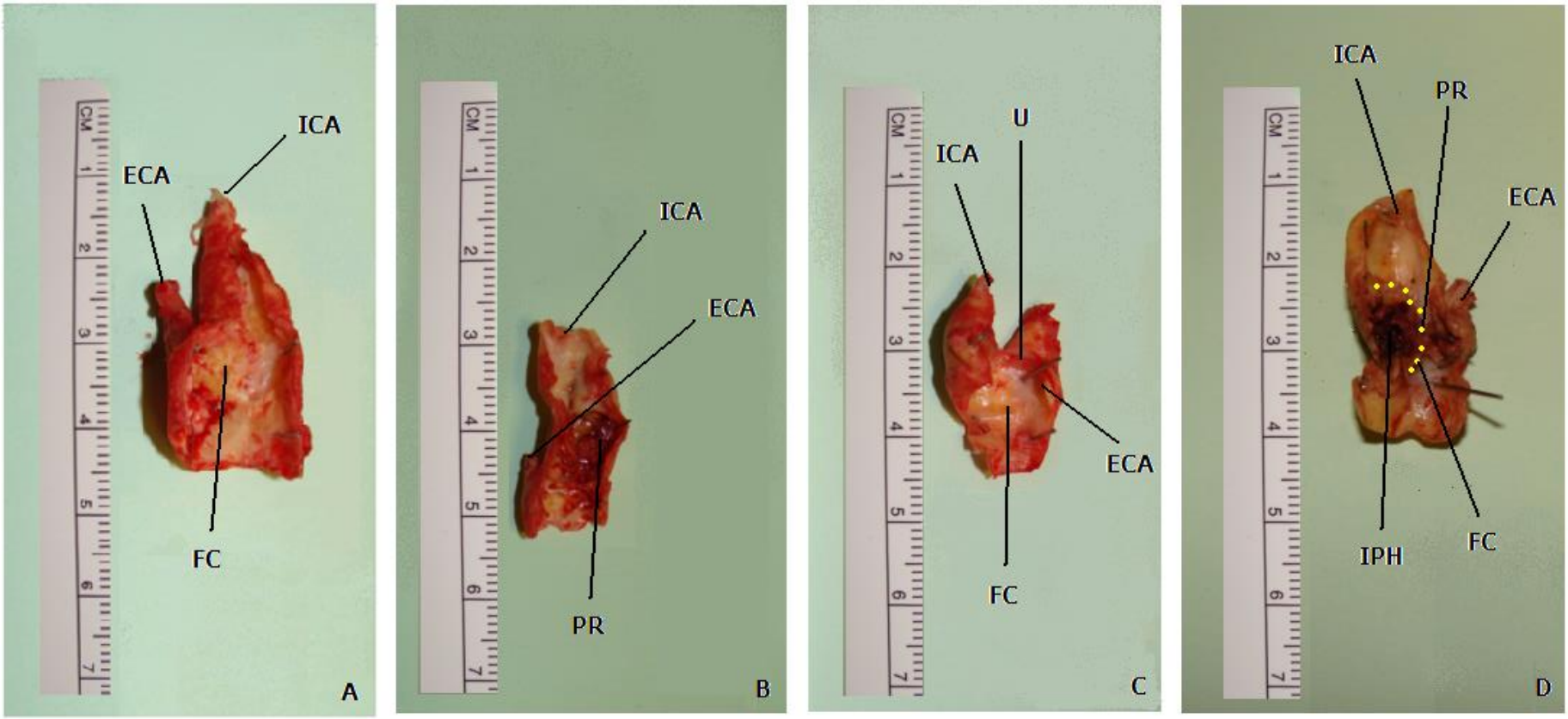

Figure 17. Plaque variability in patients. Representative images of plaques extracted from patients in the operating room showing gross differences in morphology. (A), Plaque from an asymptomatic patient. (B), Plaque from a symptomatic patient with retinal embolic disease. (C), Plaque from a symptomatic patient with amaurosis fugax (D), Plaque from a symptomatic patient who had an acute stroke, showing intra-plaque haemorrhage and a ruptured fibrous cap (dotted line).**FC**- Fibrous cap, **PR**- Plaque rupture, **U**- Ulceration, **IPH**- Intra-plaque haemorrhage, **ICA-** Internal carotid artery, **ECA-** External carotid artery

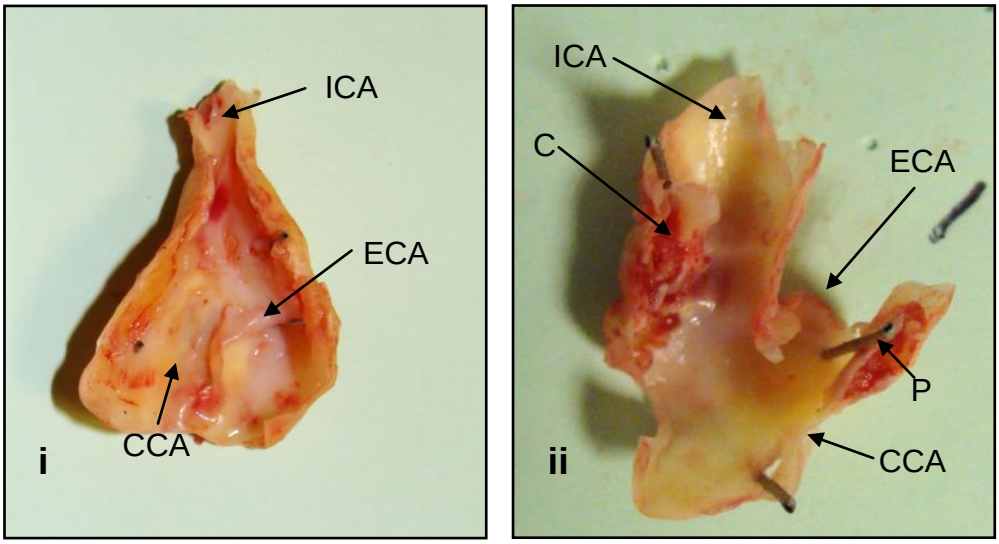

Figure 18. Plaques form two asymptomatic patients. (i) shows a stable plaque with a smooth fibrous cap compared to (II) which shows a friable unstable non- ruptured plaque, P.

P- plaque, **ICA-** Internal carotid artery, **ECA-** External carotid artery**, CCA-** External carotid artery

3.3 Histological analysis

Table 7 shows the difference in microscopic plaque characteristics between the symptomatic and asymptomatic plaques. Plaque ulceration was significantly greater in the symptomatic plaques, 90%, compared to 26% in asymptomatic group. Intra-plaque haemorrhage was noted in 82% of symptomatic and 26% in the asymptomatic group.

	Asymptomatic (n=19)	**Symptomatic (n=51)**	**P Value***
Plaque rupture	2 (10%)	40 (80%)	0.00
Fibrous cap thinning	5 (26%)	47(93%)	0.00
Intra-plaque haemorrhage	5 (26%)	42 (82%)	0.003
Necrotic core	15 (79%)	40 (78%)	0.96
Ulceration	5 (26%)	46 (90%)	0.01
Thrombus	2 (10%)	45 (88%)	0.00
Calcification	18 (95%)	40 (80%)	0.05

Table 7 Microscopic plaque characteristics

*Mann-Whitney U test used (non-parametric and not normally distributed)

Figures 19 and 20 show images captured using a DF320 camera mounted on a Leica 5000B microscope of asymptomatic and symptomatic plaques stained with different immunohistological techniques.

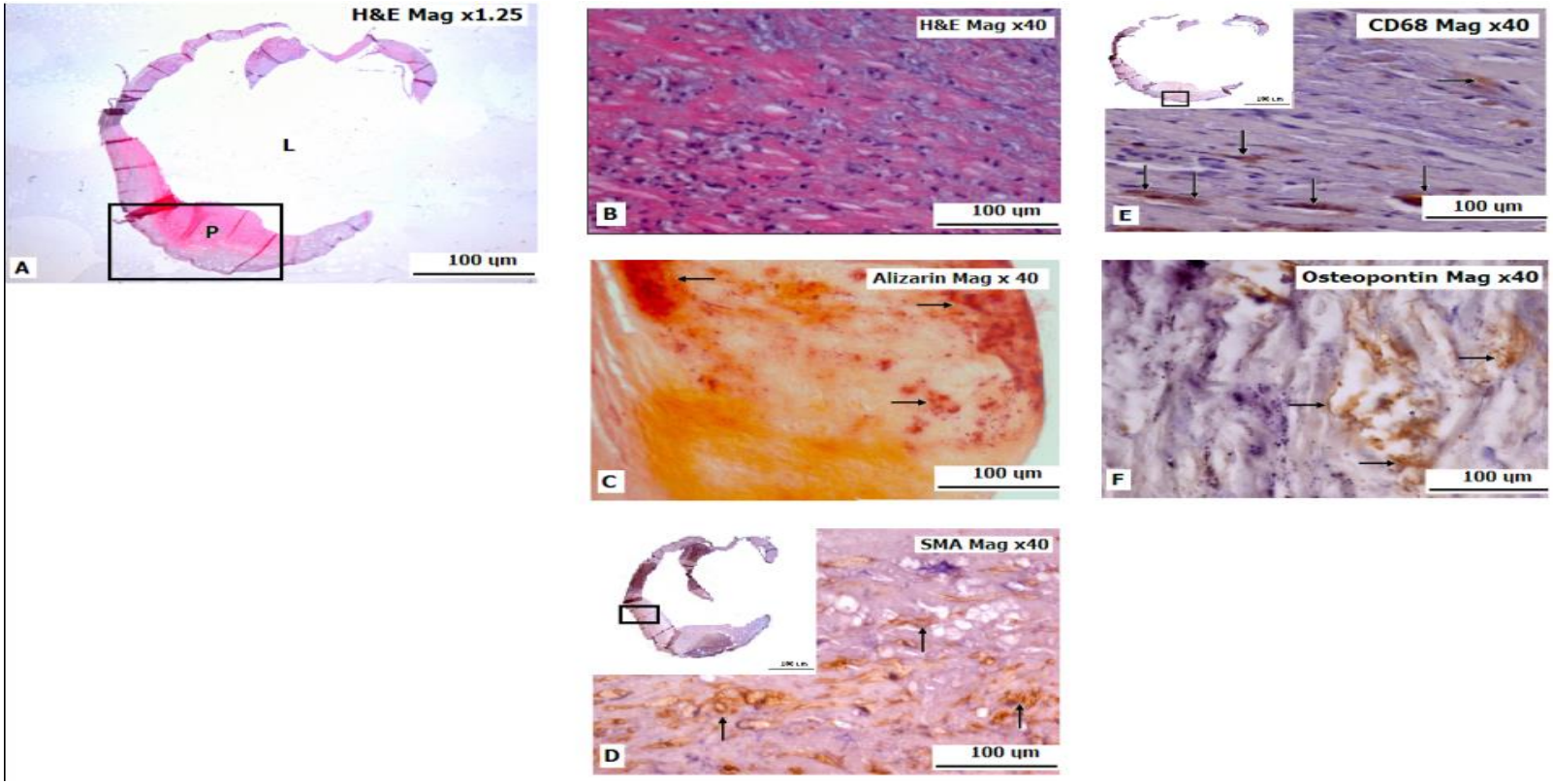

Figure 19. **Representative staining of a asymptomatic carotid plaque**. The black box highlighted in (A) shows the region from which the higher magnification micrographs are taken and stained with different antibodies. (A) & (B) show H&E staining. (C) & (F) show areas which were stained with different stains for calcium denoted in black arrows.(P) represents the area of plaque (L) represents the vessel lumen. (E) Shows the presence of $CD68^{+}$ macrophages in black arrows (D) Smooth muscle actin (SMA) stain showing SMA cells in the media.

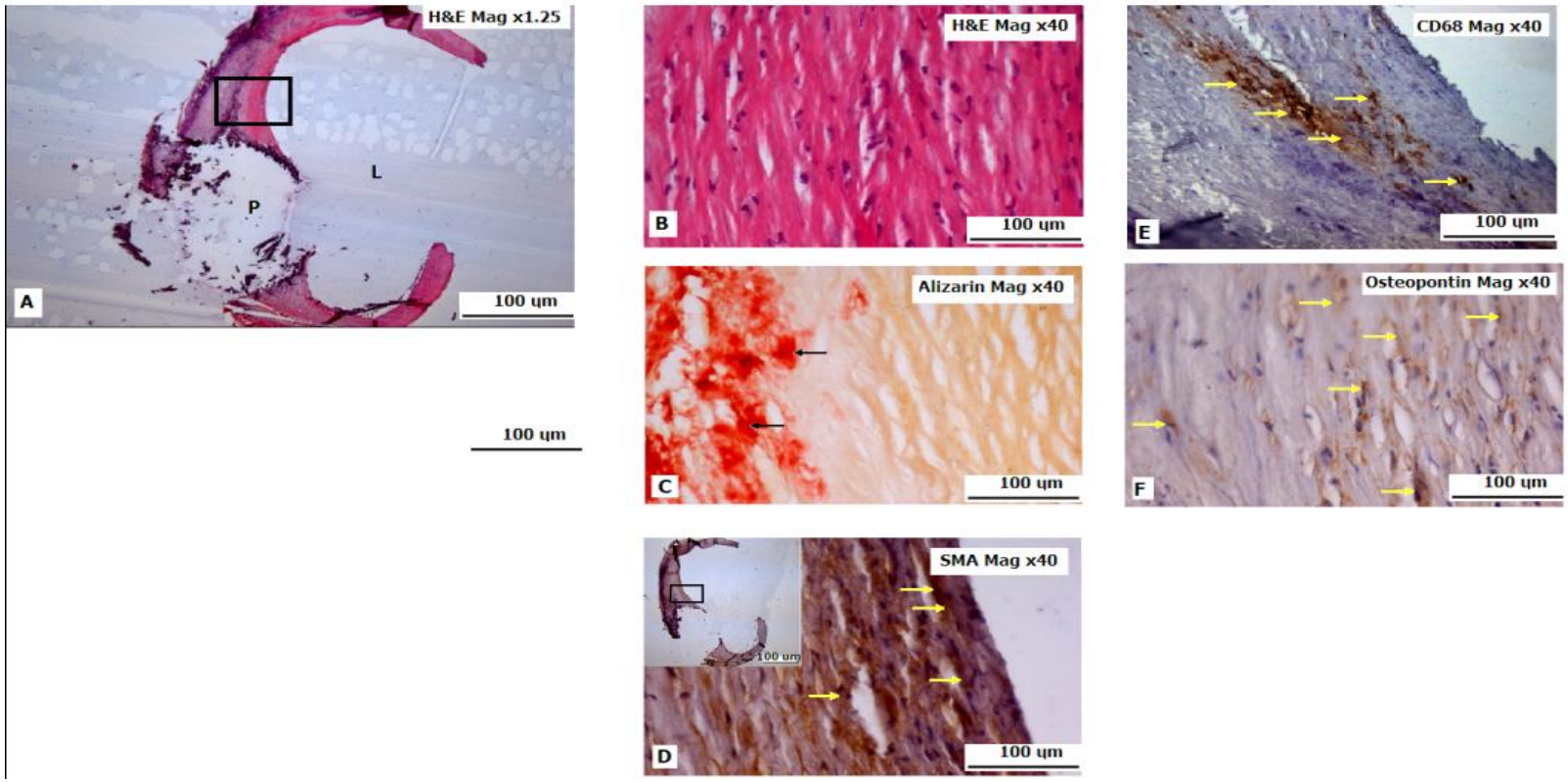

Figure 20. Representative staining of a symptomatic carotid plaque. The black box highlighted in (A) shows the region from which the higher magnification micrographs are taken and stained with different antibodies. (A) &(B) show H&E staining. (C)&(F) show areas which were stained with different stains for calcium denoted in black and yellow arrows respectively (P) is the region where plaque and a large area of calcium were present and (L) represents the vessel lumen. (E) Shows the presence of CD68 +ve macrophages which is the same area as the dark purple stain in (A) shown in yellow arrows. (D) Smooth muscle actin (SMA) stain showing SMA cells close to the endothelial surface shown in yellow arrows.

Figure 21 is a representative section through a plaque from a symptomatic patient with an acute stroke. This plaque was heavily stained with CD68 antibody showing infiltration by CD68 positive macrophages (inflammatory) in the 'shoulder region' of the plaque at the junction with the fibrous cap which is the most vulnerable region in a plaque. (Boyle *et al.*, 2003)

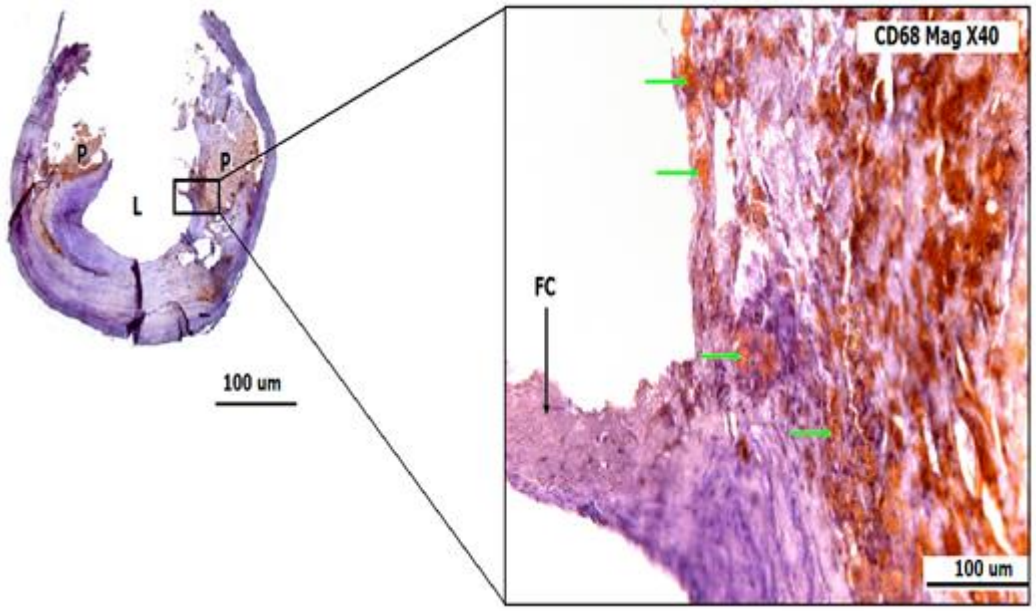

Figure 21. Symptomatic plaque from a patient with an acute stroke stained for CD68^{+} macrophages. (P) denotes the plaque and (L) the lumen. The green arrows point at the brown areas of plaque positive for CD68 macrophages. (FC) – fibrous cap.

The Figure 19 and 20 show a difference in the location of SMCs in the two different plaque types. In the asymptomatic plaques, smooth muscle cells were located within the media whilst excessive amounts of SMC were found close to the endothelial surface in symptomatic plaques. Proliferation and intimal migration of SMCs is an important factor in plaques progressing to become unstable since SMCs are responsible for the release of inflammatory cytokines that are capable of degrading the fibrous cap that leads to plaque instability and eventually rupture. (Stintzing *et al.*, 2009).

Unstable plaques from both groups of patients were shown to have significantly higher levels of the CD68 macrophage marker. See Figure 22.

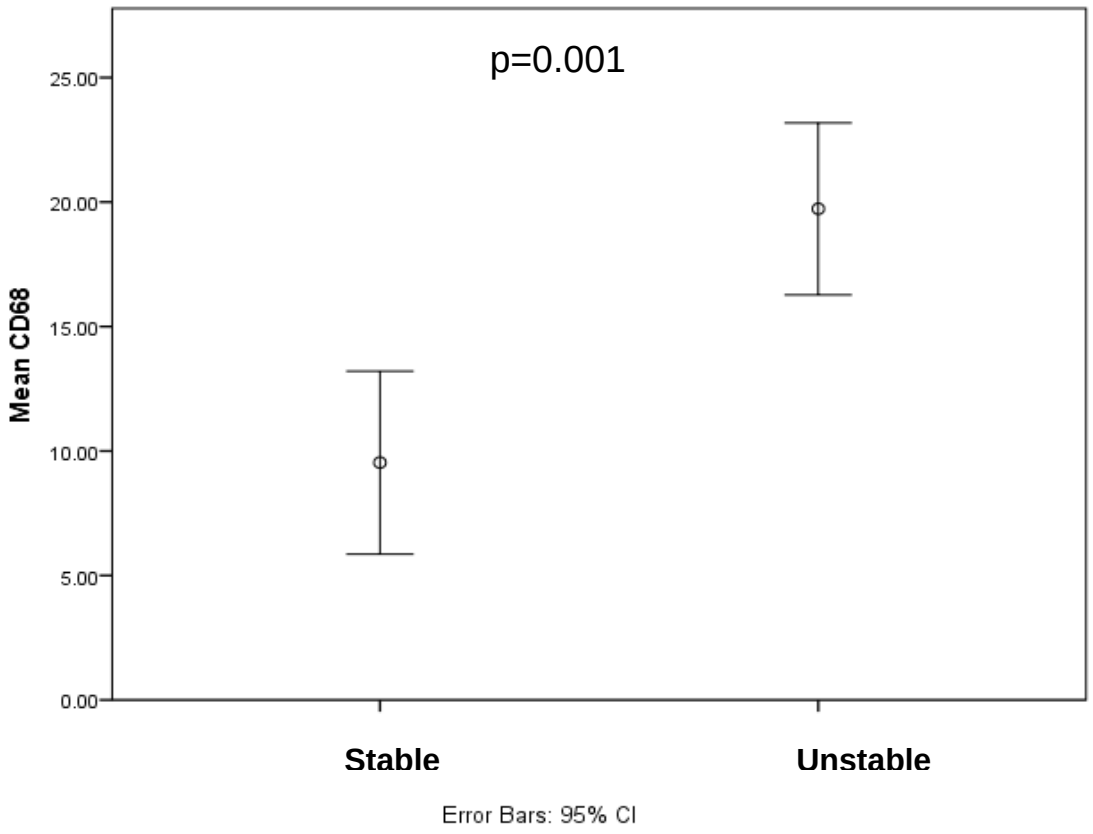

Figure 22. Graph of mean CD68 staining vs plaque stability. Staining quantification was carried out using Image J software (NIH, Bethesda, MD).Intact CD 68+ve cells were counted in the entire plaque section showing the maximal disease. Absolute count was divided by the plaque area to produce a value of counts per mm^2.

Asymptomatic and symptomatic patients groups were analysed with respect to intra-plaque calcification. Plaques from both patient groups exhibited substantial amounts of calcification, evident as spicules and as large blocks, highlighted by the alizarin red staining in Figure 23. Intense mineralisation of plaques seems to be an important contributor to plaque stability (Davaine *et al.*, 2014) Quantification of the alizarin red staining revealed that although the difference between groups was small,

plaques from asymptomatic patients exhibited significantly more alizarin red staining than the symptomatic group , see Figure 23

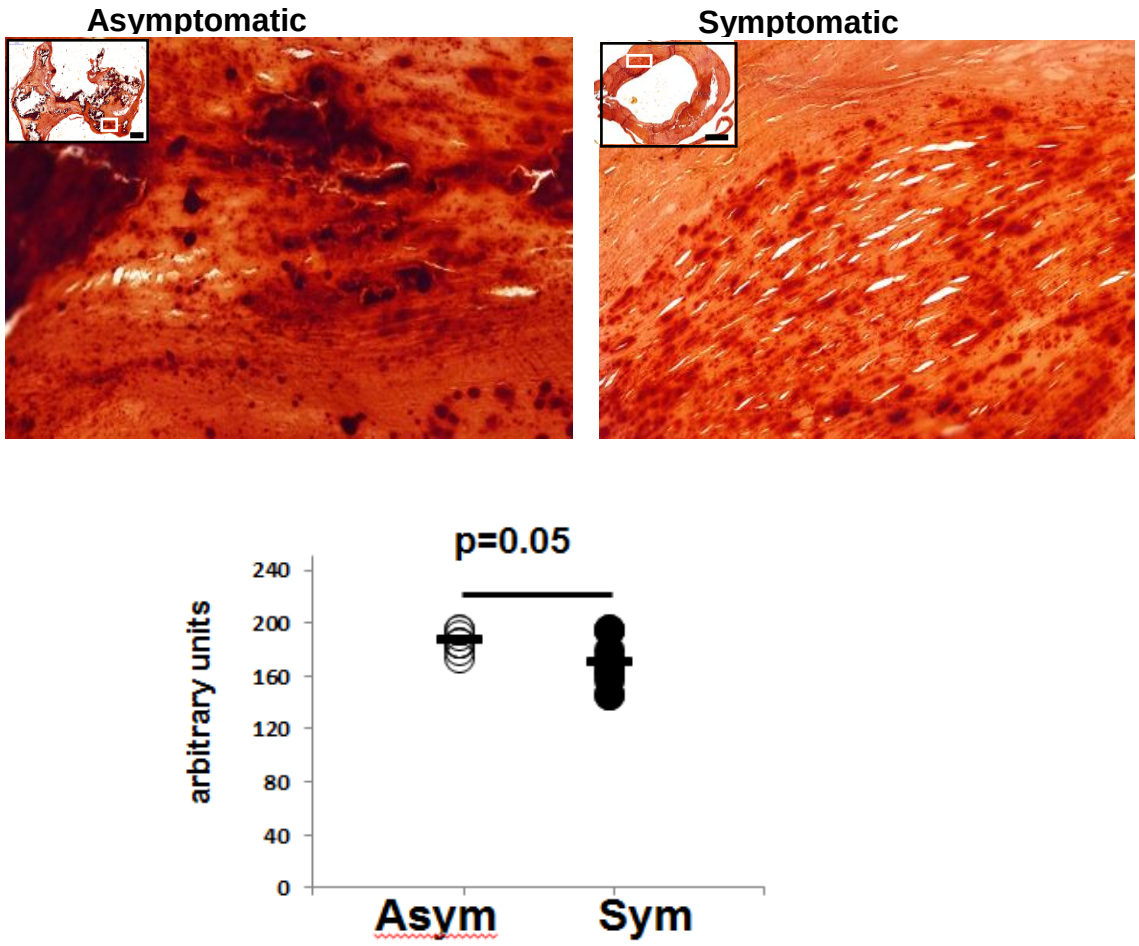

Figure 23. Alizarin red highlighting areas of calcification. Plaques from asymptomatic patients exhibited significantly more alizarin red staining compared to plaques from symptomatic patients. Calcification is shown as dark brown stains. Data were analysed using Mann Whitney U statistical test. Bars represent the median. Inset image; bar=1000µm. Main image; bar=100µm.

3.4 EMP analysis

3.4.1 EMP levels in symptomatic, asymptomatic and control patients

Annexin V^+/$CD31^+CD42b^-$ EMPs were detected in platelet poor plasma samples using flow cytometry. EMP levels in each of the patient groups were plotted see Figure 24. EMPs were significantly elevated in asymptomatic and symptomatic groups when compared to healthy controls ($p=0.03$ and $p=0.001$ respectively) No differences were detected between the asymptomatic and symptomatic groups ($p=0.54$).

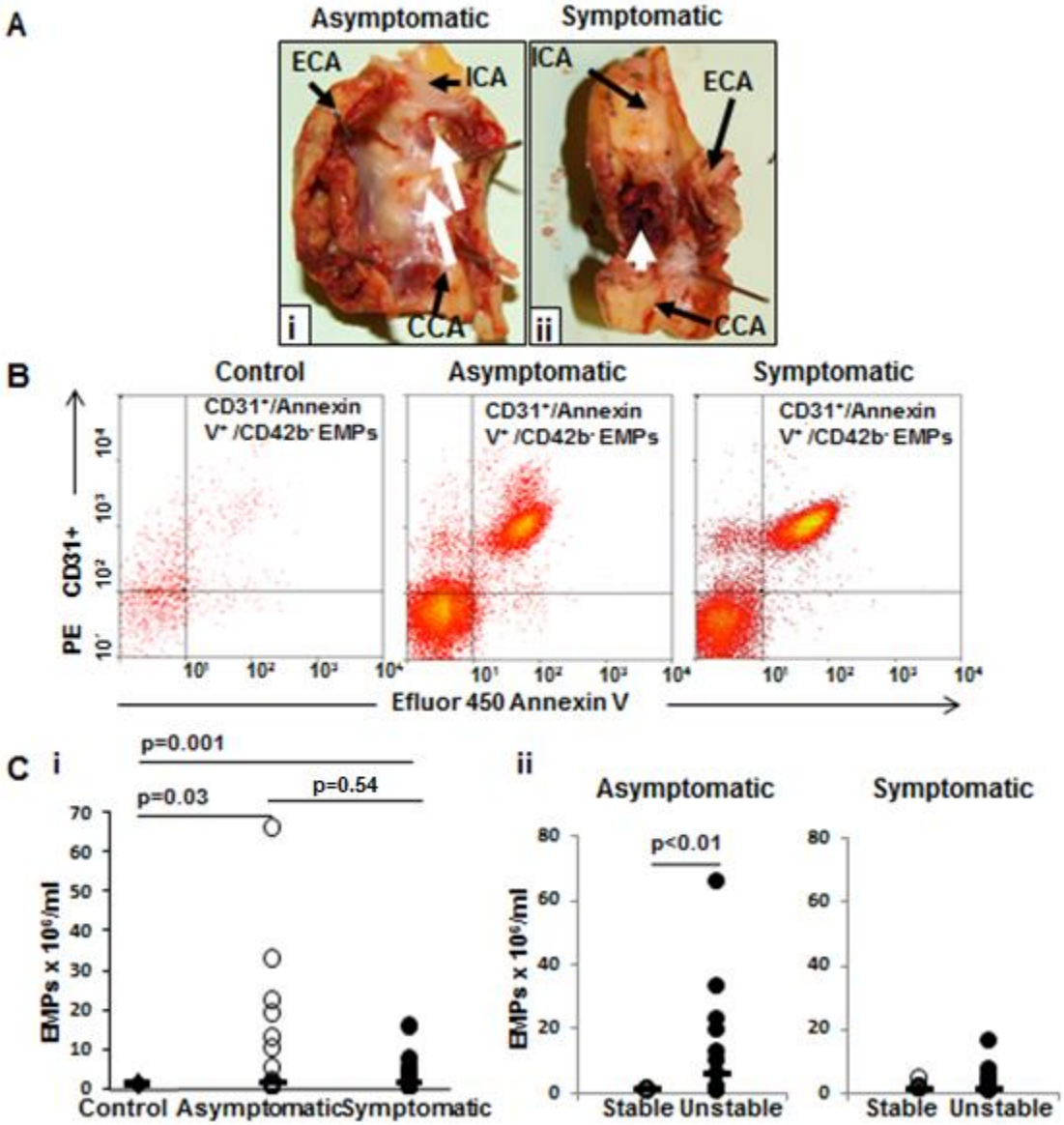

Figure 24. EMPs are significantly elevated in patient plasma compared to healthy controls.

(A) Representative images of the gross morphology of plaques harvested from i) asymptomatic and ii) symptomatic plaques highlighting a plaque ulcer (arrow) and an intra-plaque haemorrhage (arrow head). (B) Representative flow plots from each group. (C) EMPS were defined as CD31+/AnnexinV+/CD42- and counted.

3.4.2 EMP levels in the symptomatic group

Further analysis was performed on the symptomatic group of patients. These patients were subdivided into three; i) acute stroke, ii) TIA and iii) retinal embolic disease, see Figure 25. Statistical analysis using the Mann-Whitney U test showed a significant difference between the acute stroke and TIA groups when compared to controls with a p value of 0.01 and 0.004 respectively but there was no significant difference in the retinal embolic disease group which had very low levels of EMPs comparable to the control patient group, p-value 0.89.

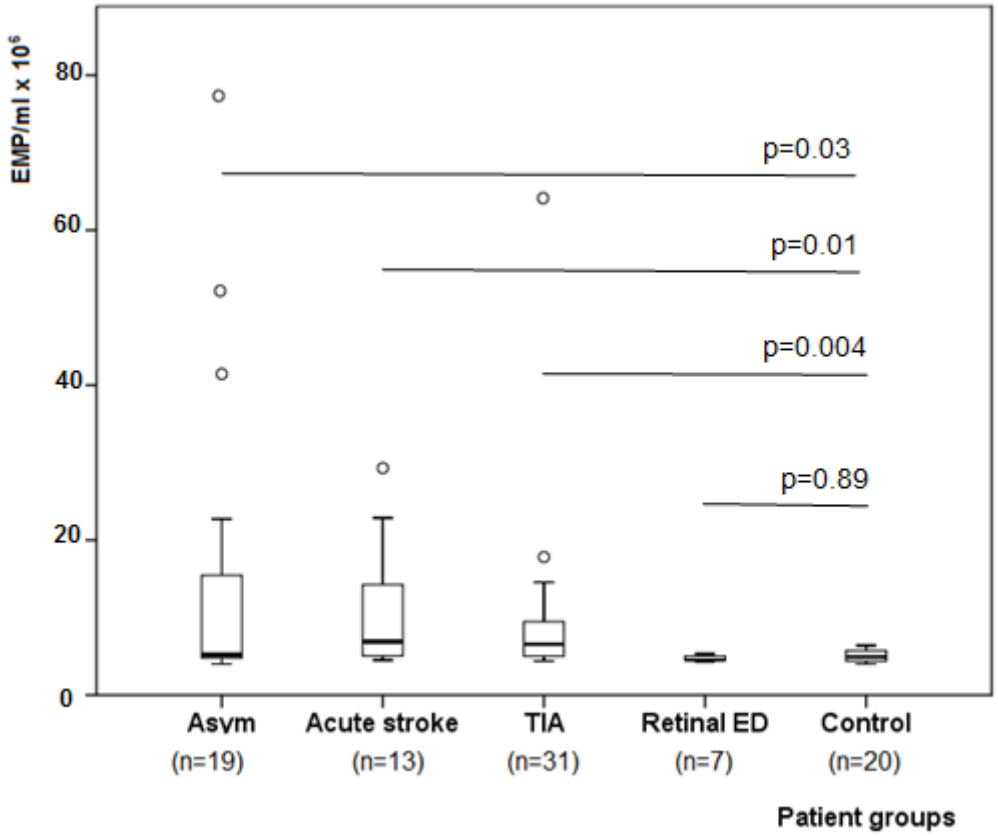

Figure 25. Box and whisker plot showing EMP/ml for each patient group and controls. Abb- Transient ischaemic attack (TIA), Retinal embolic disease (Retinal ED) EMP levels in symptomatic and asymptomatic stable and unstable plaques

EMP levels in stable and unstable plaques from both symptomatic and asymptomatic patients were analysed. Significantly elevated EMPs were detected in unstable plaques compared to stable plaques in the asymptomatic patient group. ($p<0.009$) No differences were observed in th symptomatic patient group, $p = 0.693$. See Figure 26

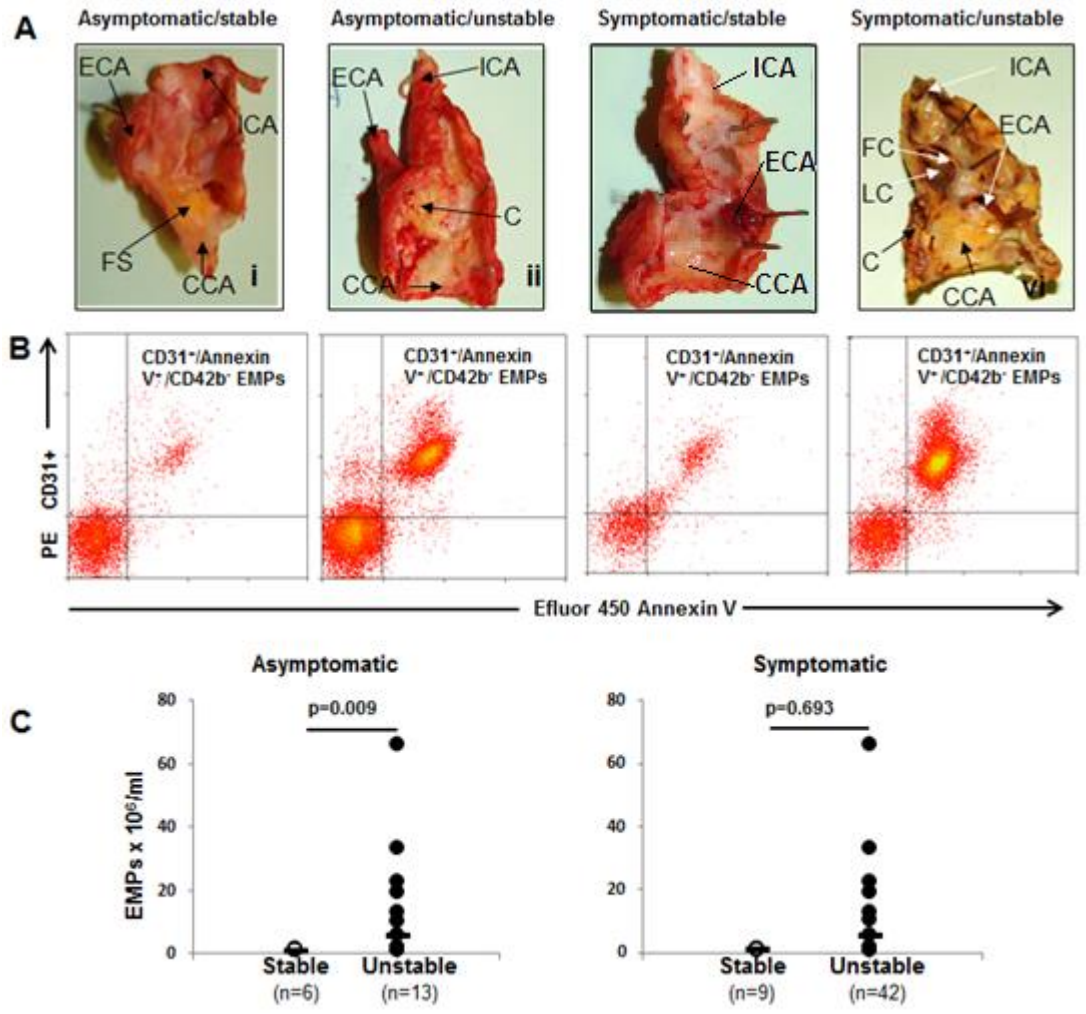

Figure 26. EMPs are significantly elevated in patients with unstable asymptomatic plaques.
(A) Representative images of the gross morphology of plaques harvested from asymptomatic and symptomatic plaques both stable and unstable i-vi. (B) Representative flow cytometry plots of each group.
(C) Bar graphs with SEM bars of patients with asymptomatic and symptomatic disease showing a significant difference in EMPs in the asymptomatic stable vs unstable group p=0.009
Abb- Common carotid artery (CCA), external carotid artery (ECA), internal carotid artery (ICA), fibrous plaque (FP), calcium (C), fibrous cap (FC), lipid core (LC)

3.4.3 EMP levels in stable and unstable plaques in symptomatic and asymptomatic groups combined together

EMP data from both stable and unstable plaques from both groups were combined and analysed. The results showed significantly higher levels of EMPs in the unstable groups with a p value of 0.035. This result confirms the importance of EMPs as potential biomarkers of carotid plaque instability. See Figure 27.

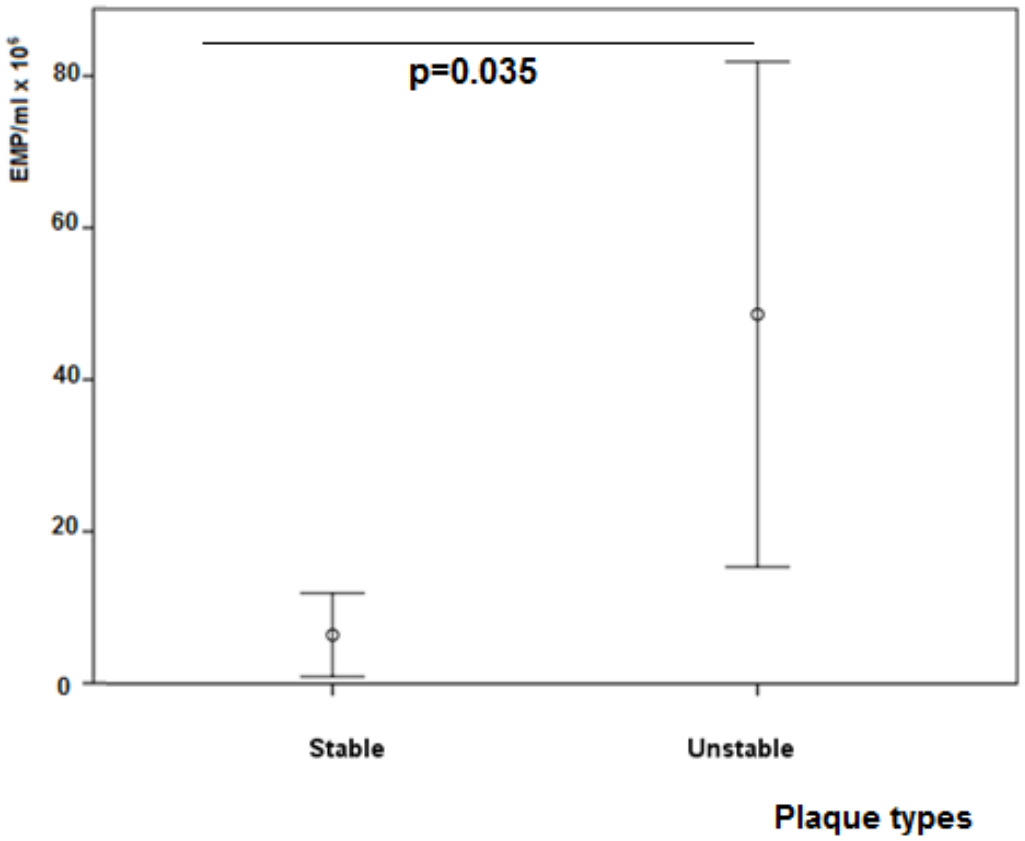

Figure 27. Graph of EMPs/ ml vs plaque stability. Significantly elevated EMPs in unstable plaques. Error Bars: 95% CI

3.5 Cytokine analysis- Systemic inflammatory profiling of patients

3.5.1 Bio-plex® analysis

Out of the 21 markers of inflammation analysed, six were differentially regulated in the asymptomatic and symptomatic groups compared to controls. See Figure 28. CTACK, HGF, IL-3, IL-16 were significantly higher in asymptomatic patient serum compared to healthy controls ($p<0.02$) with a trend towards elevation of MIG a significant reduction in MIF. CTACK, IL-3, IL-16 and MIG were significantly elevated in symptomatic patient serum compared to healthy controls with a trend towards an increase in HGF. No significant differences were detected between asymptomatic and symptomatic patients or in the levels of IL-2Rα, SCGF, IL-18, SDF-1 or SCF between any of the groups. Il-1α, GROα, IFN-α2, LIF, MCP-3, M-CSF, β-NGF, SDF-1α, TNF-β and TRAIL were below the level of detection and not data was available to perform any analysis.

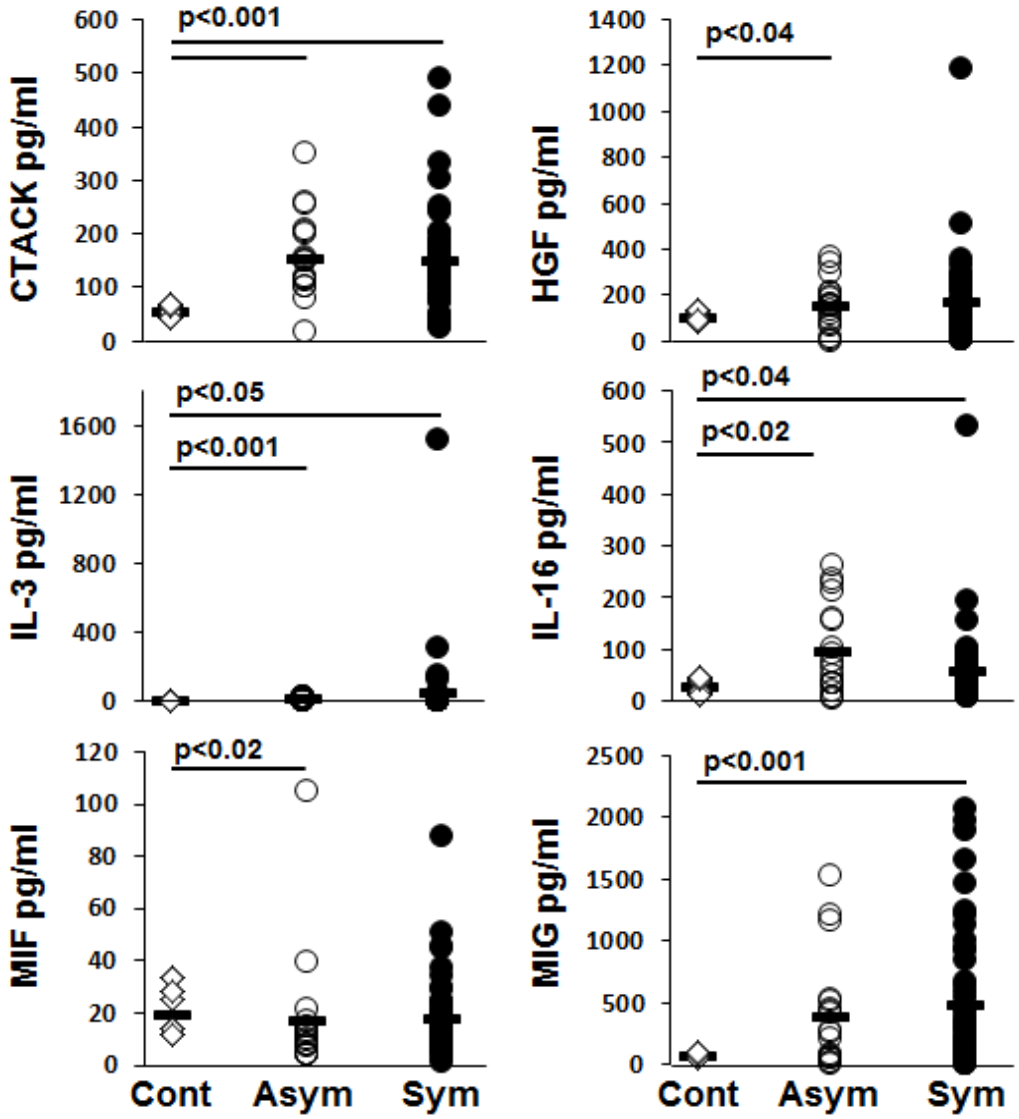

Figure 28. CTACK, IL-3 and IL-16 were significantly elevated in both asymptomatic and symptomatic groups compared to healthy controls.
Data were analysed using Kruskal Wallis statistical test. Bars represent the means. Cont- healthy controls (n=20); Asym-asymptomatic (n=19); Sym-symptomatic (n=51).

3.5.2 Inflammatory markers in patients with stable and unstable plaques

Inflammatory markers between unstable and stable plaques in both patient groups were compared. In the asymptomatic patient group, MIG and SCGF-b were significantly elevated ($p=<0.005$) in patients with unstable compared to stable plaques, whereas IL-16 and MIF were significantly elevated in the stable plaque group see Figure 29. HGF was significantly elevated in symptomatic patients with unstable plaque ($p<0.05$) with no other differences detected within the symptomatic group (Figure 4). No significant differences were detected in IL-2Rα, IL-12P40, CTACK, IL-3, IL-18, or SCF-1 between stable and unstable plaques in either the asymptomatic or symptomatic group.

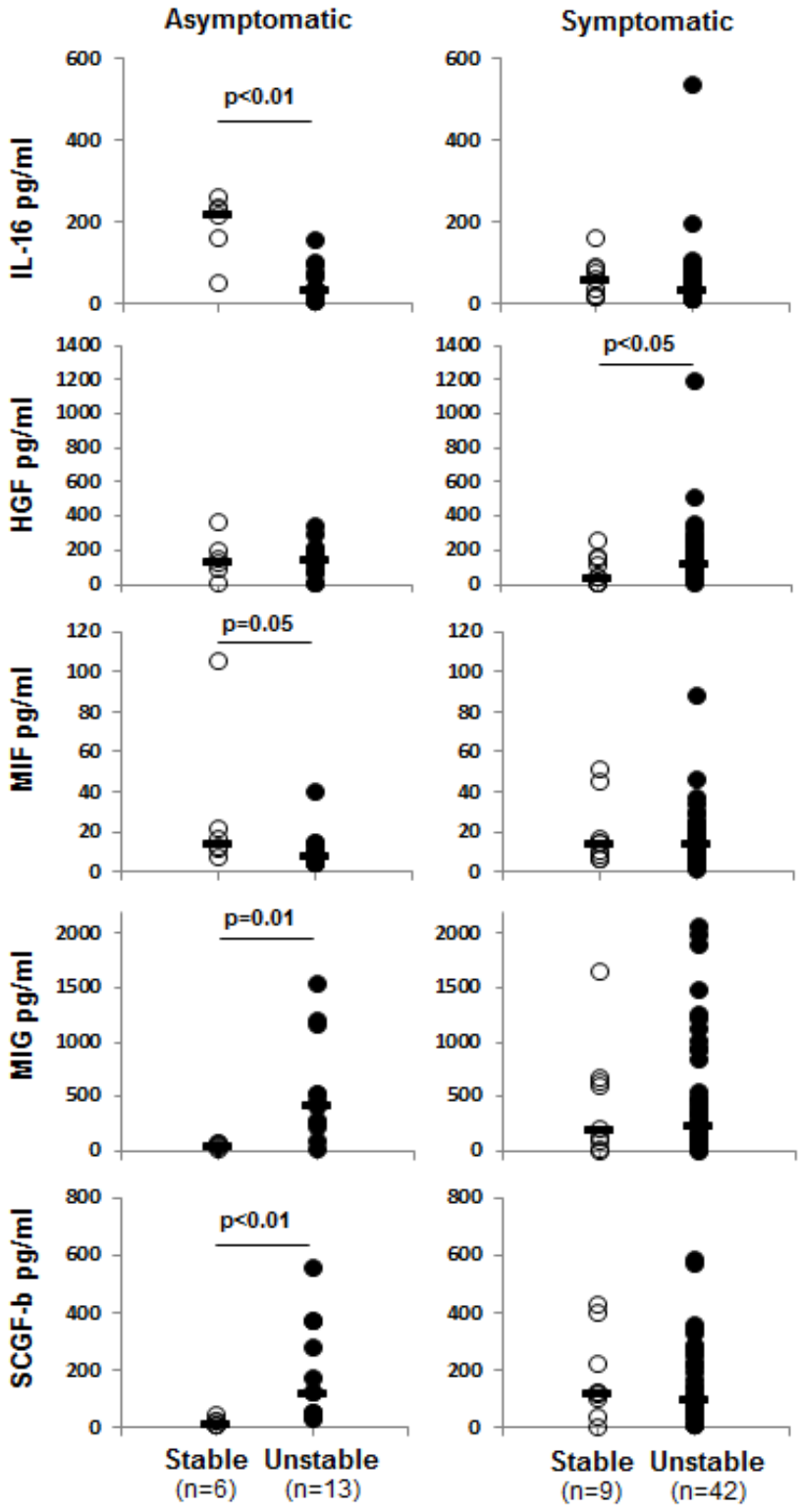

Figure 29. MIG (CXCL-9) and SCGF-b are elevated in asymptomatic patients with unstable plaques. Data were analysed using Mann Whitney U statistical test. Bars represent the median.

3.5.3 Cytokine levels in all stable and unstable plaques from the two groups combined

Following the previous analysis, stable and unstable plaques from both groups were combined with a view to determine whether we could correlate serum bi-omarkers to plaque vulnerability. When all cytokines are compared to plaque stability IL-16 was raised in the stable plaques, p=0.01 see Figure 30, whilst MIG (CXCL9) was raised in the unstable plaques, p=0.04, see Figure 31.

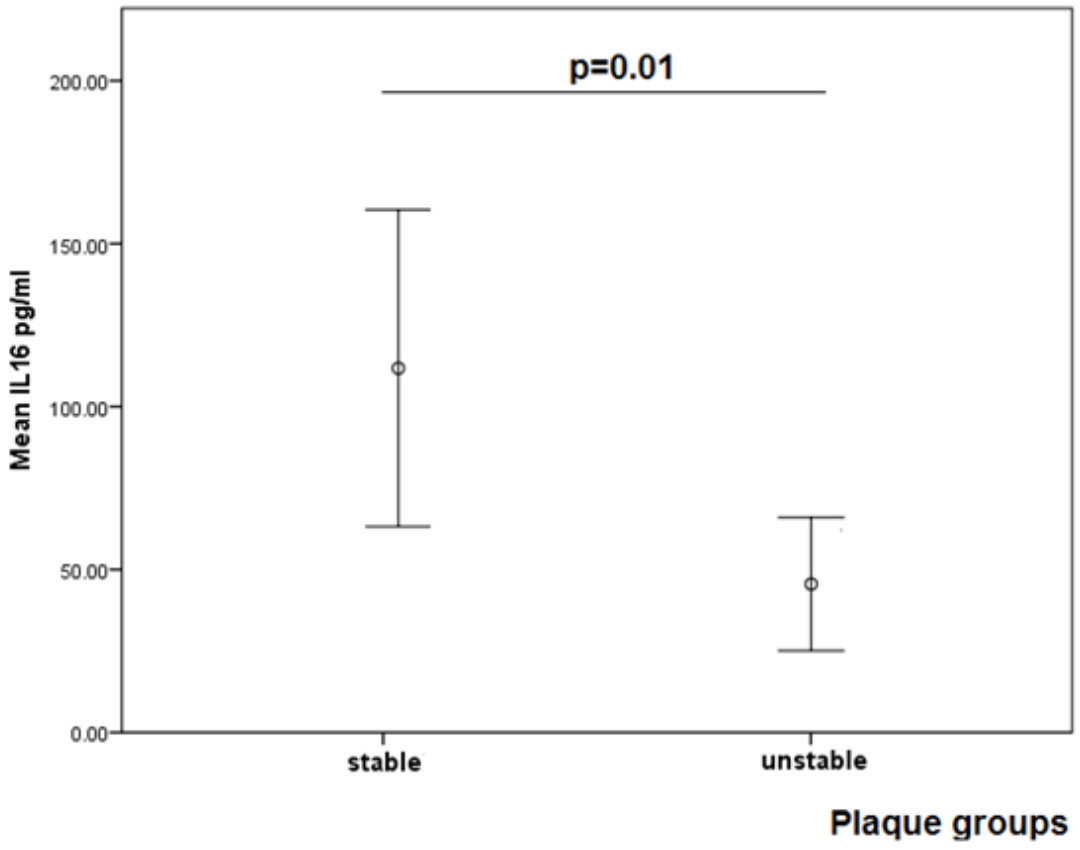

Figure 30. Graph of mean IL-16 levels vs plaque stability.
Error Bars: 95% CI

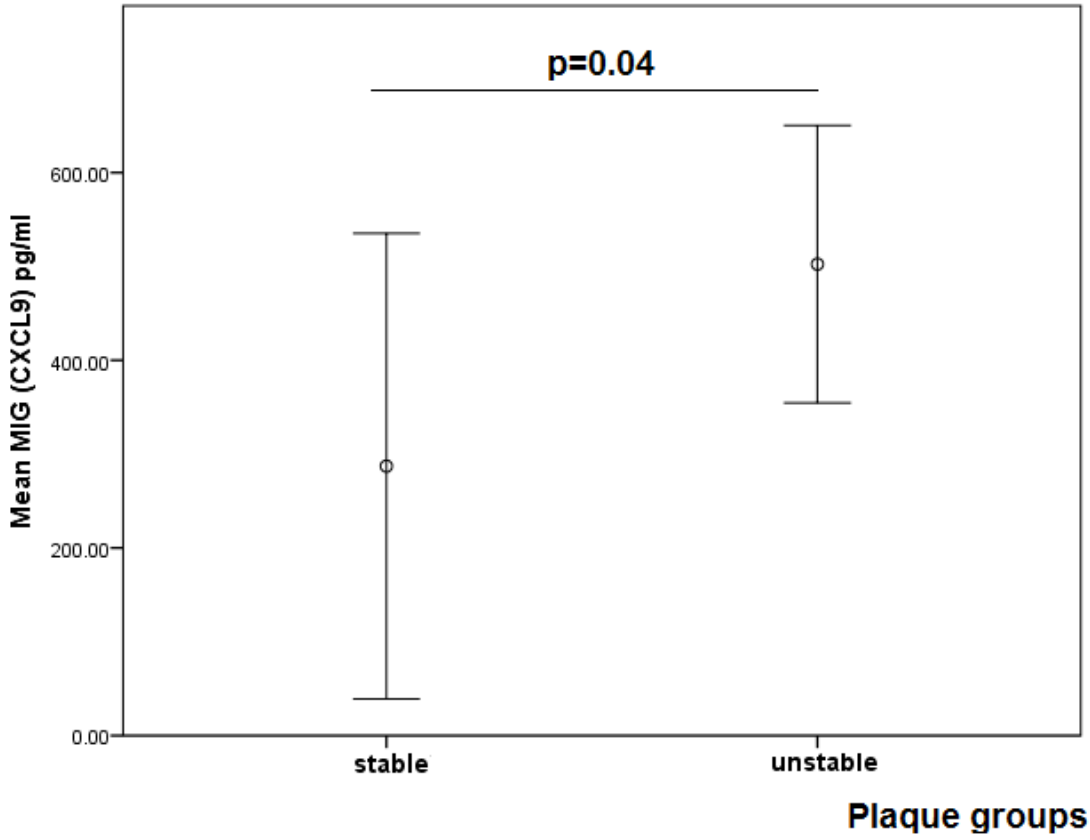

Figure 31. Graph of mean MIG (CXCL-9) levels vs plaque stability.
Error Bars: 95% CI

3.5.4 Immuno-histochemical analysis of intra-plaque cytokines

Further immuno-histochemical analysis was performed on carotid plaques with a view to locating their presence within plaques. MIG and SCGF were shown to be present in plaques in close proximity to CD68 stained macrophages. This finding indicates that these macrophages may be releasing these cytokines as part of the inflammatory response. Furthermore, there may be an ‘over spill’ of these cytokines into the circulation hence the reason why they were detected with Bioplex® analysis of serum. See Figure 32.

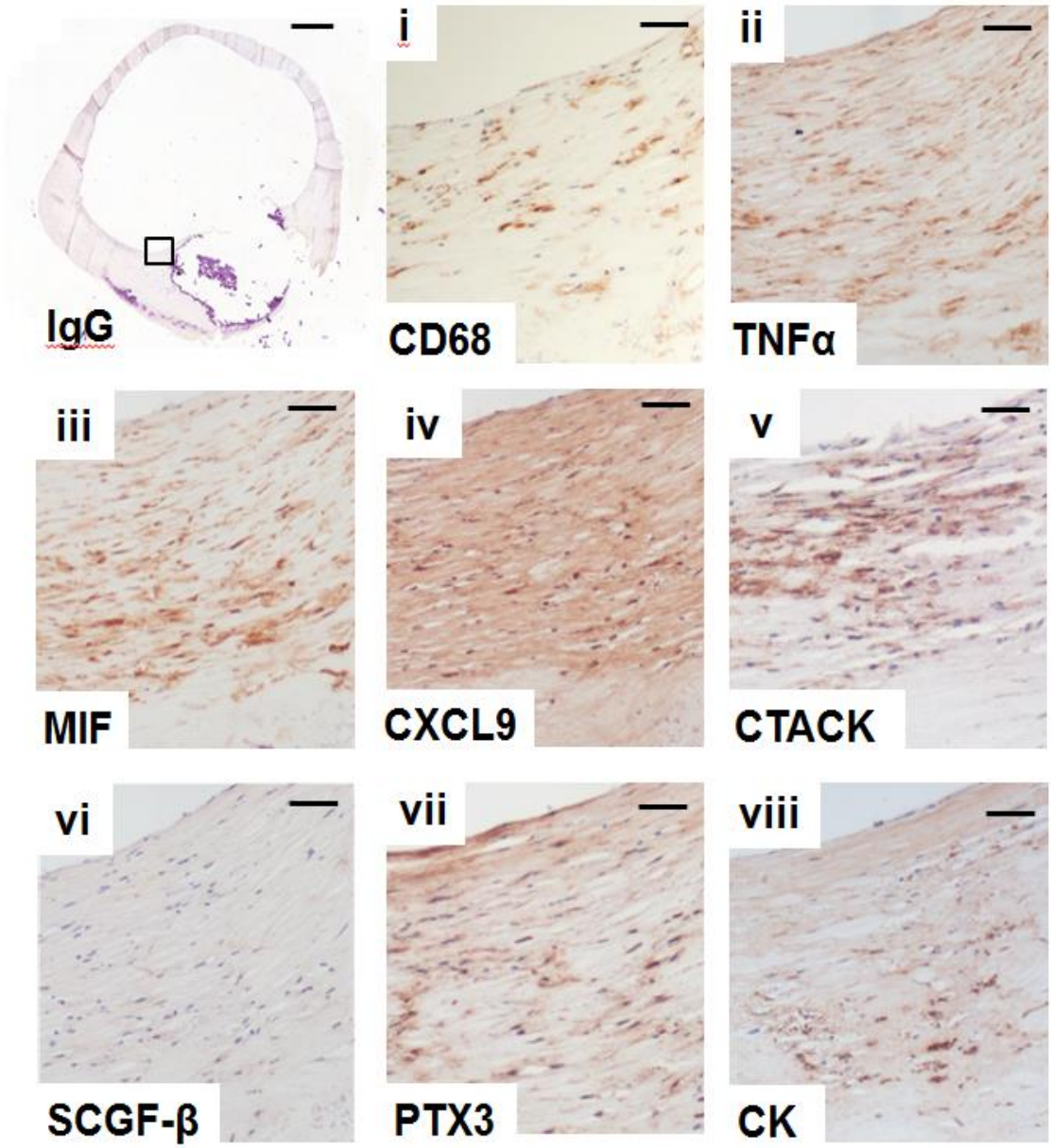

Figure 32. Inflammatory markers are expressed within carotid endarterectomy plaques. Square box in the negative IgG control is enlarged in the serial sections presented in micrographs i-viii. Consecutive sections from a representative patient sample stained positive for inflammatory markers (i) CD68 and (ii) TNFα. The expression of a panel of cytokines, (iii) MIF, (iv) CXCL9, (v) CTACK, (vi) SCGF-b, (vii) PTX3 and (viii) cathepsin K was also detected within the plaque. IgG bar=1000μm; i-viii bars =50μm.

3.6 PTX3 ELISA results

The results from the PTX3 ELISA are shown in the scatter plots below, see Figure 33. No PTX3 levels were detected in controls, asymptomatic patients with both stable and unstable plaques or the patients with stable symptomatic plaques. However, PTX3 was detected in approximately a fifth (9 out of 42 patients) of symptomatic patients with unstable plaques although this was not statistically significant, p=0.10.

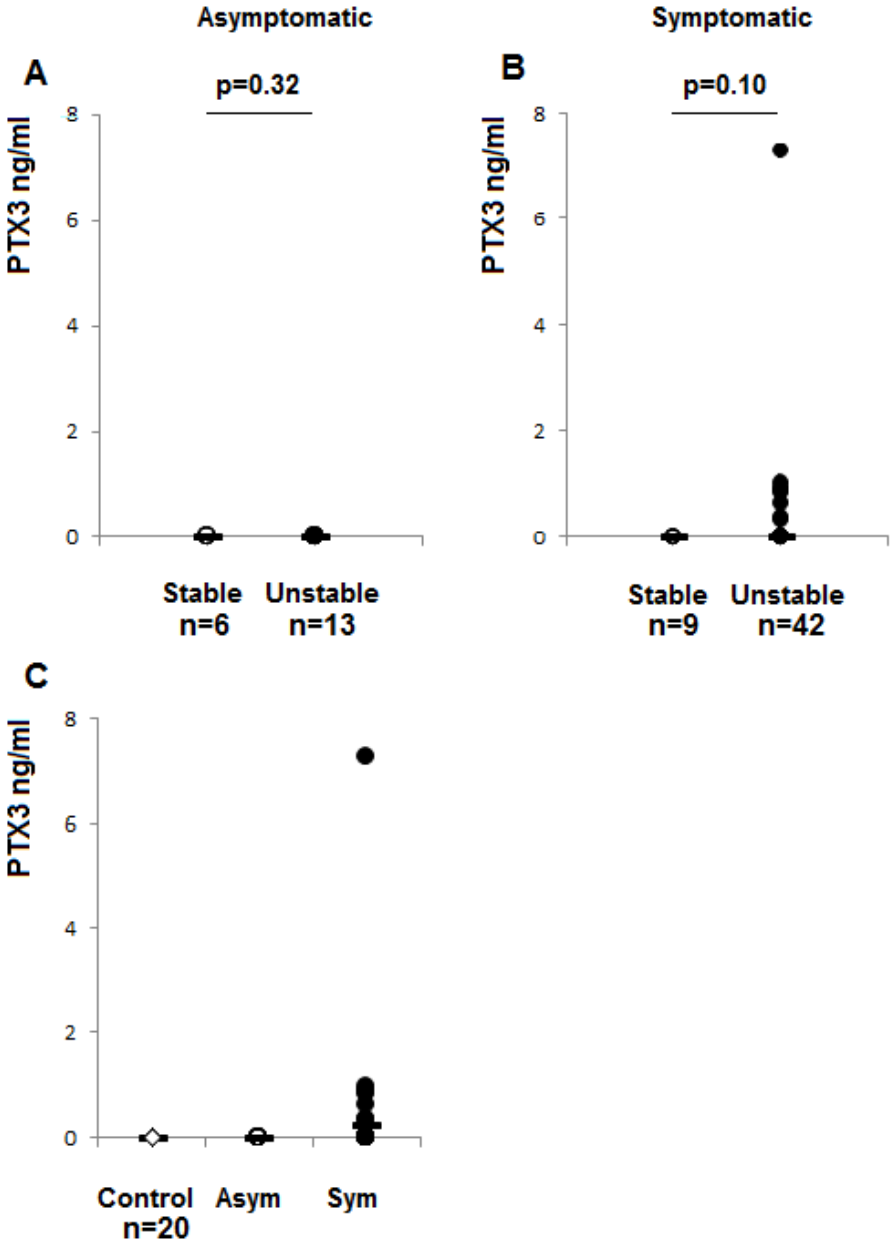

Figure 33. Scatter plots showing PTX3 levels in patient groups.

3.7 PDGF ELISA results

The results from the PDGF ELISA are shown in the scatter plots below, See Figure 34. No significant differences were detected in the asymptomatic and symptomatic patients who had stable and unstable plaques as well as controls.

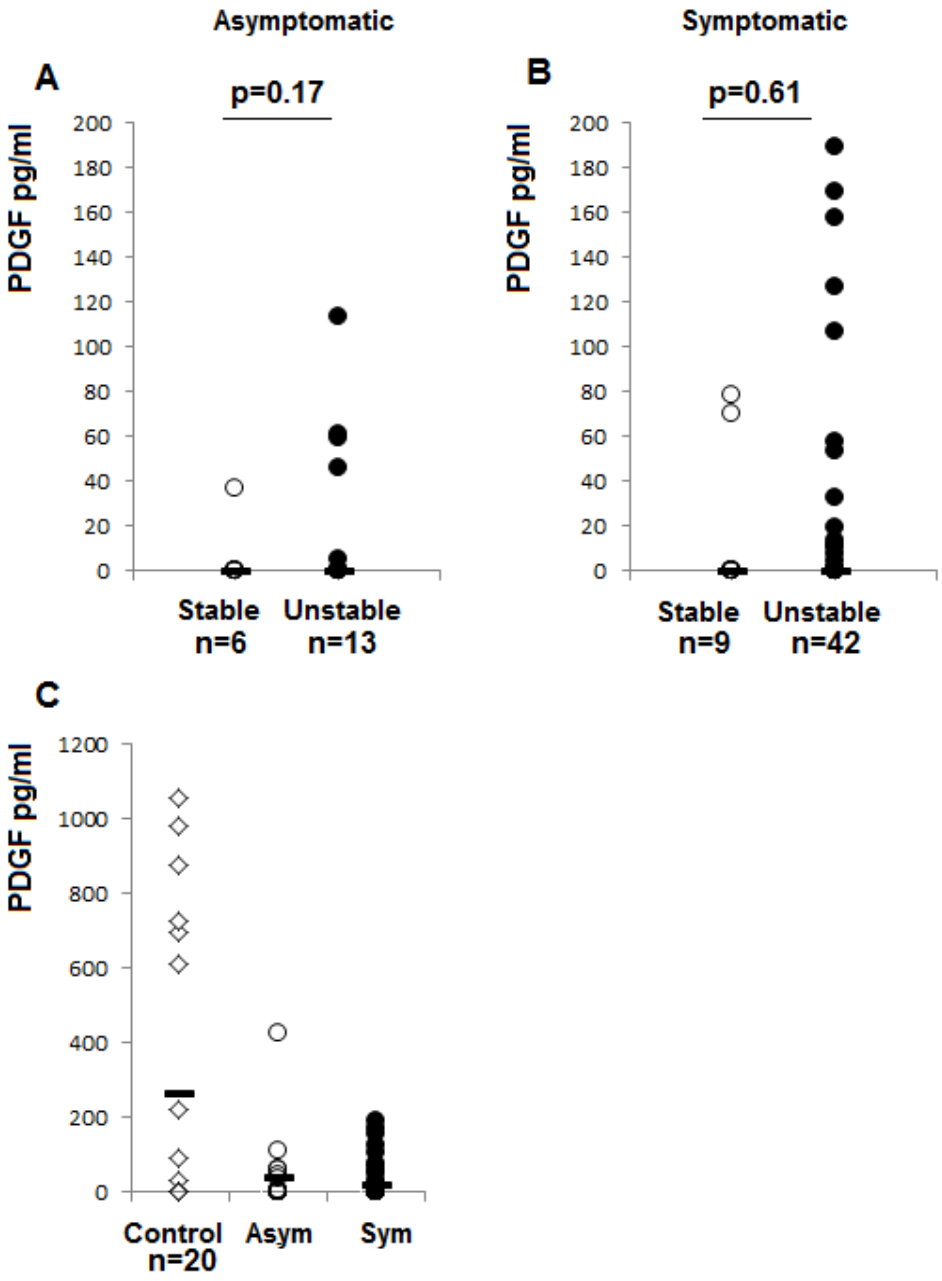

Figure 34.Scatter plots showing PDGF levels in patient groups.

3.8 Results from Human Bone magnetic bead panel.

Since asymptomatic and symptomatic patients exhibited substantial amounts of vascular calcification and stained positively for OPN this prompted us to investigate for the presence of bone related markers in blood. See Figure 35. OPN and OPG, inhibitors of bone formation, were both elevated in the serum of symptomatic patients compared to asymptomatic patients ($P<0.01$), with no difference detected between stable and unstable plaques in either group. Osteocalcin (OC) involved in bone formation, was found to be elevated in stable plaques in the asymptomatic group ($P<0.05$) but not in the symptomatic group. There was no difference between the symptomatic and asymptomatic groups, p=0.12. No differences were found in the levels of the remaining bone markers IL-6, TNF-α, or sclerostin.

3.8.1 Plaques from asymptomatic patients' exhibit extensive calcification

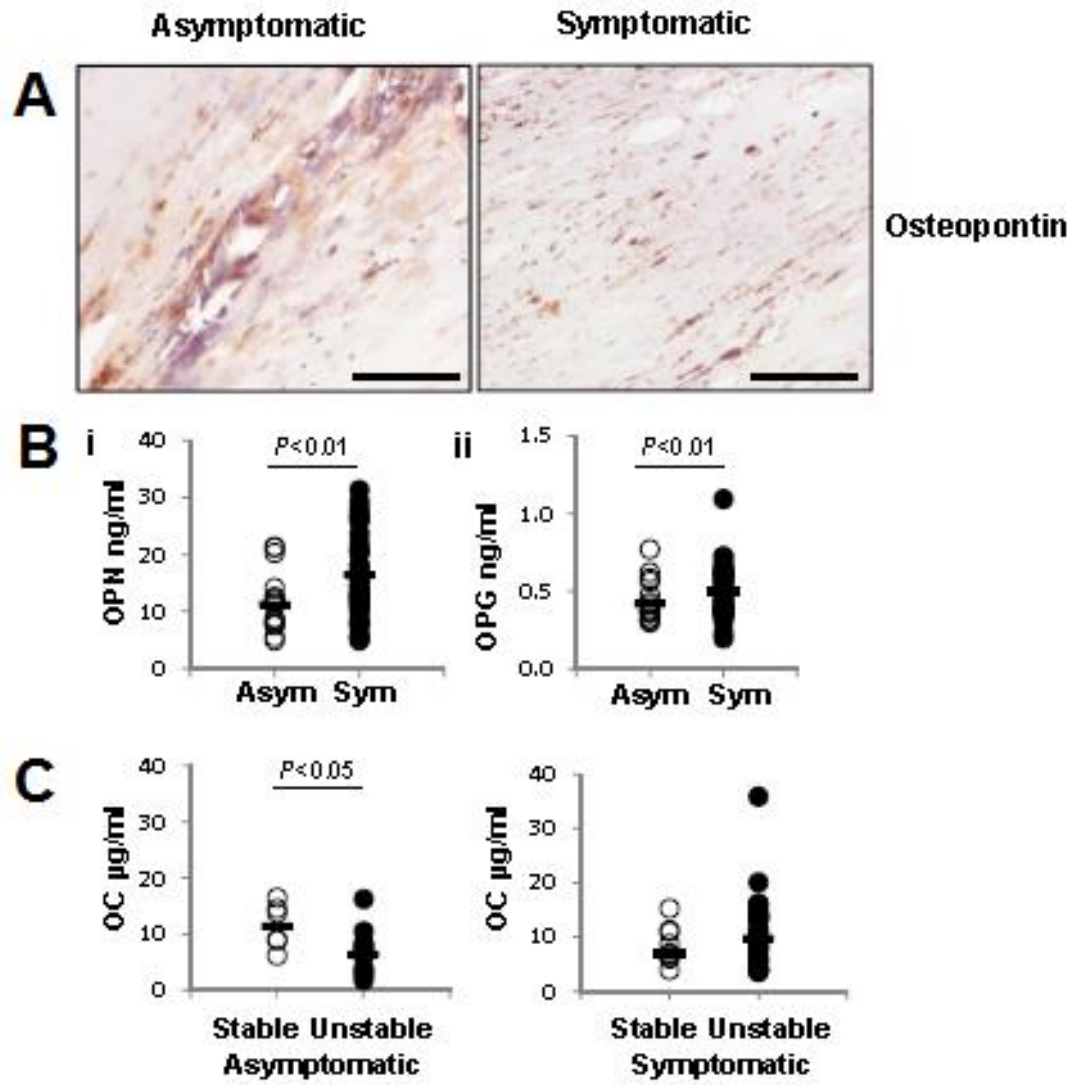

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Figure 35. Plaques from asymptomatic patients were more calcified compared to symptomatic patients. (A) (OPN) was detected in areas of calcification. (B) and (C) Bone-related markers OPN and OC were differentially regulated in patient serum. Data were analysed using Mann-Whitney U statistical test. Bars represent the median. Inset image; bar=1000µm. Main image;
bar=100µm

CHAPTER 4

Discussion

4.0 Discussion

Current clinical practice relies on the patient history and medical imaging in the form of Duplex ultrasound, computed tomography angiography and magnetic resonance angiography to select patients with carotid disease that may benefit from surgery. The role of inflammatory markers associated with cardiovascular disease has yet to gain a role in the selection process, when trying to decide which patients will benefit from intervention. It is likely, that in the future, serum biomarkers may become an important clinical tool that could revolutionise the treatment of patients with cardiovascular disease such as those patients with asymptomatic carotid stenosis.

This study has shown that EMPs were significantly increased in patients with symptomatic and asymptomatic carotid disease when compared to healthy age matched controls but there were no differences in EMP levels between the two patient groups. This is in keeping with work of others.(Leroyer *et al.*, 2007) In the unit from where we obtained our carotid plaques, surgery is not routinely offered to all patients with a ≥70% asymptomatic stenosis. Surgery is offered to the subgroup of asymptomatic patients who have a higher chance to succumb to a stroke and those patients who have a life expectancy of more than 5 years. This included patients who had unstable plaque characteristics on ultrasound, patients with a history of TIA >6 months prior to their clinic visit or patients pre or post CABG. 68% (13 out of 19) of asymptomatic plaques were unstable whilst 82% (42 out of 51) of the symptomatic plaques were unstable so it's not surprising that we showed no significant difference between symptomatic and the asymptomatic patient groups. Following this finding most data analysis focused on comparing stable and unstable plaque groups.

When patient groups were subdivided according to their plaque stability we detected a significantly higher level of EMPs in the asymptomatic patient group with unstable plaques compared to the stable group although no differences were detected in the symptomatic plaques based on their morphology. Furthermore, when stable

and unstable plaques from both groups were combined significantly higher numbers of EMPs were present in patients with unstable plaques. In contrast, another study by Wekesa (Wekesa *et al.*, 2014) *et al has* shown that elevated levels of EMP were detected in patients with symptomatic unstable plaques. This difference may be explained by the fact that they had a small patient cohort (25 patients) and also it's a known fact that working with human specimens is very variable and may also accounts for differences. These data could have a significant impact, since EMPs act as silent indicators of endothelial damage and may be used as biomarkers of plaque instability in the asymptomatic group. There is a growing need for the use of biomarker discovery in plasma as a means of predicting stroke susceptibility. We hypothesised that patients with a high risk of stroke could be identified by a combination of unstable plaque characteristics together with levels of circulating microparticles, inflammatory cytokines and bone markers.

Next we investigated levels of different cytokines to establish an inflammatory profile of the patient groups as well as their correlation to plaque stability. We identified several cytokines which were differentially regulated in the patient groups. The chemo-attractants CTACK, IL-3 and IL-16, involved in the recruitment of immune mediated cells involved in plaque development, were significantly elevated in both symptomatic and asymptomatic patients compared to controls while MIF was significantly down-regulated in asymptomatic group of patients compared to the controls. This is an interesting finding since MIF is involved in monocyte recruitment as well as clot modulation achieved by destabilising clot by promoting fibrin degradation. (Wirtz *et al.*, 2015) A significant elevation of HGF in the symptomatic group reconfirms our previous study on HGF in symptomatic patients which has been similarly reported by others as key factor in the pathophysiological role in plaque progression. (Bussolino *et al.*, 1992; Chowdhury *et al.*, 2010) MIG (CXCL9) has also been found to be significantly elevated in the symptomatic group compared to controls and in asymptomatic patients

with unstable plaques. This is an important finding since CXCL9 has been associated with severe coronary plaque calcification in keeping with our results and may also be used as a novel biomarker of plaque burden. (Yu *et al.*, 2015)

We now know that the presence of activated macrophages within plaques is a sign of plaque inflammation and a feature of plaques that are prone to rupture. Current imaging modalities cannot reliably detect unstable plaques in clinic. Therefore a combination approach using imaging and serum biomarkers for detecting high risk plaques could be used in clinic to identify patients that are more likely to develop acute cardiovascular events and hence be treated earlier on. This in theory could be achieved by the development of a device similar in principle to a multi ELISA with a panel of biomarkers to detect for the presence of biomarkers in blood in clinic. Therefore we interrogated the patient groups to determine whether we could correlate serum biomarkers with plaque vulnerability. We now demonstrate that, CXCL9 and SCGFβ were significantly elevated in serum from patients with unstable plaques compared to those with stable plaques, in the asymptomatic group, potentially increasing the invasion of inflammatory cells into the plaque. When all stable and unstable plaques from both groups were analysed with regards to inflammatory markers, CXCL9 was the only cytokine significantly elevated in the unstable group. In addition, IL-16 was down-regulated in serum of patients with unstable *vs* stable plaques, the reason for this is unclear, however, the Interleukins are known to be regulatory proteins which can either accelerate or inhibit inflammatory processes, thus acting as damaging, protective or neutral agents, most likely determined by their concentration and other regulatory factors being modulated in the disease process.(Fisman *et al.*, 2003)

The transition from a stable to an unstable plaque puts the patients at risk of thromboembolism and stroke. It is characterised by increased neovascularization and foam cell infiltration, as shown in our previous study.(Chowdhury *et al.*, 2010) Although intensive medical therapy and carotid endarterectomy have been shown to reduce stroke and death rates in asymptomatic stenosis, it is recognised that patients with low to intermediate artery stenosis can still succumb to ischemic events, providing the impetus to consider plaque composition as an important feature of atherosclerosis. The findings from studies of this nature could in the future contribute to the selection of patients for CEA surgery. This fits with the current strategy for precision medicine. See Figure 36.

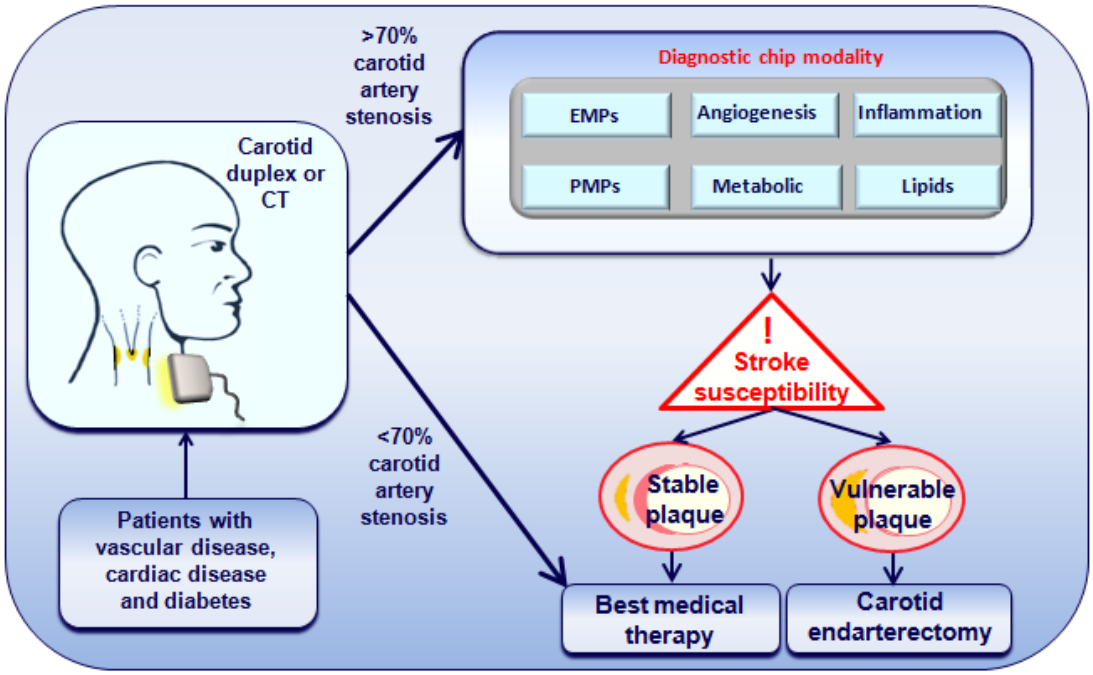

Figure 36. Proposed working model of how EMPs together with inflammatory cytokines may be of use in clinic to determine patient treatment (Schiro *et al.*, 2014)

We investigated the presence of the same biomarkers in plaques to establish if we would find a correlation between systemic and local expression with EMPs and plaque morphology to enable an identification of patients at high risk of future events. The inflammatory markers were present in both types of plaques as expected, adding

further strength to the value of systemic markers for the distinction of stable and unstable plaques. In addition, we investigated the collagenase, Cathepsin-K, since it has been described as an attractive target for plaque regression and selective inhibitors are already being used in phase III clinical trials.(Newby, 2014) It is not surprising to find the presence of this collagenase in all plaques, since haemodynamics play such a key role in atherosclerotic development and the carotid endarterectomy specimens are taken from regions of low and oscillatory shear stress, where endothelial cells have been shown to contribute to proteolytic vascular remodelling by upregulating cathepsins.(Platt *et al.*, 2007) These findings are in accordance with the results previously seen by Lutgens *et al*, who demonstrated Cathepsin-K expression is upregulated in advanced lesions up to 28 fold.(Lutgens *et al.*, 2006) In addition, it is interesting to note that both Cathepsin-K and macrophage migration inhibitory factor (MIF) have been shown to be involved in bone metabolism(Mandelin *et al.*, 2006) and we find the presence of both molecules in association with mineral deposition in plaques with evidence of calcification. PTX3 expression, an acute phase protein, was also detected in the lesions, alongside other mediators of inflammation.

Elevated calcification was apparent in the plaques from the asymptomatic group compared to the symptomatic group, whether this affords increased stability remains debatable. In light of these findings, we investigated circulating bone markers and found significantly raised levels of OPN and OPG in the symptomatic group *vs* asymptomatic patients, which adds strength to our histological findings, as both OPN and OPG are inhibitors of mineralisation, supporting the reduced mineralisation detected in the symptomatic patients. Furthermore OC, involved in bone formation, was elevated in asymptomatic patients with stable plaques, lending credence to the idea that calcification could have a stabilising influence. Although tissue analysis of these pro-inflammatory cytokines will not be used clinically, identifying specific cellular and signalling pathways in plaque development will provide a deeper insight into mecha-

nisms of the disease process and could provide a target to be used with non-invasive imaging to enhance the diagnostic and predictive opportunities in this patient group. See Figure 37. Although the data set in this study are small, it highlights the challenges associated with patient-to-patient variability and the need to collect not only serological, cellular and molecular analyses of clinical samples but to include genetic information and utilise large scientific data sets, obtained from state-of-the-art omic technologies.

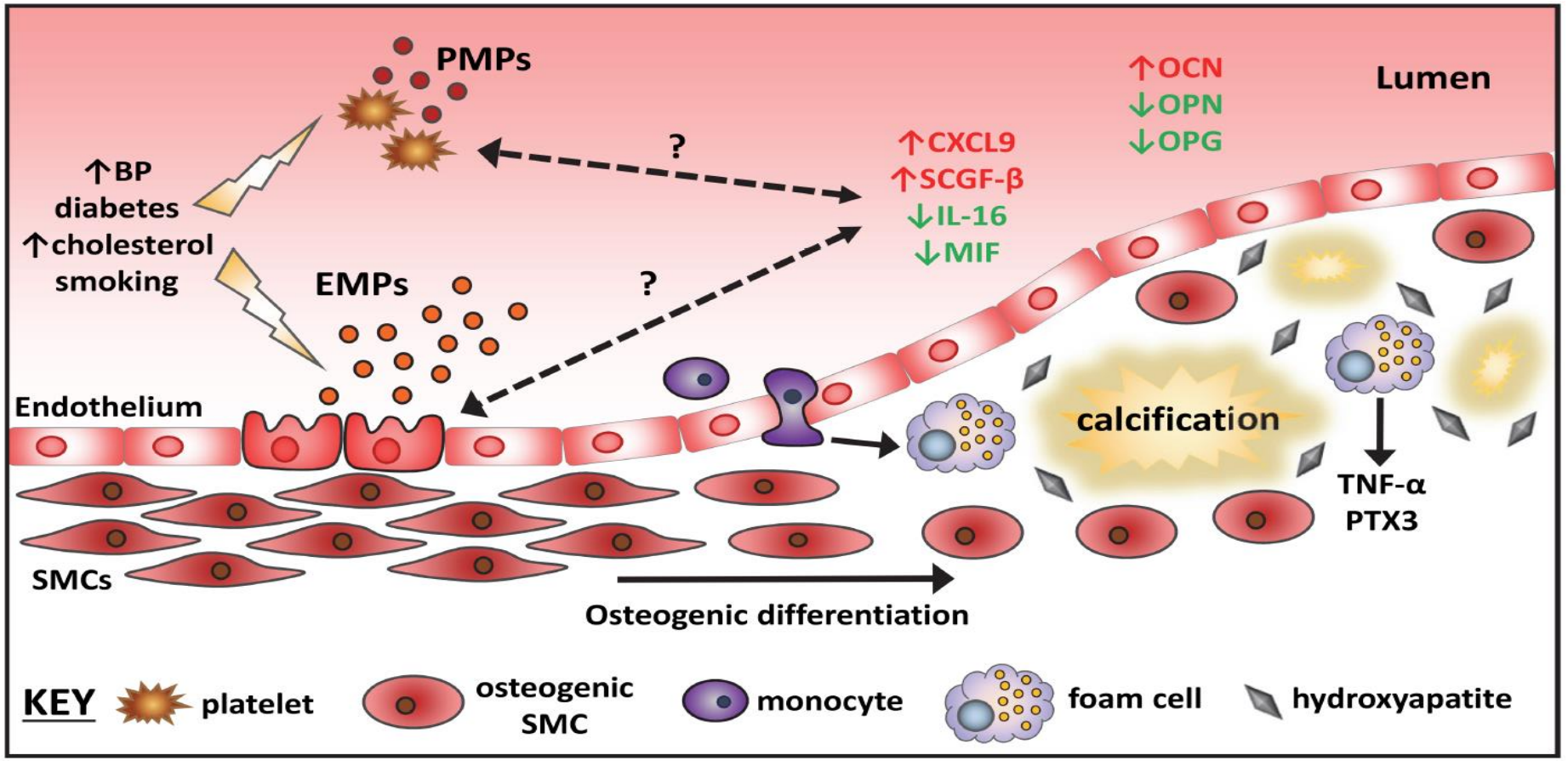

Figure 37. A schematic representation of the key factors involved in plaque instability in asymptomatic patients. Endothelial cells and platelets are activated by various cardiovascular disease risk factors and release microparticles into the circulation. During plaque development, smooth muscle cells (SMCs) can differentiate from a contractile to a secretory phenotype, depositing a calcified matrix. Both processes may be exacerbated by the cytokines modulated during disease pathology. OC osteocalcin; OPN-osteopontin; OPGosteoprotegerin; TNF-tumour necrosis factor-alpha; PTX3-pentraxin-related protein; SCGF-β-stem cell growth factor; SMC-smooth muscle cell; EMP-endothelial microparticle; PMP-platelet microparticle.

4.1 Future studies

There are two potential studies that can be carried out in the future. The first study following this research project could look at patients with asymptomatic disease who are not treated surgically. This would involve the recruitment of a large amount of patients from multiple sites. Patients' levels of EMPs and cytokines will be assessed over a predetermined number of years as well as identifying those patients who stroke. A large number of patients will be needed since the stroke rate in this patient cohort is very low especially now since the introduction of best medical treatment which wasn't available to all patients when the ACAS and ACST were carried out many years ago.

The second study could involve the proteomic assessment of EMPs extracted from symptomatic, asymptomatic and control patients. This expensive process will help identify whether the protein molecules in EMPs from these patient groups are different and if so identify these proteins. This will be revolutionary to how we treat patients with asymptomatic carotid disease.

4.2 Future goals

Future goals will be aimed to develop a blood test based on the principles of enzyme-linked immunosorbent assay (ELISA) for routine clinical analyses of EMPs and relevant inflammatory cytokines in clinic. These tests will be technically easy to be carried out in clinic, cost-effective and suitable for high-output screening. This method will be of benefit to many patients, prevent many unnecessary operations and cut health care costs.

4.3 Limitations of the study

i) *Numbers*- patient numbers are small and a potential limiting factor in this study. On average our unit performs around 80 carotid endarterectomies per year. Not all patients could be recruited. Some patients were missed. Such patients included those operated over weekends or those who had their plaques completely destroyed by the operating surgeon making it impossible to analyse histologically.

ii) *Flow cytometry*- Although flow cytometry is widely used to identify and quantify MPs, there are no agreed universal protocols so the protocol we used for analysis was created by our lab taking into account the multitude of MP protocols available from different studies.

iii) *Sample Storage*- Storage of plasma samples for a long time may affect their MP content. This was avoided as much as possible by analysing samples early throughout the course of the study. Thawing and refreezing of samples was avoided. This was achieved by storing samples into several eppendorf containers. Each time an experiment was performed an eppendorf was thawed, used and discarded. With regards to serum, cytokine analysis was only performed once to include all the samples since the kit was very expensive. This mean that some samples were a year old before analysis was carried out.

iv) *EMPs and inflammatory cytokines levels.* Measured EMPs may originate from other disease processes so they could essentially be skewing the results of these investigations. This is one of the reasons why other studies that include proteomic assessment of EMPs are required. In this way we could identify the origin of MP depending on their membrane proteins and this could help trace their origin to selected patients and diseases.

4.4 Conclusion

This thesis has investigated the use of EMP and cytokines in detection of plaque instability in patients with carotid disease. We have demonstrated high levels of EMPs, SCGF and CXCL9 in patients with asymptomatic unstable disease.

Asymptomatic patients with stable plaques have been shown to have significant levels of OC that may be a contributing factor to plaque stability. We have demonstrated significant levels of bone formation inhibitors OPG and OPN in serum of symptomatic patients which may play a role in plaque instability in this patient group.

These data could have major implications for the development of a diagnostic tool whereby EMPs together with markers of macrophage activity could act as biomarkers of plaque vulnerability and stroke susceptibility. Immuno-modulation of atherosclerosis is the way forward with respect to the treatment and or reversal of the atherosclerotic process.

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Appendix 1: Ethics approval documents

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds West
First Floor
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Telephone: 0113 3050122
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17 February 2012

Dr Yvonne Alexander
Lecturer in Molecular medicine
University of Manchester
Core Technology Facility
46 Grafton Street
Manchester
M13 9NT

Dear Dr Alexander

Study title: **Microparticles in patients with vascular disease**
REC reference: **12/YH/0107**

Thank you for your letter of 14 February 2012 responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

The question regarding mortality should be removed from the telephone interview schedule, the protocol states that the researcher will contact the participants GP to check they are still alive before telephoning.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
GP/Consultant Information Sheets	2	14 February 2012
Investigator CV		
Other: CV for Mr Serracino-Inglott		
Other: Cv for Mr Schiro		
Other: Data collection sheet	1	16 December 2011
Other: Follow up reminder letter	1	16 December 2011
Other: Reference from Mr Serracino-Inglott		24 January 2012
Other: Reference from Mr Smyth		26 January 2012
Other: Telephone interview schedule	1	14 February 2012
Participant Consent Form	2	14 February 2012
Participant Information Sheet	2	14 February 2012
Protocol	2	14 February 2012
REC application		25 January 2012
Response to Request for Further Information		
Summary/Synopsis	1	16 December 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/YH/0107	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Rhona Bratt
Chair

Email: Elaine.hazell@nhs.net

Enclosures: *"After ethical review – guidance for researchers"*

Copy to: *Miss Carla Barrett*

Lynn Webster, Central Manchester University Hospitals NHS Foundation Trust

Appendix 2: Patient information sheet

Central Manchester University Hospitals NHS
NHS Foundation Trust

Regional Vascular and Endovascular Unit
Manchester Royal Infirmary
Oxford Road
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M13 9WL

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PATIENT INFORMATION SHEET

Serum microparticles as markers of active unstable atherosclerotic carotid plaques

Dear Patient,

We would like to invite you to take part in a research study on carotid disease. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

Blood vessels are lined by a layer of cells called the endothelium. If this layer gets damaged fatty material builds up in the blood vessel and is called a plaque. The disease is known as atherosclerosis. Sometimes the plaque breaks down and causes a stroke. Some patients can develop plaques which will never break open and some have plaques that will break. We are trying to understand why this happens and how we can identify the patients that are at most risk of the plaque rupturing and blood vessels breaking open. These are the patients most likely to suffer from a future stroke.

Blood carries certain substances called microparticles which are breakdown products of damaged arteries. We would like to compare these microparticle levels in patients that are symptomatic and asymptomatic from carotid disease i.e. have no symptoms of stroke. In particular, we wish to calculate how concentrated they are in blood. This study is an early step in the complicated process of identifying factors that might indicate why certain patients with carotid disease are at risk of having mini-strokes.

Why have I been chosen to take part?

You have been chosen because you have been diagnosed with carotid disease or because you are a healthy individual in the same age group of our study patients. Data from healthy individuals is invaluable as it gives researchers as base line onto which to compare results to.

What will happen to me if I agree to take part?

If you agree to take part you will be asked to donate a 20 ml (4 teaspoons) sample of blood. We will use this blood sample to extract microparticles. We may also look at substances such as cytokines (inflammatory markers) in the blood that may indicate inflammation. To clearly identify the fatty layer coating the inside of the carotid artery we will organise a duplex scan which will quantify the extent of your carotid plaque and pin point exactly where surgery will be performed. We would also like to perform a Transcranial Doppler test on the ward. This is a non-invasive (painless) test which will monitor the flow of blood going to your brain. This will be carried out for an hour pre and post operatively. Should for some reason you feel uncomfortable during this procedure you can withdraw from the study at any time.

We would like to use a piece of the fatty material that we scrape out of your blood vessel during surgery (if you are a vascular patient going to have surgery). We would normally incinerate this fatty tissue that is blocking your blood vessel, but if we take it to the lab we can investigate it with detail. The tissue will be analysed by our team of highly-dedicated and qualified researchers.

Subsequently, with your permission, you will undergo an ultrasound scan (done routinely) and Transcranial Doppler assessment) prior to surgery or in outpatients if you have no vascular disease. We will then separate the blood sample and do the measurements described above in our laboratory. We will also use some of the tissue that would be taken during surgery and transport it to the lab for detailed examination. With your permission, and if it is relevant to your participation in the study, we will also review your notes and medical history to see if any aspects of carotid disease or its treatment are associated with what we find in the lab. All this information will be strictly confidential, no personal details will be given to the researchers and none of our samples or forms will identify you personally.

What are the possible risks of taking part?

We do not envisage that you will be harmed by taking part in this study. Your treatment will not be affected by a decision not to take part. There are no risks associated with this study other than the normal risks associated with surgery for which you have already given consent.

Do I have to take part?

No, taking part is voluntary. If you would prefer not to take part you do not have to give a reason and your medical care will not be affected in any way.

What are the expected benefits of taking part?

This study will help us to better understand atherosclerosis in symptomatic and asymptomatic patients who have carotid disease and may suggest new ways to prevent heart disease and stroke. Your participation in this study will not affect or delay your treatment in any way. Patients that have carotid disease in the future may benefit from this research.

One year Follow-up

If you are a vascular patient undergoing an operation then in one year's time from your operation, you will be contacted by phone by Mr Andrew Schiro (research student) who will ask you some questions about your general health and address any issues you have with your current medication.

What study results are expected, and when will these be available?

We plan to involve 60 patients in this study. 30 patients will have an operation to their carotid artery because they would had symptoms from it such as stroke whilst the other 30 would be patients whose carotid disease would have been picked up incidentally. The results from this study will be available in about two to three year's time.

Will the information I provide be kept confidential?

Any information you provide us with will be confidential. We will store all personal / clinical data on two computers in the Vascular and Diabetic research offices connected to secure servers. Your data will be anonymised and allocated with a patient ID number which is only known to the researcher.

What happens if you change your mind about the research?

If for some reason you change your mind regarding your participation into the study you can withdraw at any time without giving any reasons for this and all data which you provided us with will be deleted from our database. Your carotid plaque and blood samples will be discarded.

What if there is a problem?

Should you have any concern or query about the study you could contact the Chief investigator, Mr Andrew Schiro on the number below, the Research and development office at the Manchester Royal Infirmary or the Patient Advice and Liaison Services

Who has reviewed the study?

The study has been reviewed and given approval by the Manchester Research Ethics Committee, internally reviewed by Physicians and Scientists within the University of Manchester. This study is also being used as a project for a clinical student registered in The University of Manchester as part of his specialised educational training.

Who is sponsoring the study?

The study is being funded by Medronic

How can I get more information?
If you wish to obtain independent advice about this research you may contact:

Name, address and telephone number of local independent adviser:

Mr Ferdinand Serracino-Inglott (Clinical advisor)

Consultant Vascular and Endovascular Surgeon
Department of Vascular Surgery,
Manchester Royal Infirmary
Oxford Road
Manchester, M13 9WL
Tel: 01612764301

Or
Dr. Yvonne Alexander PhD (Scientific Advisor)
Dept. of Medicine,
Cardiovascular Research Group
Core Technology Facility,
46 Grafton St.
Manchester. M13 9NT
Tel: 0161 275 1224

Mr Andrew Schiro (Clinical research student involved in the study) Contact telephone number 01612768524. Email: andrew.schiro@cmft.nhs.uk

What do I do now?
The research sister organising the study will contact you in a few days. She can answer any questions and you can let her know if you are interested in taking part.

Thank you very much for considering taking part in our research.

Appendix 3: Informed consent form

Central Manchester University Hospitals NHS
NHS Foundation Trust

INFORMED CONSENT FORM

Serum microparticles as markers of active unstable atherosclerotic carotid plaques

Study Number:
Name of Researcher**:** Mr Andrew Schiro

		Please initial box
1.	I confirm that I have read and understood the information sheet dated ……2013 for the above study. I have had the opportunity to consider the Information, ask questions and have had these answered satisfactorily.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3.	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from The University of Manchester, regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
4.	I agree to my GP being informed of my participation in the study.	☐
5.	I agree for my blood samples to be stored for future use in this research study	☐
6.	I agree to gift my blood and blood vessel tissue samples for future similar research	☐
7.	I agree to take part in the above study.	☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

NB: when completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical note

Appendix 4: Letter to GP

Central Manchester University Hospitals NHS
NHS Foundation Trust

Regional Vascular and Endovascular Unit
Manchester Royal Infirmary
Oxford Road
Manchester
M13 9WL

Tel: 0161 276 8524
Fax: 0161 276 6828

General Practice Information Sheet

Study Title: The use of microparticles as biomarkers for plaque instability in patients with carotid disease

Dear Colleague,

Patient name: _____
Date of birth:_____/_____/_____

Your patient wishes to take part in our study named above.
This study aims to identify a high risk stroke population undergoing carotid artery endarterectomy and healthy patients who volunteer to donate a blood sample for research purposes.
The study involves:
1. Patient interview for eligibility and consent for the study after routine vascular clinic appointment and carotid duplex scan.
2. Patients will also undergo Transcranial Doppler assessment for microemboli and a blood tests to measure serum biomarkers including microparticles pre and post-operatively.
3. Patients that undergo surgery will have their carotid plaque analysed in the Core Technology facility at the University of Manchester.
4. Healthy patients (controls) who volunteer to donate a blood sample for research.
Patients recruited in this study will not be treated differently from non-participants. This study has been reviewed and approved by the ethics committee in accordance with local regulations.

I will be happy to discuss any queries you might have about this study.

Yours sincerely

Mr Andrew Schiro MD, MRCS (Edin)
Clinical Vascular and Endovascular Research Fellow
Email: andrew.schiro@cmft.nhs.uk

Appendix 5: Data collection sheet

Central Manchester University Hospitals NHS
NHS Foundation Trust

DATA COLLECTION SHEET

DERMOGRAPHICS

Patient Name: …… DOB…… HOSPNO……

AGE:…… SEX…… Height: …… BMI:……

CONTROL : Y / N

PMH

IHD: Y / N Diabetes: Y / N cholesterol : Y / N ↑BP: Y / N

AF: Y / N MI : Y / N TIA: Y / N STROKE: Y/ N CABG Y / N

CEA Y / N PVD: Y / N KIDNEY DISE Y / N COPD Y / N ASTHMA Y/ N

SH

SMOKING: Y / N NEVER SMOKED ( ) STOPPED ( )

ALCOHOL: ( ) UNITS/ WEEK

PRE-OP

Date referred:…….. Referred by:…….. Date seen :……..

Symptoms > 14 days prior to surgery Y / N

- ASYMPTOMATIC Y / N SYMPTOMATIC Y / N

Date of symptoms …….. CT DATE……

SYMPTOMS

	UL- MO-TOR	LL-MOTOR	UL-SENSORY	LL-SENSORY		EYE
RIGHT						
LEFT						

Baseline Modified Rankin score: Stroke Score

		Pre- event	**Post event**	**Post op**	**@ 1 YR**
0	No symptoms at all.				
1	No significant disability despite symptoms; able to carry out all usual duties and activities.				
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance.				
3	Moderate disability; requiring some help, but able to walk without assistance				
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance.				
5	Severe disability; bedridden, incontinent and requiring constant nursing care and attention.				
6	Dead.				

MEDICATION

……
……
……

BLOODS

Hb……., WCC…….PLT……..

Na……..K……Ur……Cr……EGFR…… Calcium…….

ALT…….ALP…….BIL…….. ALB……. CRP……..

Se Cholest……..LDL……..HDL……..TRIG…….Chol/HDL ratio…….

CAROTID DUPLEX

1. Duplex on presentation Date: ……% stenosis Right: …….. % Left: …… %

2. Preoperative duplex (if repeated) Date: ……% stenosis Right: …….. % Left: …… %

3. Disease length: ……

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

5. Carotid outer diameter (cm): ……...

6. Plaque volume index (mm3): ……

OPERATION Date of Operation: ……

Operation side: RIGHT / LEFT

Diameter of the Internal carotid artery (mm): …….……

Carotid closure: Graft? YES / NO

Shunt YES NO

Blood loss: …….. Mls

POST OP

COMPLICATIONS	**YES**	**NO**
Post-operative stroke If 'YES': how long after operation?		
Post-operative TIA If 'YES': how long after operation?		
Post-operative MI If 'YES': how long after operation?		
Post-operative haematoma If 'YES': how long after operation?		
Cranial Nerve injury If 'YES': how long after operation?		
Death If 'YES': how long after operation?		

ONE YEAR FOLLOW-UP :

1. Date of follow up:……

2. GP contacted: YES / NO

3. Mortality: ALIVE / DEAD if 'DEAD' cause of death: ……..

4. Stroke/TIA: YES NO if 'YES' date of onset: ……

5. Any further cardiac/vascular intervention: YES / NO

Appendix: 6 Copy of paper publication in Atherosclerosis Journal- March 2014

Accepted Manuscript

Endothelial microparticles as conveyors of information in atherosclerotic disease

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Endothelial microparticles as conveyors of information in atherosclerotic disease

Authors:

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ABSTRACT

Endothelial microparticles (EMPs) are complex submicron membrane-shed vesicles released into the circulation following endothelium cell activation or apoptosis. They are classified as either physiological or pathological, with anticoagulant or pro-inflammatory effects respectively. Endothelial dysfunction caused by inflammation is a key initiating event in atherosclerotic plaque formation. Athero-emboli, resulting from ruptured carotid plaques are a major cause of stroke. Current clinical techniques for arterial assessment, angiography and carotid ultrasound, give accurate information about stenosis but limited evidence on plaque composition, inflammation or vulnerability; as a result, patients with asymptomatic, or fragile carotid lesions, may not be identified and treated effectively. There is a need to discover novel biomarkers and develop more efficient diagnostic approaches in order to stratify patients at most risk of stroke, who would benefit from interventional surgery. Increasing evidence suggests that EMPs play an important role in the pathogenesis of cardiovascular disease, acting as a marker of damage, either exacerbating disease progression or triggering a repair response. In this regard, it has been suggested that EMPs have the potential to act as biomarkers of disease status. In this review, we will present the evidence to support this hypothesis and propose a novel concept for the development of a diagnostic device that could be implemented in the clinic.

MICROPARTICLES

Microparticles (MPs) are anucleoid submicron fragments (50nm-1µm diameter) of plasma membranes made up of oxidised phospholipids and specific proteins that represent the cells

from which they originate. To date, MPs have been identified as particles released from vascular and blood-related cells such as endothelial cells, platelets, erythrocytes, leukocytes and smooth muscle cells, as demonstrated by their cell-specific surface proteins[1]. MPs were once referred to as 'cell debris'[2], however, it is now established that MP release is a regulated process and they are not randomly released into the circulation[3], but are endocrine and paracrine effectors involved in intercellular communication and potentially plaque vulnerability[4]. These effects will be discussed in this review.

MICROPARTICLE RELEASE

A number of inflammatory or physical stimuli are able to induce cell activation or apoptosis via different signalling pathways resulting in disruption of lipid membrane asymmetry and cytoskeleton proteins[5]. In turn, this causes budding of the plasma membrane and release of MPs[6] (Figure 1). Plasma membrane remodelling results from an imbalance between the cytoplasmic and outer plasma membrane aminophospholipids. Cytoplasmic aminophospholipids include phosphatidylserine (PS) and phosphatidylethanolamine (PE), whilst phosphatidylcholine (PC) and sphingomyelin (SM) constitute the outer part of the plasma membrane. Following cell stimulation, PS translocates into the outer surface of the membrane resulting in MP release[5] (Figure 1).

An increase in intracellular calcium results in the inhibition of enzymes involved in lipid transport and translocation of phospholipids between the two monolayers of a cell's plasma membrane[7]. Calpain, a calcium dependent cytosolic cysteine protease, is subsequently activated leading to cleavage of gelsolin, an actin binding protein necessary for filament assembly and disassembly, causing cleavage of long actin filaments[7]. When actin-capping proteins are cleaved the cytoskeleton is disrupted. Rho-associated kinases (ROCK I and ROCK

II) are proteins responsible for regulating the shape and movement of cells by acting on the cytoskeleton. When ROCK I or ROCK II are cleaved and activated by caspases[8], actin-myosin contraction is induced, resulting in disruption of the cell membrane, leading to a loss of plasma membrane asymmetry and the formation of MPs. Together, an increase in intracellular calcium and activation of Rho-associated kinases initiate the process of cytoskeletal reorganisation and membrane blebbing.

A number of different stimuli and pathways have been reported to activate endothelial microparticle (EMP) release eg, shear stress triggers release of MPs *via* activation of the Rho kinase and the ERK 1/2 signalling pathway[9]; angiotensin II-mediated EMP release also requires Rho kinase and intact lipid rafts[10]; TNF-α is a well characterised inducer of EMP formation and acts through p38 mitogen-activated protein kinase[10, 11] and thrombin acts on caspase-2 which cleaves ROCK II leading to cytoskeletal rearrangement and EMP release independent of apoptosis[12]. In addition, Liu ***et al*** have shown that cellular cholesterol accumulation in human monocytes and macrophages generates biologically active, phosphatidylserine (PS)- and tissue factor (TF) -positive MVs, causing an increase in procoagulant activity, thus contributing to atherosclerotic plaque development[13].

ENDOTHELIAL MICROPARTICLE (EMP) COMPOSITION AND STRUCTURE

MPs are complex vesicular structures, that consist of an inner and outer phospholipid bilayer containing membrane proteins and receptors[14, 15] (Figure 1). The protein constituents of MPs are dependent on the cell type they were derived from, and the nature of the stimuli which cells are subjected to, as shown by proteomic analysis of MPs released *in vitro* from cultured cells[15].

EMPs have also been shown to contain mRNA and micro RNAs (miRs)[16-18]. MiRs are short non-coding RNAs involved in tissue- and cell type-specific gene regulation. They are thought to be transferred from EMPs to target cells via interaction with α4 and β1 integrins on the cell surface[17] and via membrane fusion involving phosphatidyl serine uptake[19]. These novel miRs thus provide a vital and novel pathway by which cells can communicate. EMP mRNA and micro RNA transfer has been shown to induce angiogenesis and proliferation of endothelial cells[17, 18]. Furthermore, differences in the micro RNA composition of MPs under disease conditions such as cardiovascular disease[16], or under different states of activation[20] may influence cell communication via this mechanism. EMPs are considered as cargo vehicles derived from parental cells, as they can also include bioactive lipids, integrins, cytokines and enzymes[21]. Overall, this work demonstrates how the molecular composition of EMPs dictates their downstream biological function.

DETECTION OF ENDOTHELIAL MICROPARTICLES

MPs are detected using flow cytometry, a technique which separates cells or particles by their size, granularity and cell-surface antigens, using antibody-conjugated fluorophores[22]. MPs can be more accurately quantified by the addition of a known volume of size and density-specific fluorescently-labelled microbeads to the sample[23].

No single marker for EMPs exists, as many antigens are also present on a number of different cell sub-sets, therefore, for accurate identification a panel of markers is used. We[23, 24] and others[3], routinely assess EMPs on size, granularity, Annexin V (to detect PS) and CD31-positivity. CD31-positive platelets are eliminated using a platelet-specific marker,

CD42b, thus generating an EMP profile of Annexin $V^+/CD31^+/CD42b^{-}$[23]. Other endothelial markers, such intercellular adhesion molecule 1 (ICAM) and endoglin, can also be used with CD45 to gate against contaminating haematopoietic cells. More specific markers, such as CD144, have been identified but they have lower sensitivity as reviewed by Dignat-George & Boulanger *et al* [25].

ATHEROSCLEROTIC PLAQUE FORMATION

Atherosclerosis is a known chronic inflammatory disease characterised by atheromatous plaque deposition in the vascular bed. Plaque formation is a complex process largely driven by high blood cholesterol, endothelial activation and inflammation-induced vascular cell remodelling with deposition of extracellular matrix (ECM), lipid and calcification. Plaque instability and possible rupture may lead to athero-emboli triggering a major cardiovascular event, such as stroke or myocardial infarction.

Atherosclerotic plaques tend to occur in areas of arterial branching such as the carotid, femoral and aortic bifurcation where turbulence is greatest[26]. In these areas, the endothelial cells are polygonal in shape and not aligned in the direction of flow, making them highly permeable to macromolecules such as LDL[27]. Excess LDL is subject to oxidation, lipolysis, proteolysis and aggregation in the vessel wall[28] where it initiates inflammation. Activation of the endothelium elevates expression of endothelial adhesion markers, such as ICAM, vascular cell adhesion molecule, P and E selectin, which bind to specific surface markers on monocytes (CD11b/CD18, CD49d, and CD162 respectively) enabling their migration into the vessel wall where they differentiate into macrophages[28, 29]. Activated macrophages secrete inflammatory molecules, such as IL-6, IL-8 and monocyte chemotactic protein-1. In turn, this perpetuates the recruitment of monocytes and other inflammatory cells including T and B

cells[30, 31], and matrix metalloproteinases (MMPs) to facilitate cellular migration through the ECM, as reviewed by Lusis[27].

The inflammatory environment created can induce apoptosis of smooth muscle cells, eventually forming a necrotic core of lipid and ECM covered by a fibrotic cap, where the newly deposited ECM provides structural rigidity[32]. Smooth muscle cell proliferation and migration from the media into the intima can also contribute to plaque development, where they assume a synthetic role producing ECM, collagen, cytokines and proteases[33, 34] and may act as a nidis for calcification in the later stages[35]. This hypoxic niche also induces angiogenesis in an attempt to nourish the damaged vessel wall, however, neovessels are often leaky, due to a lack of cell recruitment and vessel maturation[36, 37].

It is thought that these remodelling processes involving the presence of a thin fibrotic cap and/or a large necrotic core, coupled with shear stress and endothelial erosion causes plaque instability and increased predisposition to plaque rupture and subsequent thrombosis[38,39] (Figure 2). It is still debatable whether the presence of calcification in the plaque exerts a stabilising effect.

CURRENT BIOMARKERS OF CARDIOVASCULAR DISEASE

The risk of developing cardiovascular disease can be assessed by calculating a Framingham risk score, which takes into account, hypertension and high blood LDL, low HDL, and smoking. Typically, atherosclerosis remains undetected until a rupture or a reduction in blood flow results in a cardiovascular event, such as transient ischaemic attack, stroke or myocardial infarction.

There are many studies identifying new biomarkers associated with standard risk factors of cardiovascular disease[40]. A comprehensive review of 53 biomarkers published in the last 10 years by different research groups was compiled by Yanan[40]. The sedimentation rate of erythrocytes was found to be a good predictor of coronary artery disease[40]. However, many mediators of inflammation, such as the interleukins and high sensitivity C-reactive protein[41], are notoriously difficult to interpret since levels can be elevated as a result of other independent co-related morbidities. Furthermore, a panel of biomarkers will provide a more comprehensive assessment of disease, although they may only be elevated in the acute phase when it is possibly too late for intervention[40, 42].

EVIDENCE OF EMPs AS A BIOMARKER OF ATHEROSCLEROSIS /CARDIOVASCULAR DISEASE

The vascular endothelium is of paramount importance in arterial wall integrity and blood flow regulation[43] and its activation can lead to cell membrane disruption by apoptosis and EMP release[25]. Our group has previously demonstrated that the pro-inflammatory cytokine, tumour necrosis factor-alpha (TNF-α), stimulated significant release of EMPs from human aortic endothelial cells *in vitro,* an effect which was abrogated by the TNF-α-binding drug, certolizumab[24]. In patients with Systemic Lupus Erythematosis (SLE), where TNF-α is a major disease protagonist, we found significantly elevated EMP levels in association with endothelial dysfunction, in addition immunosuppressive therapy was able to significantly reduce EMP levels to that comparable with controls[23]. These data strengthen the findings of others demonstrating elevated levels of EMPs in SLE[44,45], supporting the suggestion that EMPs could act as a biomarker of SLE treatment strategies[23] but also other cardiovascular diseases with a dysfunctional endothelial component.

Our recent studies demonstrate significantly elevated EMP levels in patients who are symptomatic for carotid artery disease compared to age-matched controls (p=0.01; Figure 3) and support studies by other groups who have also demonstrated elevated circulating EMPs in association with coronary artery disease[16,46,47,48] and stroke[49, 50]. There is evidence to suggest that elevated EMPs not only correlate with coronary endothelial dysfunction[47], but are associated with an increased risk of a major cardiovascular event[46,48]. Bernal-Mizrachi *et al*, found that in patients undergoing coronary angiography, EMPs were significantly elevated in patients with high-risk eccentric type II lesions with multiple irregularities and thrombi compared to low-risk concentric lesions containing no thrombi[46]. Interestingly, although patients with 20-45% stenosis demonstrated 5-fold more EMPs than those with no stenosis, they also had 3-fold more EMPs compared to patients with >45% stenosis. The authors postulate that the level of EMPs may peak during remodelling of the vessel wall when inflammatory stimuli are at their greatest. Furthermore, plasma leukocyte-derived MPs have recently been correlated with carotid plaque instability in a cohort of patients undergoing carotid endarterectomy thus advocating elevated MPs as a potential marker of unstable plaques[3].

These findings correlate with recent work in a larger cohort of patients where EMPs were significantly higher in patients who had experienced their first major cardiovascular/cerebral event[48] with a similar finding in acute stroke[51]. Other work has shown that elevated EMP levels were not useful in distinguishing between acute stroke and stroke mimic patients[50].

MPs are being assessed as one of many biomarkers for treatment outcomes in various clinical trials for cardiovascular disease, as reviewed by Martinez *et al*[52]. Statins, which are given to treat hyperlipidaemia, have been shown to not only lower cholesterol, but also

reduce the number of total MPs in the circulation[53] including those derived from platelets, leukocytes as well as endothelial cells. This is in agreement with other groups who have demonstrated a reduction in MPs derived from monocytes[54] and platelets[55] after statin treatment. Other cardio-protective drugs such as angiotensin II receptor blockers[54], calcium blockers[56], aspirin[57] and clopidrogrel[58] have also been found to reduce levels of MPs and are summarised in Table 1. Whether circulating MPs are reduced by a direct effect of these drugs or as a consequence of decreased cholesterol, inflammation or overall cardiovascular risk remains to be elucidated. Interestingly, digoxin was shown to increase EMPs and PMPs in patients treated for atrial fibrillation, potentially predisposing patients to thrombosis and vascular events[59]. To our knowledge, there are no published data to date which correlate the use of endothelin antagonists and beta blockers with MP levels.

Patients with pulmonary hypertension exhibit significantly elevated EMPs, compared to normal controls. In addition, no significant difference exists between pulmonary hypertension patients with coronary artery disease and those without, suggesting a link between EMP release[60] and pulmonary artery dysfunction. High levels of circulating EMPs have also been observed in smokers compared to healthy individuals, however, even in healthy individuals, baseline levels may fluctuate under pathological conditions, for example stress and inactivity[61, 62]. It is interesting to note that raised EMP levels have been detected in coronary artery disease, peripheral vascular disease, cerebral ischemia, and congestive heart failure, all of which have been described as examples of age-related vascular disease, however, it is still unclear whether microparticles play a role in the aging process *per se*.

In order to demonstrate that an increased EMP level is a robust biomarker of cardiovascular disease, EMP levels should be established as independent of other risk factors. This

independence has been shown[46,47], but a link between high EMPs, diabetes and gender has also been detected[48] which may reflect the higher number of patients investigated in the latter study. Taken together, these findings support the use of EMPs as potential biomarkers of cardiovascular disease and as a potential predictor of high-risk events. Although technically difficult to measure with some variations between laboratories, steps have been taken to standardise the detection protocol[63] which will be necessary before adoption for clinical diagnostic use.

DETRIMENTAL EFFECT OF EMPs IN ATHEROSCLEROTIC DISEASE

A number of studies have provided evidence on how MPs may be interacting with plaque initiation, formation and stability, although the exact mechanism remains largely unknown. *S*hear stress and turbulent flow, potential plaque-initiating factors, have been shown to stimulate endothelial cell apoptosis in endarterectomy plaques, which may favour EMP release. EMPs may also attenuate the bioavailability of endothelial nitric oxide, a well-known vasodilator, by stimulating the formation of free radicals[64, 65], thus disrupting normal function. Densmore ***et al*** also reported pulmonary oedema in rats injected with EMPs[65].

MPs have also been detected in atherosclerotic plaques, where their composition may elicit different functions to those observed in the blood[66,67,68]. It has been shown that MPs isolated from plaques exhibit MMP activity that may, in turn, perpetuate inflammation via TNF-α shedding[67]. Detailed analysis of plaque MPs revealed that MPs derived from leukocytes and erythrocytes constitute the greatest proportion, whilst those derived from smooth muscle cells and endothelial cells, were present in smaller amounts[68]. No differences in MP composition were detected between asymptomatic and symptomatic patients[68]. However, the plaque MPs exhibited tissue factor (TF) activity and were able to

generate thrombin in *in vitro* assays, suggesting that these MPs exhibit pro-thrombogenic activity[68].

Plaque thrombogenicity is dependent on the substrate present within the plaque itself. Initiation of the coagulation cascade requires the activation of coagulation factors, influx of calcium ions within cells and negatively charged phospholipids. Both PS present in the outer layer of the plasma membrane of apoptotic cells and MPs, together with TF, have procoagulant properties[69]. The negatively charged PS is thought to interact with clotting proteins, resulting in the formation of clotting factors and thrombin, as reviewed by Owens and Mackman[70]. TF is a transmembrane glycoprotein found on the surface of certain MPs and is also expressed in large amounts in atherosclerotic plaques[70, 71]. It is involved in the early stages of coagulation forming complexes with Factor VII/VIIa leading to the initiation of the coagulation cascade, which plays a major role in endothelial repair after injury as part of a normal haemostatic mechanism[70]. Plaque-derived MPs, mainly of macrophage origin, have been shown to be responsible for most of the TF activity in atherosclerotic plaque, indicating a relationship between MPs and TF concentration[66, 68].

MPs may provide the substrate for secretory phospholipase A_2($sPLA_2$) producing lysophosphatidic acid (LPA), a known platelet agonist and potent bioactive phospholipid involved in inflammatory pathways[72]. LPA has been shown to accumulate in human atherosclerotic plaques and is formed by the autotoxin-mediated hydrolysis of lysophosphatidylcholine derived from oxidation of low density lipoprotein (LDL)[73, 74]. When atherosclerotic plaques become damaged, procoagulant material is exposed to circulating blood. The thrombogenic potential of the exposed plaque is a key feature in vascular thrombosis and luminal narrowing[75].

Rautou *et al*, 2011 isolated MPs from plaques and demonstrated that they were able to transfer ICAM-1 to the membranes of endothelial cells *in vitro* through membrane fusion involving phosphatidyl serine uptake[76]. It was suggested that high levels of plaque MPs could transfer ICAM molecules to endothelial cells of leaky neovessels in close proximity and induce recruitment of immune cells from the peripheral blood, thus exacerbating disease[19]. EMPs may facilitate angiogenesis by releasing MMPs promoting the degradation of interstitial matrix, allowing endothelial cell invasion[77, 78]. Leroyer *et al* have shown that MPs extracted from human atherosclerotic plaques promote and regulate endothelial cell proliferation and neovascularisation *in vitro*[11].

The detrimental effect that other types of MPs in the circulation may have on endothelial cell activation should not be ruled out. MPs of different cellular origin, including leukocytes and platelets have been shown to stimulate the release inflammatory cytokines which may exacerbate the underlying inflammation in atherosclerotic disease, and stimulate thrombotic events[60, 79-81].

A PROTECTIVE ROLE FOR EMPs IN ATHEROSCLEROSIS

Reports show that EMPs could play a protective role in disease by carrying proteins that possess anti-coagulant and anti-inflammatory properties. For example, Perez-Casal *et al* demonstrated that EMPs were released when human endothelial cells were exposed to activated protein C (APC)[82]. Protein C has been shown to have an anti-coagulant and anti-inflammatory role since it can reduce the production of i) thrombin by inactivating factors Va & VIII, ii) tissue factor and iii) cytokines IL-1, IL-6 and TNF-α[82]. APC binds to the protein C receptor (EPCR) on endothelial cells and the EMPs released, retain the EPCR-APC complex, resulting in an anti-coagulant effect and anti-inflammatory effect by reducing IL-1 and TNF-

α[82]. Furthermore, it has also been shown that prevention of EMP release is associated with endothelial cell apoptosis and cell detachment *in vitro*[83] supporting a possible protective function for EMPs.

Gasser ***et al***, found that MPs released from polymorphonuclear neutrophils blocked the innate immune response of macrophages to compounds found in bacterial (lipopolysaccharide) and yeast cell walls (zymosan)[84]. Polymorphonuclear neutrophil MPs display PS on their outer membrane, which can attach to the PS receptor on the cell surface membrane of macrophages inducing the release of transforming growth factor beta 1 and suppressing the release of pro-inflammatory mediator cytokines IL-8 and IL-10[85, 86]. These data suggest that EMPs may also be involved in the homeostatic mechanism that controls the anti-coagulant / coagulation equilibrium.

To determine a function for EMPs in atherosclerosis, Zernecke and colleagues generated apoptotic bodies from HUVECs and injected them into the ApoE-/- atherosclerosis mouse model. Increased CXCL12/CXCR4-mediated signalling in vascular cells was detected thus triggering recruitment of progenitor cells to sites of damage[87]. Indeed, other groups have demonstrated that MPs have the potential to induce endothelial differentiation of progenitor cells[17, 88, 89]. These promising studies suggest a potential role for MP-stimulated regeneration of the endothelium[52,89,88] but their function may be dependent on the composition of the MPs and the conditions under which they were generated.

SUMMARY

Despite the current understanding of atherosclerotic plaque development and rupture and the ominous outcome of thrombosis in asymptomatic patients, the question of what causes

the transition from a stable to an unstable plaque has yet to be elucidated. Identifying a way of predicting patients' susceptibility to plaque rupture could have enormous cost-saving benefits in the clinic. Although research to explore the use of EMPs as biomarkers of disease has progressed substantially over the last few years, further work is still required to develop standardised methods of EMP analysis and quantification. Validation of normal EMP ranges and a specific set of EMP markers for diagnostic testing needs to be established, as discussed at the International Society on Thrombosis and Haemostasis (ISTH) Vascular Biology Standardisation Subcommittee (VB SSC) workshop, and highlighted in the report by Lacroix ***et al***, (2013)[63]. A recent study has discussed the design of a more refined procedure for the isolation of circulating EMPs, suggesting this method removes contamination of other cell fragments and platelets using imaging flow cytometry[90].

As methodology is developed with more precision, EMP levels and content analysis may be combined with other markers of atherosclerosis, including inflammatory, lipid, angiogenic and metabolic profiling, allowing the development of an adjunct to current clinical practice that would have the potential to stratify patients according to their risk of carotid disease and stroke (Figure 4). We propose the innovation of a high throughput multiplex assay or a fluorescent chip detection modality. The device would identify specific MPs, based on protein content, and could be used in parallel to a device that identifies an associated cytokine/growth factor profile, which correlates with the initial stages of endothelial damage, allowing the development of an accurate and speedy point-of-care tool for routine use in diagnostic laboratories.

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FIGURE LEGENDS

Figure 1. Schematic representation of endothelial cell activation and EMP release. Oxidised LDL, sheer stress and other inflammatory stimuli induce cell activation and apoptosis. Caspase-mediated Rho-associated kinase (ROCK1) activation and increased intracellular calcium result in the translocation of phosphatidylserine and cytoskeletal protein disruption. Together, these processes cause membrane budding and EMP release.

Figure 2. Stages of atherosclerotic plaque development. Endothelial cells (ECs) in areas of turbulent blood flow become permeable to oxidised LDL. In response to activation, increased adhesion marker (intercellular adhesion molecule 1 [ICAM] and vascular cell adhesion molecule [VCAM]) expression leads to monocyte recruitment, migration and differentiation into macrophages. Inflammatory stimuli induce EC activation and apoptosis, resulting in EMP release. Macrophages, in addition to releasing lipids into the extracellular matrix, also secrete pro-inflammatory cytokines which promote the recruitment of monocytes and other immune cells. These processes, in combination with smooth muscle cell (SMC) migration and collagen production, lead to plaque development.

Figure 3. EMPs are significantly elevated in carotid artery disease (CAD) patients compared to controls. EMP content (Annexin $V^+/CD31^+/CD42b^-$) was measured using standard flow cytometric techniques in blood samples taken from symptomatic patients with carotid artery disease (n=51) and age-matched controls (n=10). (Ethics reference: 12/YH/0107) Subject range was from 33yrs to 75yrs of age. Data were analysed using a Mann-Whitney test and the error bars represent the standard error of the mean. * denotes p=0.01.

Figure 4. Schematic diagram of proposed model illustrating how microparticles and biomarkers could be used to stratify patients at risk of stroke. A chip could be developed with a panel of markers associated with the respective pathological processes as shown, that will define a patient's disease status and ability to respond to a treatment strategy.

Drug used	Effect on Microparticles				Reference
	EMP	PMP	LMP	MMP	
Statins	↓	↓	↓	↓	Suades *et al*, 2013[53]
	-	-	-	↓	Nomura *et al*, 2004[54]
	-	↓	-	-	Mobarrez *et al*, 2011[55]
Ang II receptor blockers	-	-	-	↓	Nomura *et al*, 2004[54]
	-	↓	-	-	Labiós *et al*, 2004[56]
Calcium channel blocker	-	↓	-	-	Nomura *et al*, 2002[91]
Aspirin	↓	↓	-	-	Bulut *et al*, 2007[57]
Clopidrogrel	-	↓	-	-	França *et al*, 2012[58]
Digoxin	↑	↑	-	-	Chirinos *et al*, 2005[59]

Table 1. Effects of commonly used cardio-protective drugs on microparticle levels. (EMP-endothelial microparticle, PMP- platelet microparticle, LMP-leukocyte microparticle, MMP-monocyte microparticle, - not investigated).

Figure 1. Schiro, Wilkinson *et al*, 2014.

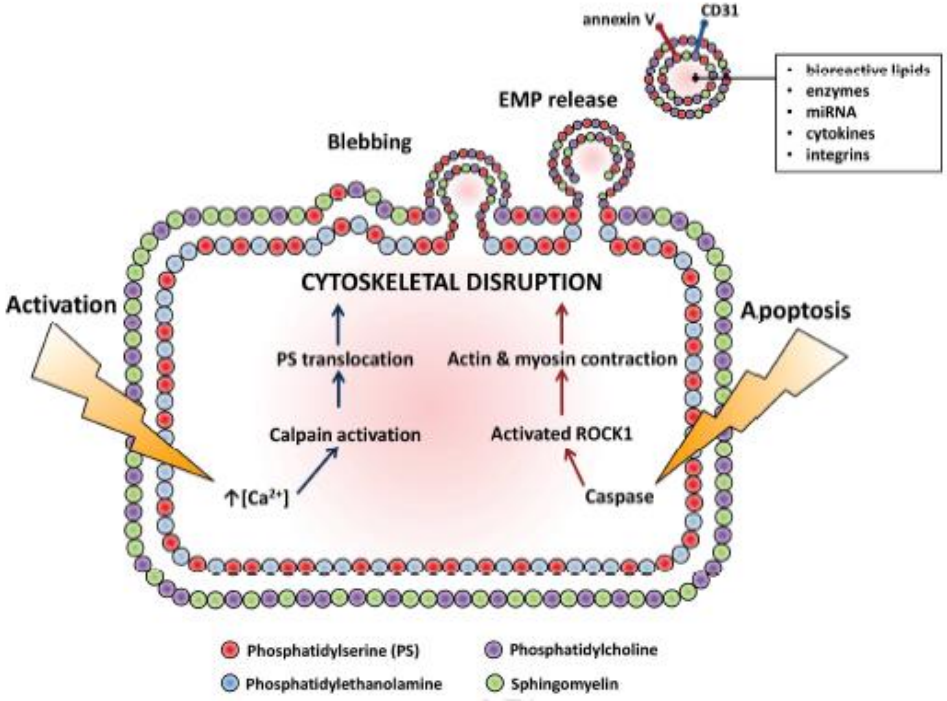


Figure 2. Schiro, Wilkinson *et al*, 2014.

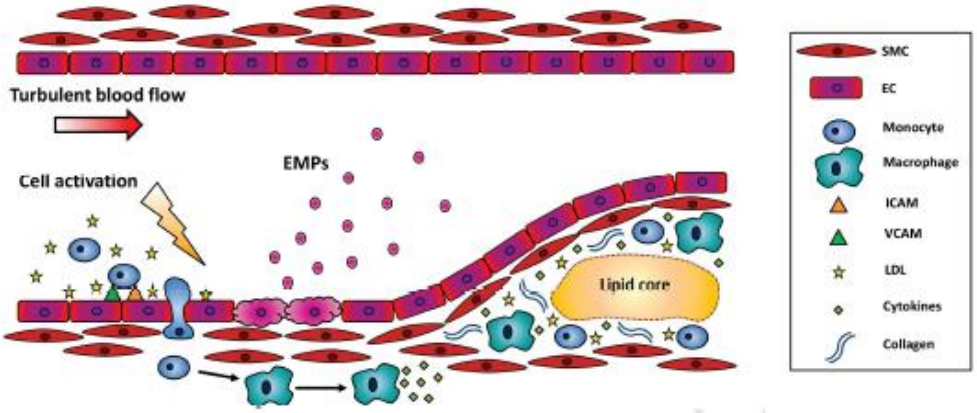


Figure 3. Schiro, Wilkinson *et al*, 2014.

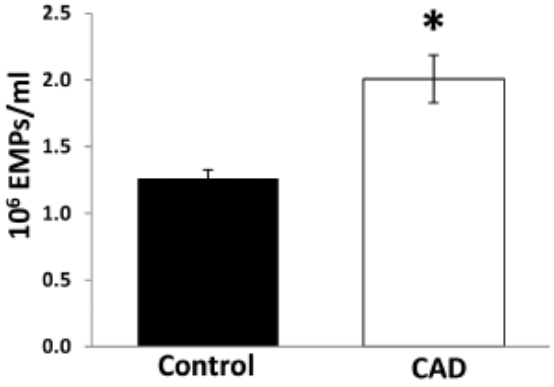
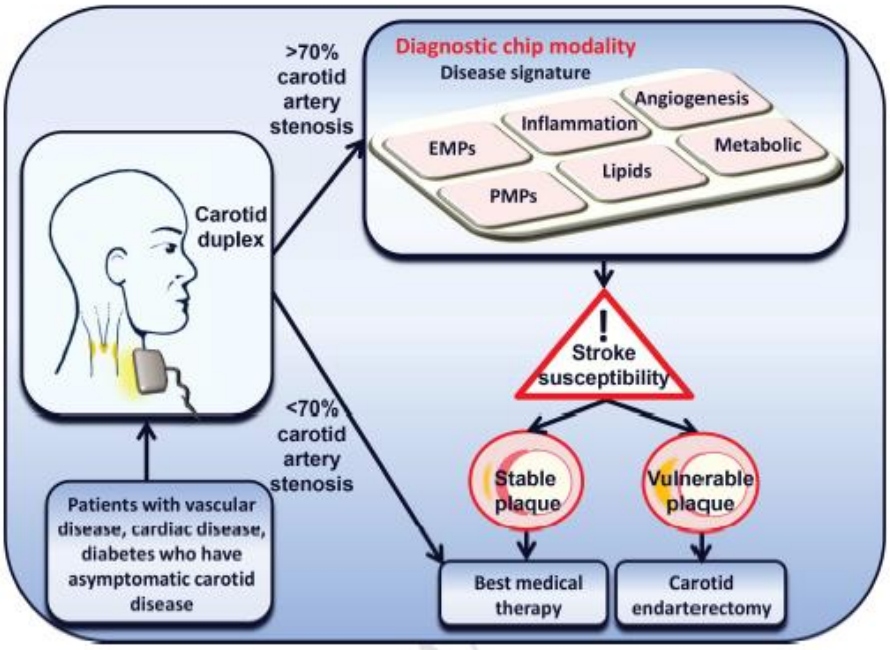


Figure 4. Schiro, Wilkinson *et al*, 2014.



Highlights

Endothelial microparticles as conveyors of information in atherosclerotic disease.

Schiro, Wilkinson *et al*, 2014.

- Review of the role of microparticles in pathogenesis of atherosclerotic disease
- Potential of endothelial microparticles as novel biomarkers of atherosclerosis
- Use of a novel diagnostic approach to stratify patients most at risk of stroke
- Microparticles as signalling molecules in cardiovascular disease
- Endothelial microparticles in atherosclerosis

Appendix: 7 Copy of paper submitted to ATVB Journal (Atherosclerosis, thrombosis and Vascular Biology) - June 2015

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Elevated levels of endothelial-derived microparticles and serum CXCL9 and SCGF-β are associated with unstable asymptomatic carotid plaques.

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RUNNING TITLE
Endothelial microparticles in carotid disease.

KEY WORDS
Endothelial microparticles, inflammation, carotid stenosis, plaque stability, endarterectomy, biomarker, stroke.

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ABSTRACT

Objective. Endothelial microparticles (EMPs) are released from dysfunctional endothelial cells. We hypothesised that patients with unstable carotid plaque have higher levels of circulating microparticles compared to patients with stable plaques, and this may correlate with serum markers of plaque instability and inflammation.

Approach. Circulating EMPs, platelet MPs and inflammatory markers were measured in twenty healthy controls and seventy patients undergoing carotid endarterectomy. EMP/PMPs were quantified using flow cytometry. Bioplex assays profiled systemic inflammatory and bone-related proteins. Immunohistological analysis detailed the contribution of differentially-regulated systemic markers to plaque pathology. Alizarin red staining showed calcification.

Results. EMPs and PMPs were significantly higher in patients with carotid stenosis (≥70%) compared to controls, with no differences between asymptomatic *vs* symptomatic patients. Asymptomatic patients with unstable plaques exhibited higher levels of EMPs compared to those with stable plaques, with a similar trend observed in symptomatic patients. CXCL9 and SCGF-β were significantly elevated in asymptomatic patients with unstable plaques, with IL-16 and macrophage inhibitory factor significantly elevated in the stable plaque group. CXCL9, CTACK and SCGF-β were detected within all plaques, suggesting a contribution to both localised and systemic inflammation. Osteopontin and osteoprotegerin were significantly elevated in the symptomatic *vs* asymptomatic group, while osteocalcin was higher in asymptomatic patients with stable plaque. All plaques exhibited calcification, which was significantly greater in asymptomatic patients. This may impact on plaque stability.

Conclusions. Circulatory EMP, CXCL9 and SCGF-β levels are raised in asymptomatic patients with unstable plaques, which could be important in identifying patients at most benefit from intervention.

INTRODUCTION

Every year around 145,000 carotid endarterectomies (CEA) and 20,000 carotid stenting procedures are performed in Europe[1] and the US.[2, 3] Of these, around 12,000 procedures are performed in asymptomatic patients in an attempt to reduce these patients' risk of a possible stroke. The two landmark randomised trials, Asymptomatic Carotid Atherosclerosis Study (ACAS)[4] and the Asymptomatic Carotid Atherosclerosis Trial (ACST)[5] demonstrated that CEA conferred a 50% relative risk reduction in a 5-year risk of stroke in patients with carotid stenosis of ≥70% from 12% to 6%. ACST, the larger study showed that immediate CEA conferred a 4.6% absolute risk reduction in stroke compared to best medical treatment. This equates to 46 strokes prevented per 1000 operations over a 10 year period. Not all patients with asymptomatic disease go on to develop a stroke, which is thought to be due to the composition of carotid plaques, as outlined in the Oxford plaque study for symptomatic plaques[5]. This study showed a correlation between presence of symptoms, timing of surgery and the morphological characteristics of plaques. Patients who had a CEA shortly after the onset of symptoms had plaques which showed features of instability that included a higher prevalence of fibrous cap rupture, a large lipid core and dense macrophage infiltration.[5]

The natural progression of atherosclerotic disease is characterised by a chronic inflammatory response in the arterial wall. It is well established that elevated systemic proteins and increased expression of inflammatory cytokines are associated with vulnerable plaque.[7, 8, 9] However, due to the complex nature of the underlying inflammatory status of these patients, it is unlikely that identifying single molecules or pathways will yield new therapeutic targets to reduce or alleviate progression of atherosclerotic plaque development. Further investigation is needed to understand the direct and specific effects of the inflammatory cytokines and their interaction with other proteins before they become applied as novel biomarkers for use in the clinic. Inflammatory cytokines, which have been shown to be associated with unstable atherosclerotic plaques in previous studies, may help to predict plaque behaviour.

Recently, there has been interest in the presence and role of circulating microparticles as biomarkers of disease.[10, 11] Microparticles are anucleoid submicron sized fragments (50nm-1μm) derived from damaged cell membranes that harbour lipids, microRNAs, and specific proteins that represent the parent cells they originate from,[12] thus acting as carriers of biological information. Endothelial microparticles (EMPs) are complex vesicular structures released from activated or apoptotic endothelial cells.[10, 13] We postulated that EMPs may be potential biomarkers of patients who have unstable plaques. These patients are thought to be at higher risk of stroke. The aim of this study was to correlate plaque morphology with circulating EMPs and inflammatory cytokines, in order to generate a panel of biomarkers to identify those patients most at risk of plaque rupture.

METHODS

Materials and methods are available in the online-only Data Supplement together with Supplemental Figures I to IV.

RESULTS

Plaques from symptomatic patients exhibit increased ulceration and haemorrhage

Asymptomatic patients (n=19), symptomatic patients (n=51) and healthy age-matched controls (n=20) with no history of cardiovascular disease, were recruited into the study.

Patients with carotid artery disease, from both groups, were matched evenly for age and various risk factors including hypertension, hypercholesterolemia and diabetes, with a high proportion of both groups receiving statins, anti-platelet and anti-hypertensive drugs (Supplemental Table II). Of the symptomatic patients, 13 patients had an acute stroke, 31 patients had a TIA, while 7 patients had retinal embolic disease on the ipsilateral side of their carotid stenosis.

Following surgical intervention on the depiction of stenosis, the plaques were graded histologically into two groups; stable and unstable (see supplemental methods). In patients with symptomatic carotid artery stenosis, 42 (82.3%) and 9 (17.7%) patients were identified with unstable and stable plaques respectively, whilst in the asymptomatic group,13 (68.3%) and 6 (31.7%) patients exhibited unstable plaques and stable plaques respectively.

Plaque ulceration was significantly greater in the plaques from the symptomatic group (48 plaques ulcerated, 96%) compared to those from asymptomatic patients (12 plaques ulcerated, 63%; $P<0.0008$). Intra-plaque haemorrhage (IPH) was defined as an area containing red cells that caused disruption of plaque architecture[14] and was greater in symptomatic plaques (42 plaques, 82%) compared with asymptomatic plaques (5 plaques, 26%; $P=0.01$).

Endothelial microparticle levels correlate with instability in asymptomatic patient plasma

Annexin V$^+$/CD31$^+$/CD42b$^-$-EMPs were detected in platelet-poor plasma samples from asymptomatic and symptomatic patients and healthy controls, using flow cytometry (Supplemental Figure I). EMPs were significantly elevated in both asymptomatic and symptomatic groups, compared to healthy controls ($P=0.03$ and $P=0.001$, respectively) but no differences were detected between the asymptomatic and symptomatic groups (Figure 1Ai).

Each patient group was subdivided based on plaque stability. Significantly elevated EMPs were detected in asymptomatic patients with unstable plaques compared to those with stable plaques ($P<0.01$), while no such distinction was apparent in symptomatic patients (Figure 1Aii). Furthermore, EMP levels were significantly higher in patients with unstable plaques compared to those with stable plaques, when both the asymptomatic and symptomatic patient groups were combined ($P=0.035$; Supplemental Figure III), as well as showing a weak correlation ($r=0.424$; $P<0.001$) between plaque grade and EMPs.

A higher EMP to PMP ratio is associated with plaque instability in asymptomatic patients

Annexin V$^+$/CD31$^+$/CD42b$^+$-PMPs were significantly higher in the asymptomatic and symptomatic patient groups compared to healthy controls ($P=0.018$ and $P=0.03$), respectively (Supplemental Figure IV). These PMP data strongly correlated with increasing EMPs ($r=0.981$; $P<0.001$), however no differences were detected between the asymptomatic and symptomatic groups. In addition, there was no distinction in PMP levels between patients with stable and unstable plaques in either patient group nor when asymptomatic and symptomatic groups were combined (data not shown). When the ratio of EMPs to PMPs (EMP:PMP) was measured, it was found that the ratio was significantly lower in the symptomatic group compared to the asymptomatic group and healthy controls ($P=<0.001$ and $P=0.02$ respectively; Figure 1Bi). In the asymptomatic patient group, the EMP:PMP ratio was significantly higher in patients with unstable plaques compared to the those with stable plaques (Figure 1Bii), but again no distinction of this nature was detected in the symptomatic group.

Systemic and carotid artery plaque inflammatory profiling

We next examined the relationship between plaque vulnerability and inflammatory biomarkers using pre-operative blood and tissue specimens. From the panel of 21 systemic markers of inflammation analysed, six were differentially regulated in the asymptomatic and symptomatic groups compared to controls. CTACK, IL-3, IL-16 and HGF were significantly higher in asymptomatic patient serum compared to healthy controls (P<0.02), with a trend towards elevation of CXCL9 and a significant reduction in MIF (Figure 2). CTACK, IL-3, IL-16 and CXCL9 were significantly elevated in symptomatic patient serum compared to healthy controls, with a trend towards an increase in HGF (Figure 2). No significant differences were detected between asymptomatic and symptomatic patients or in the levels of IL-2Rα, SCGF-β, IL-18, SDF-1α or SCF between any groups. IL-1α, GRO-α, IFN-α2, LIF, MCP-3, M-CSF, β-NGF, TNF-β and TRAIL were below the level of detection (data not shown).

Furthermore, inflammatory markers were compared between unstable and stable plaques in each patient group. In asymptomatic patients, CXCL9 and SCGF-β were significantly elevated (P=<0.01) in patients with unstable compared to stable plaques, whereas IL-16 and MIF were significantly elevated in the stable plaque group (Figure 3). In addition, EMPs exhibited a strong negative correlation with the level of SCGF-β (r=-0.943; P<0.005) and IL-16 (r=-0.829; P<0.05) in asymptomatic patients with stable plaques. HGF was significantly elevated in symptomatic patients with unstable plaque (P<0.05) with no other differences detected within the symptomatic group (Figure 3). IL-2Rα, IL-12P40, CTACK, IL-3, IL-18, or SCF-1 showed no difference in patients with stable and unstable plaques in either the asymptomatic or symptomatic group. When we compared all patients with stable versus unstable plaques, CXCL9 (P=0.040) was significantly elevated and IL-16 (P=0.014) was significantly lower in patients with unstable plaques (data not shown).

The plaque composition, cellularity, severity of inflammation, and atheroma-associated macrophages and foam cells were analysed. CD68-positive macrophages were detected in all of the CAE specimens analysed, particularly within the shoulders of the plaque (Figure 4), and of note, the inflammatory nature of these macrophages could be inferred by the presence of TNF-α immunoreactivity in the same vicinity in consecutive sections. To determine whether the up-regulated systemic markers of inflammation present in blood samples of these patients might also influence localised plaque pathology, we assessed their presence within the plaques. MIF, CXCL9, CTACK and SCGF-β were detected in lesions from both asymptomatic and symptomatic groups with no differences between stable and unstable plaques, nor between asymptomatic and symptomatic groups. All IgG controls were negative. Given the close association between thin plaque atheroma,[15] macrophage activity and cathepsin K (CatK) expression, we compared the carotid atheroma specimens for CatK staining and demonstrated expression within the fibrous cap of all specimens with little, if any distinction between groups. In addition, given the recent interest in pentraxin (PTX3) as a new biomarker for inflammatory vascular disease, our data support the findings of others[16] where we demonstrate, not only a positive correlation between the expression of PTX3 in the plaque and the presence of macrophage cells, but also with a range of inflammatory mediators (Figure 4).

Plaques from asymptomatic patients exhibit extensive calcification

Plaques from asymptomatic and symptomatic patients exhibited substantial amounts of vascular calcification, evident both as small spicules and as large blocks, highlighted by the Alizarin red staining (Figure 5A). Quantification of the Alizarin red staining revealed that the plaques from asymptomatic patients had significantly more positive mineralisation than the symptomatic group (P=0.05; Figure 5A). Osteopontin (OPN), a bone related protein, was also detected in close proximity to the calcified areas (Figure 5B), thus confirming our

previous observations, of the association between OPN positive staining within the vicinity of calcification in the vessel wall.[17, 18]

Therefore, we questioned whether proteins involved in bone metabolism may be differentially regulated in the circulation of these patients. OPN (Figure 5Ci) and OPG (Figure 5Cii), inhibitors of bone formation, were both elevated in the serum of symptomatic patients compared to asymptomatic patients (P<0.01), with no difference detected between stable and unstable plaques in either group (data not shown). Osteocalcin (OC), involved in bone formation, was found to be elevated in stable plaques in the asymptomatic group (P<0.05) but not in the symptomatic group (Figure 5D). No differences were found in the levels of IL-6, TNF-α, or sclerostin.

DISCUSSION

Current clinical practice relies on the patient history and medical imaging in the form of Duplex ultrasound, computed tomography angiography and magnetic resonance angiography to select patients with carotid disease that may benefit from surgery. The role of inflammatory markers associated with cardiovascular disease has yet to gain a role in the selection process, when trying to decide which patients will benefit from intervention. It is likely, that in the future, serum biomarkers may become an important clinical tool that could revolutionise the treatment of patients with cardiovascular disease such as those patients with asymptomatic carotid stenosis.

In this study no distinction could be made on actual EMP or PMP numbers between symptomatic and asymptomatic patient groups. In the unit from where we obtained our carotid plaques, surgery is not routinely offered to all patients with asymptomatic disease with ≥70% stenosis. Surgery is only performed to the subgroup of patients who we feel are at high risk of developing a stroke compared to the standard asymptomatic group of patients, for example, patients with ultrasound features of unstable plaques, patients who have a history of a transient ischaemic attack (>6 months) or patients requiring coronary artery bypass graft surgery. 68% of patients in our asymptomatic group had unstable plaques compared to 82% in the symptomatic group. It is therefore not surprising that we found no differences in EMP and PMP levels between the two groups. In view of this finding, the remainder of our analysis largely focused on comparing stable and unstable plaque groups.

Reports show that EMPs are elevated in response to endothelial damage.[7, 19] as well as other cardiovascular-related diseases.[20,21] When we subdivided patients based on the nature of their plaques being stable or unstable, we detected a significant elevation of EMP in the unstable plaques. When this analysis was repeated within the asymptomatic group a difference in EMPs between stable and unstable plaques was still present and significant. This is in keeping with the findings of Wekesa *et al*, (2014).
We found no changes in PMP levels in relation to stable or unstable plaques in either patient group, consistent with Wekesa *et al*.[22] Interestingly, the ratio of EMPs to PMPs was lower in the symptomatic patients compared to asymptomatic patients and controls. However, further analysis showed that the EMP:PMP ratio was significantly higher in asymptomatic patients with unstable plaques.

These data could have significant impact, since EMPs are not thought to give rise to symptoms *per se,* but instead act as a silent indicator of internal damage to the integrity of the endothelium, thus adding strength to the concept of EMPs being a biomarker of plaque instability in the asymptomatic group. There is a growing need for the use of biomarker discovery in plasma as a means of developing a non-invasive diagnostic/prognostic test for stroke susceptibility. We hypothesized that patients at high risk of future events could be

identified prospectively by a combination of high risk plaque features, levels of microparticles and a set of circulating blood biomarkers.

Next, we investigated levels of different serum cytokines to establish the inflammatory profile of the patients. Using a cytokine bead array-approach, we identified several novel biomarkers, which were differentially up and down-regulated in the different patient groups. CTACK, IL-3 and IL-16, which serve as chemo-attractants for different immune cells that are involved in plaque development, were significantly elevated in both symptomatic and asymptomatic patient serum compared to healthy controls, while MIF, was shown to be significantly down-regulated in the asymptomatic group *vs* controls. This is an interesting finding, given that MIF shows properties associated with clot retraction.[23] The finding that HGF was raised in the symptomatic group supports our previous studies on elevated expression of HGF in carotid disease patients,[24] and other reports where hepatocyte growth factor (HGF) has been shown to have a pathophysiological role in disease progression.[24] Serum CXCL9 levels have also been associated independently with coronary artery calcification score after adjusting for traditional cardiovascular risk factors, suggesting that CXCL9 may be used in a panel as a novel biomarker of atherosclerotic plaque burden in humans.[25]

Since the presence of activated macrophages and inflammation is a feature of rupture-prone plaques, and the fact that current imaging techniques cannot be reliably used for the detection of a vulnerable plaque in the clinic, a combination approach using imaging and serum biomarkers for detecting high-risk plaques and preventing acute cardiovascular events, could have clinical benefit for the future development of a point-of-care prognostic device. Therefore, we interrogated the patient groups to determine whether we could correlate serum biomarkers with plaque vulnerability. We now demonstrate that, CXCL9 and SCGF-β were significantly elevated in serum from patients with unstable plaques compared to those with stable plaques, in the asymptomatic group, potentially increasing the invasion of inflammatory cells into the plaque, thus contributing to the instability. When all stable and unstable plaques from both groups were analysed with regards to inflammatory markers, CXCL9 was the only cytokine significantly elevated. Furthermore, there was a strong negative correlation between the level of SCGF-β and EMPs in asymptomatic patients with stable plaque, supporting the concept of an elevated inflammatory/EMP profile, for the unstable group of patients at most risk of stroke. In addition, IL-16 was down-regulated in serum of patients with unstable *vs* stable plaques, the reason for this is unclear, however, the Interleukins are known to be regulatory proteins which can either accelerate or inhibit inflammatory processes, thus acting as damaging, protective or neutral agents, most likely determined by their concentration and other regulatory factors being modulated in the disease process.[26]

The transition from a stable to an unstable plaque puts the patients at risk of thromboembolism and stroke. It is characterised by increased neovascularization and foam cell infiltration, as shown in our previous study.[24] Although intensive medical therapy and carotid endarterectomy have been shown to reduce stroke and death rates in asymptomatic stenosis, it is recognised that patients with low to intermediate artery stenosis can still succumb to ischemic events, providing the impetus to consider plaque composition as an important feature of atherosclerosis. The findings from studies of this nature could in the future contribute to the selection of patients for CEA surgery. This fits with the current strategy for precision medicine.

We investigated the presence of the same biomarkers in plaques to establish if we would find a correlation between systemic and local expression with EMPs and plaque morphology to enable an identification of patients at high risk of future events. The inflammatory markers were present in both types of plaques as expected, adding further strength to the value of systemic markers for the distinction of stable and unstable plaques. In addition, we

investigated the collagenase, cathepsin K, since it has been described as an attractive target for plaque regression and selective inhibitors are already being used in phase III clinical trials.[27] It is not surprising to find the presence of this collagenase in all plaques, since hemodynamics play such a key role in atherosclerotic development and the carotid endarterectomy specimens are taken from regions of low and oscillatory shear stress, where endothelial cells have been shown to contribute to proteolytic vascular remodelling by upregulating cathepsins.[28] These findings are in accordance with the results previously seen by Lutgens *et al*, who demonstrated cathepsin K expression is up-regulated in advanced lesions up to 28 fold.[29] In addition, it is interesting to note that both Cathepsin-K and macrophage migration inhibitory factor (MIF) have been shown to be involved in bone metabolism[30] and we find the presence of both molecules in association with mineral deposition in plaques with evidence of calcification. PTX3 expression was also detected in the lesions, alongside other mediators of inflammation.

Elevated calcification was apparent in the plaques from the asymptomatic group compared to the symptomatic group, whether this affords increased stability remains debatable. In light of these findings, we investigated circulating bone markers and found significantly raised levels of OPN and OPG in the symptomatic group *vs* asymptomatic patients, which adds strength to our histological findings, as both OPN and OPG are inhibitors of mineralisation, supporting the reduced mineralisation detected in the symptomatic patients. Furthermore OC, involved in bone formation, was elevated in asymptomatic patients with stable plaques, lending credence to the idea that calcification could have a stabilising influence. Although tissue analysis of these pro-inflammatory cytokines will not be used clinically, identifying specific cellular and signalling pathways in plaque development will provide a deeper insight into mechanisms of the disease process and could provide a target to be used with non-invasive imaging to enhance the diagnostic and predictive opportunities in this patient group. Although the data set in this study are small, it highlights the challenges associated with patient-to-patient variability and the need to collect not only serological, cellular and molecular analyses of clinical samples but to include genetic information and utilise large scientific data sets, obtained from state-of-the-art omic technologies.

CONCLUSION

A biomarker-based risk stratification for carotid disease may greatly assist clinicians to determine which patients are more likely to have a cerebrovascular event. In turn, this reduces the number of prophylactic carotid endarterectomies performed and their associated complications. Our data, summarised in Figure 6, may contribute to the future development of a risk-profiling tool for assessment of EMPs and a specific inflammatory marker signature, which can be used in combination with current imaging to detect plaque vulnerability and stroke susceptibility.

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DISCLOSURES

None.

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SIGNIFICANCE

Carotid artery atheroma with >70% stenosis is associated with a high risk of restroke, but is common in the asymptomatic population as well, resulting in a large number of operations being performed prophylactically, with a high number needed to treat to prevent each stroke and an associated risk of perioperative stroke. We have found that circulatory endothelial microparticles (EMPs) and markers of inflammation CXCL9 and SCGF-β levels are elevated in asymptomatic patients with unstable plaques, which are the most likely to produce ab initio stroke. These biomarkers could be used to determine plaque vulnerability, stroke

susceptibility and stratify treatment regimens accordingly, avoiding a large number of unnecessary interventions and minimising iatrogenic events.

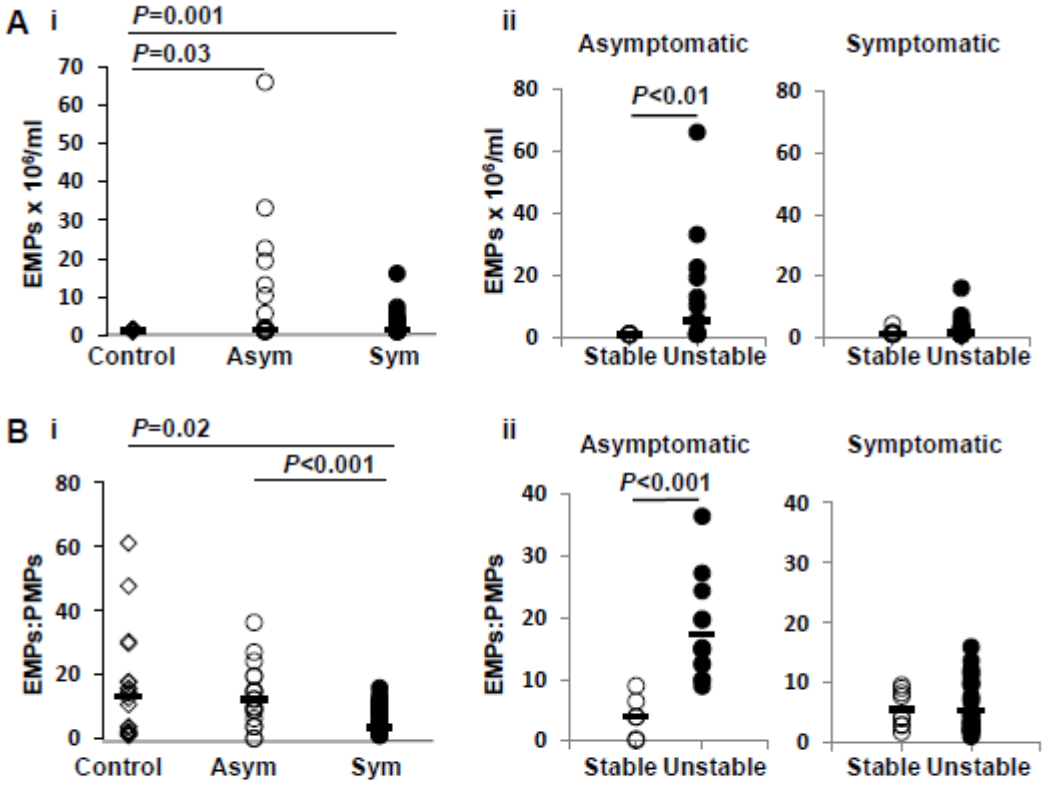

Figure 1. EMPs and EMP:PMP ratio are significantly elevated in asymptomatic patients with unstable plaques. (Ai and ii) EMPs were defined as CD31⁺/AnnexinV⁺/CD42⁻ and counted. (Bi and ii) The ratio of EMPs to PMPs (CD31⁺/AnnexinV⁺/CD42⁺) was also determined. Bars represent the median. Asym-asymptomatic; Sym-symptomatic.

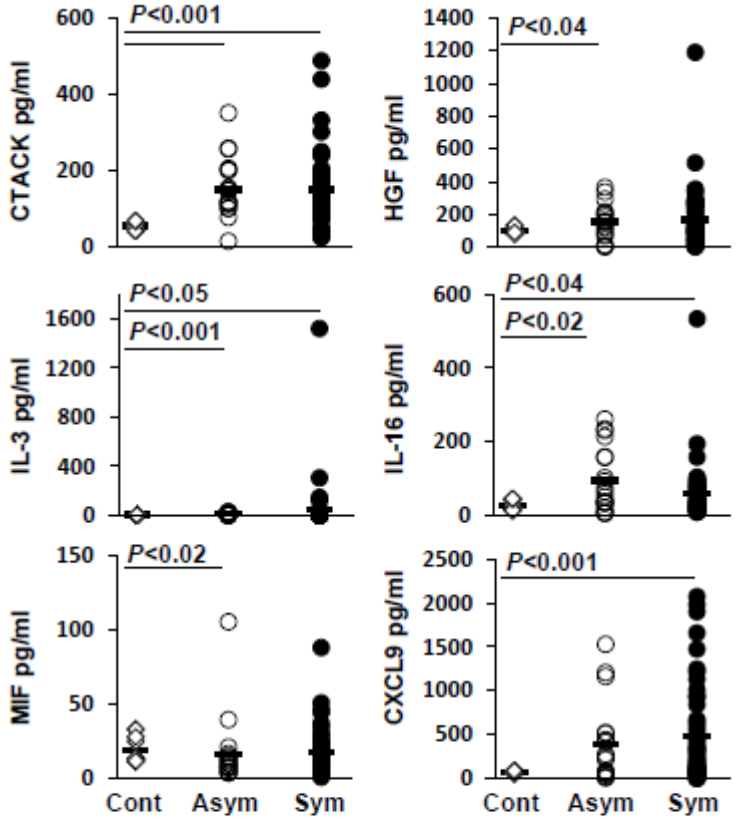

Figure 2. CTACK, IL-3 and IL-16 were significantly elevated in both asymptomatic and symptomatic groups compared to healthy controls. Data were analysed using Kruskal Wallis statistical test. Bars represent the median. Cont; healthy control (n=20), Asym; asymptomatic (n=19), Sym; symptomatic (n=51).

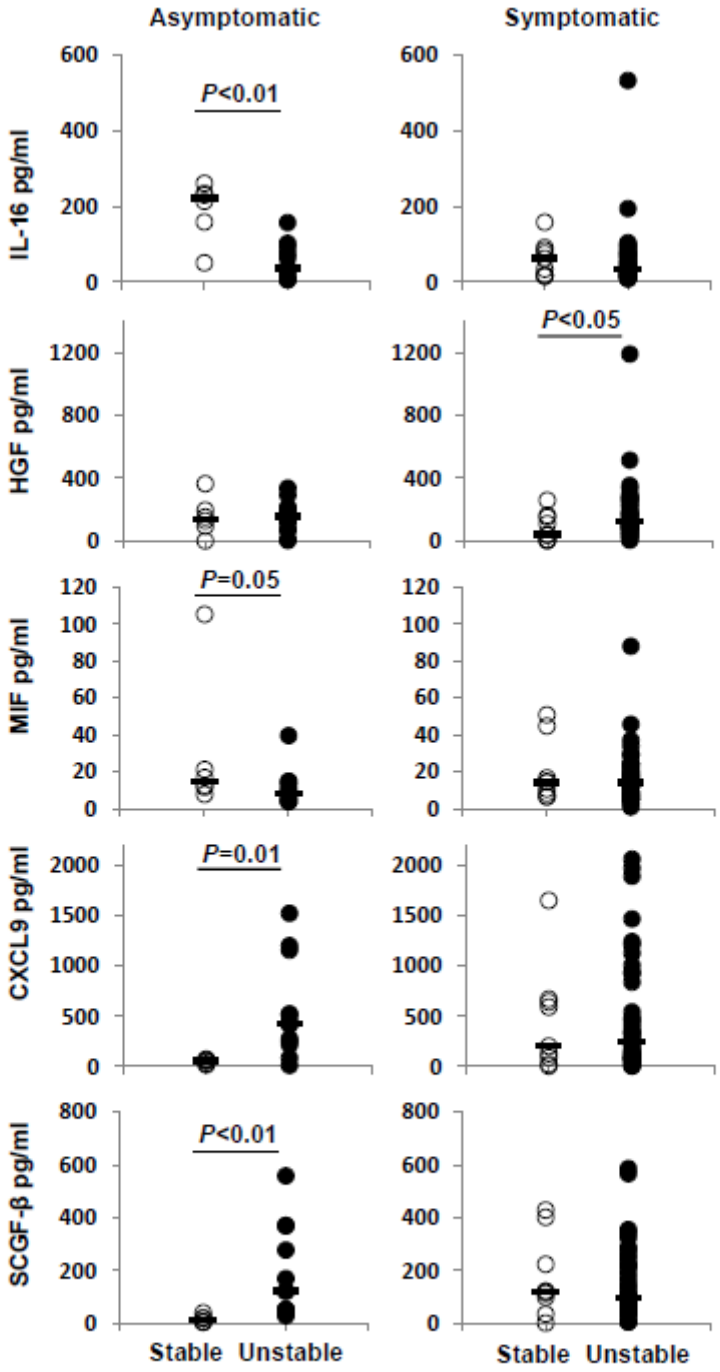

Figure 3. Bioplex analysis of patient serum. CXCL9 and SCGF-β are elevated in asymptomatic patients with unstable plaques, whereas IL-16 and MIF were reduced. HGF was increased in symptomatic patients with unstable plaque. Data were analysed using Mann-Whitney U statistical test. Bars represent the median.

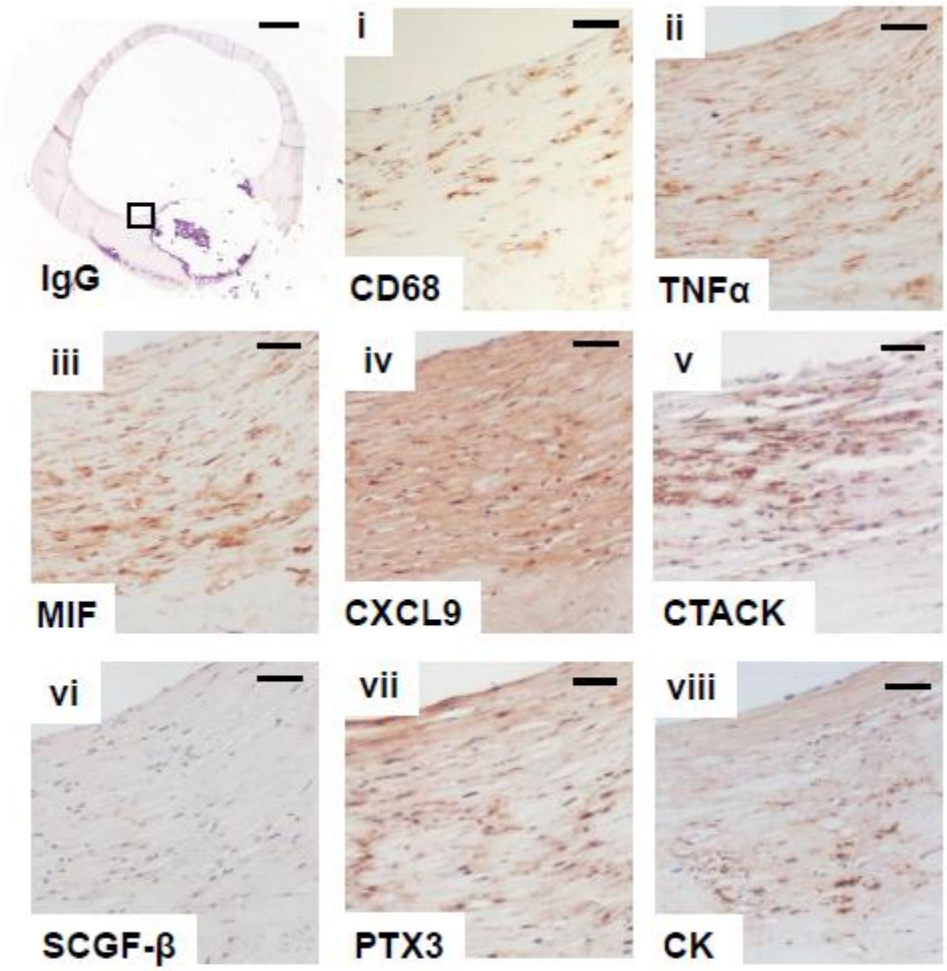

Figure 4. Inflammatory markers are expressed within carotid endarterectomy plaques. Square box in the negative IgG control is enlarged in the serial sections presented in micrographs i-viii. Consecutive sections from a representative patient sample stained positive for inflammatory markers (i) CD68 and (ii) TNFα-tumour necrosis factor-alpha. The expression of a panel of cytokines, (iii) MIF-macrophage migration inhibitory factor, (iv) CXCL9, (v) CTACK-cutaneous T-cell-attracting chemokine, (vi) SCGF-β-stem cell growth factor beta, (vii) PTX3-pentraxin-related protein and (viii) cathepsin K was also detected within the plaque. IgG bar=1000μm; i-viii bars =50μm.

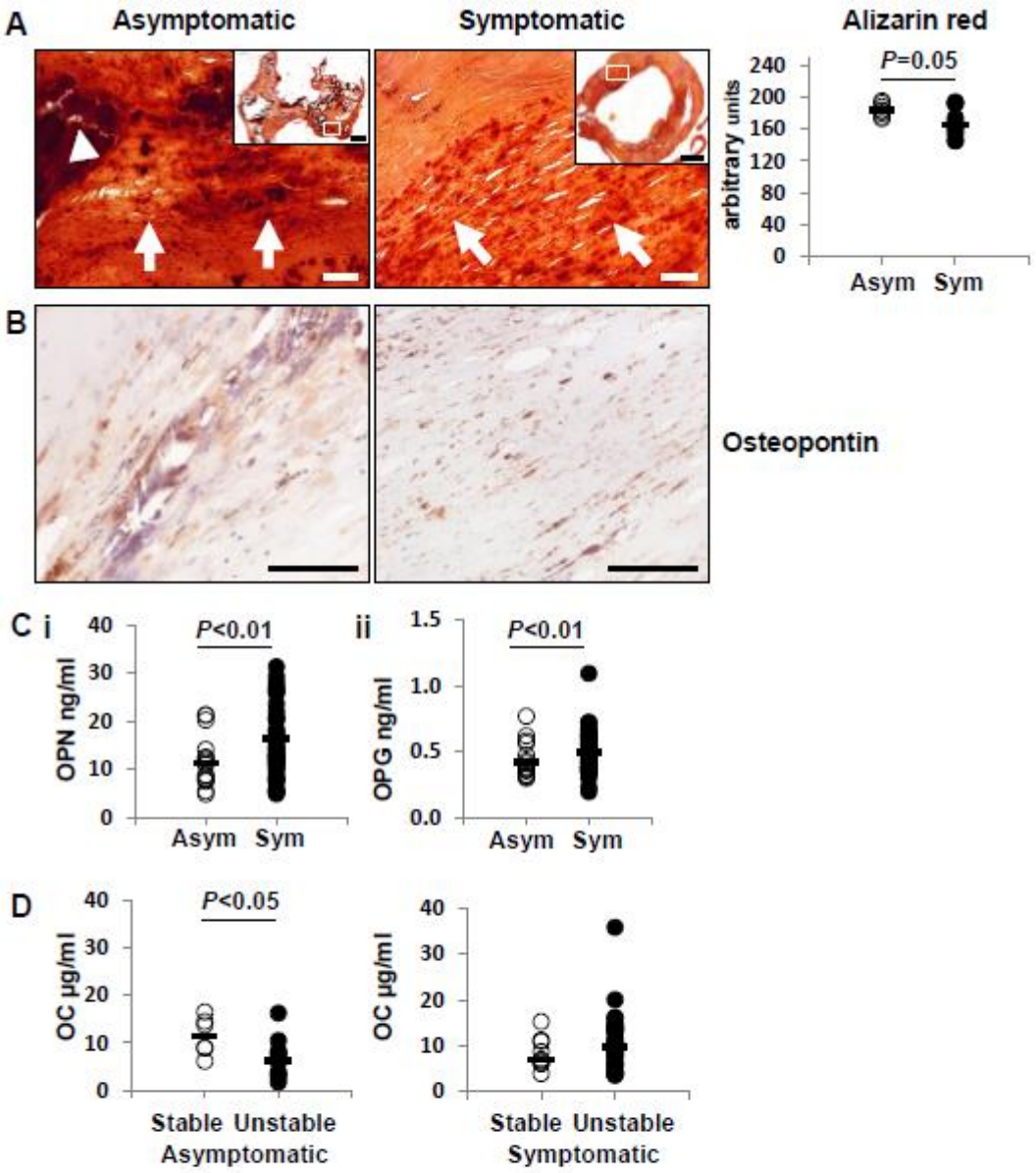

Figure 5. Plaques from asymptomatic patients were more calcified than from symptomatic patients. (A) Tissue sections were stained with Alizarin red to highlight areas of calcification, which appeared as spicules (arrows) or as large blocks (arrow head). Plaques from asymptomatic patients (Asym; n=8) exhibited significantly more Alizarin red staining compared to plaques from symptomatic patients (Sym; n=11). (B) Osteopontin (OPN) was detected in similar areas of consecutive sections. (C) Bone-related markers were differentially regulated in patient serum. Data were analysed using Mann-Whitney U statistical test. Bars represent the median. Inset image; bar=1000µm. Main image; bar=100µm. Asym-asymptomatic; Sym-symptomatic.

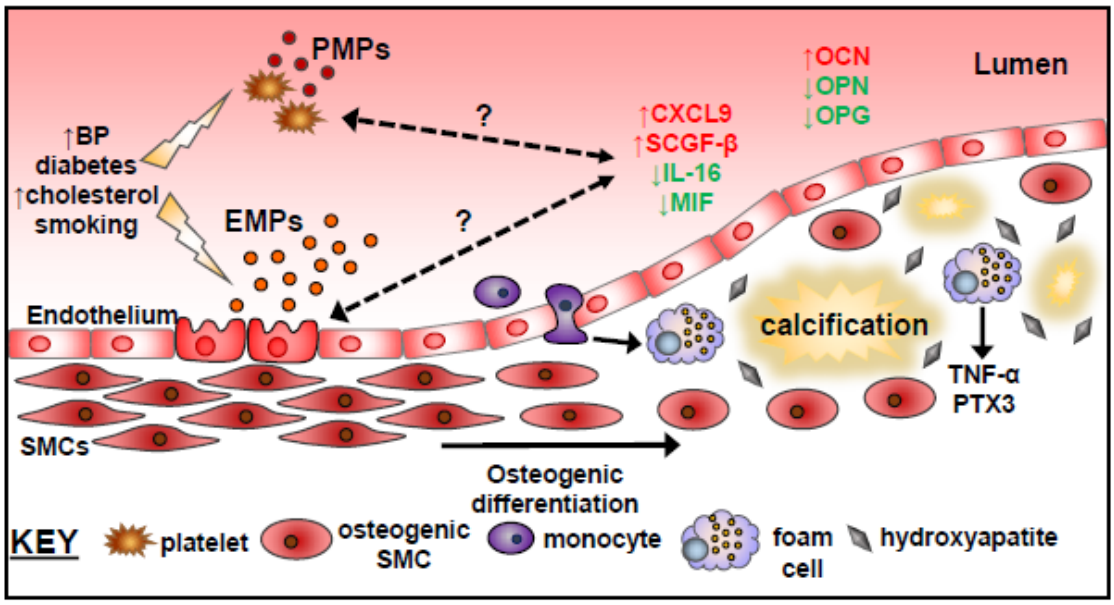

Figure 6. A schematic representation of the key factors involved in plaque instability in asymptomatic patients. Endothelial cells and platelets are activated by various cardiovascular disease risk factors and release microparticles into the circulation. During plaque development, smooth muscle cells (SMCs) can differentiate from a contractile to a secretory phenotype, depositing a calcified matrix. Both processes may be exacerbated by the cytokines modulated during disease pathology. OC-osteocalcin; OPN-osteopontin; OPG-osteoprotegerin; TNF-tumour necrosis factor-alpha; PTX3-pentraxin-related protein; SCGF-β-stem cell growth factor; SMC-smooth muscle cell; EMP-endothelial microparticle; PMP-platelet microparticle.

ELEVATED EMPs IN ASYMPTOMATIC PATIENTS: MARKERS OF UNSTABLE PLAQUES?

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Introduction

Endothelial microparticles (EMPs) are released from dysfunctional endothelial cells. We hypothesised that patients with unstable carotid plaque have higher levels of circulating microparticles compared to patients with stable plaques which could be related to a specific cytokine profile.

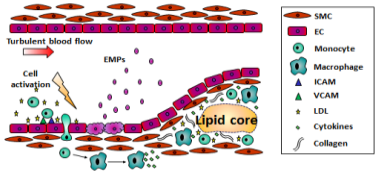


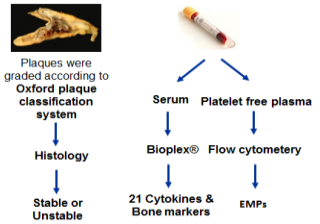
Figure 1. Stages of endothelial cell (EC) activation leading to EMP release. Endothelial cells (ECs) in areas of turbulent blood flow become permeable to oxidised LDL. In response to activation, increased adhesion marker expression leads to monocyte recruitment, migration and differentiation into macrophages. Inflammatory stimuli induce EC activation and apoptosis, resulting in EMP

Aims

- To establish levels of EMPs in patients with carotid disease and whether they could act as a biomarker of plaque instability.
- To determine whether EMP levels reflect plaque morphology and stroke risk.
- Correlate inflammatory cytokine profile with plaque morphology.

Methods

51 symptomatic and 19 asymptomatic patients with a stenosis of >60% and ≥ 70% respectively were listed for a carotid endarterectomy. Fasting pre-op bloods were centrifuged- serum & platelet free plasma was stored at -80 ^{0}C.



Results; 1. EMPs are elevated in asymptomatic patients with unstable plaques

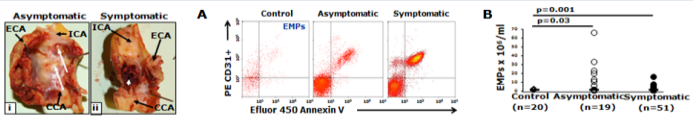


Figure 2. EMPs are significantly elevated in patient plasma compared to healthy controls. Plaques from **i)** asymptomatic and **ii)** symptomatic patients highlighting a plaque ulcer (white arrows) and an intra-plaque haemorrhage (white arrow head). (A) Representative flow plots from each group. (B) EMPS were defined as CD31+/AnnexinV+/CD42-.

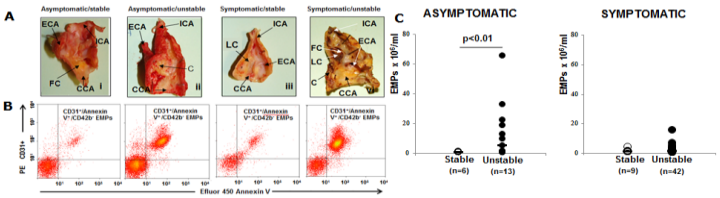


Figure 3. EMPs are significantly elevated in asymptomatic patients with unstable plaques.

(**A**) Representative images of the gross morphology of stable and unstable plaques harvested from asymptomatic and symptomatic patients. (**B**) Representative flow cytometry plots of each group. (**C**) EMPs in patient subgroups. **Abbreviations**: CCA- common carotid artery, ECA- external carotid artery, ICA - internal carotid artery C- calcium, LC-lipid core, FC- fibrous cap

2. Serum cytokine levels

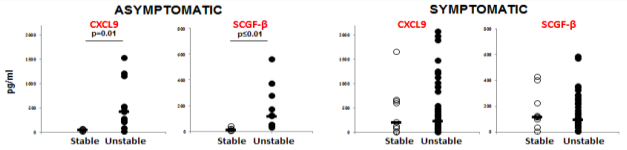


Figure 4. CXCL9 and SCGF-β are significantly elevated in asymptomatic patients with unstable plaques. Bars represent medians.

3. MIG and SCGF are detected within plaques

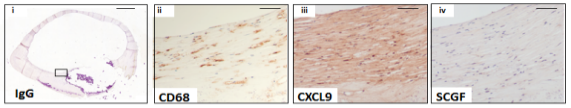


Figure 5. CD68+ve macrophage infiltration was detected within most plaques, with CXCL9 and SCGF-β localised in the same vicinity. Plaques macrophages may contribute to the elevation of both localised and systemic inflammation. i) Bar=1000μm, ii-iv) Bar=50μm.

4. Asymptomatic plaques are significantly more calcified

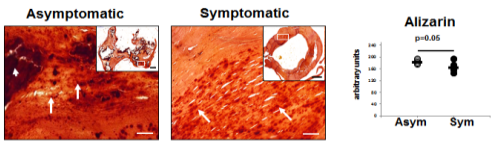


Figure 6. Alizarin red highlights the areas of calcification. Plaques from asymptomatic (Asym) exhibited significantly more alizarin red staining compared to plaques from symptomatic patients (Sym). Data were analysed using Mann Whitney U statistical test. Bars represent the median. Inset image; bar=1000μm. Main image; bar=100μm.

Factors involved in plaque stability in asymptomatic patients

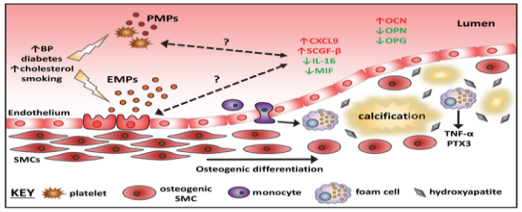


Figure 7. Key factors involved in plaque instability in asymptomatic patients.

Conclusion-EMPs as a biomarker of plaque stability

In summary, EMPs are significantly up-regulated in carotid artery disease compared to healthy controls. EMP levels, CXCL9 and SCGF were significantly increased in asymptomatic patients with unstable plaques. Large areas of inflammation and calcification were detected within the plaques of both asymptomatic and symptomatic patients.

These data could have implications for the development of a diagnostic tool whereby EMPs and markers of macrophage activity are combined to act as biomarkers of plaque vulnerability and stroke susceptibility.

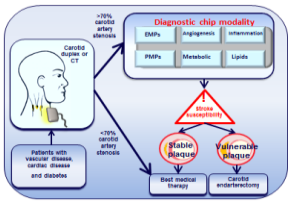


Figure 8. Proposed working model of how microparticles and biomarkers could be used to identify asymptomatic patients at risk of stroke.