Comparative analysis of atherosclerotic disease in the carotid and femoral arteries

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List of abbreviations

ABCA1	Cholesterol efflux regulatory supporter
ABPI	Ankle brachial pressure index
AHA	American Heart Association
AMITIE	Acute Myocardial Infarction Trends in Europe
apoB	Apolipoprotein B
Ca	Cancer
CAD	Coronary arterial disease
CAPRIE	Clopidogrel versus Aspirin in Patients at Risk of Ischemia Events
CEA	Carotid endarterectomy
Cf.	Compared to
CFEA	Common femoral endarterectomy
COPD	Chronic obstructive pulmonary disease
CRF	Clinical record form
CRP	C reactive protein
CT	Computerised tomography
DM	Diabetes mellitus
ECG	Echocardiogram
EtOH	Alcohol
HDL	High density lipoprotein
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
Hs-CRP	High sensitivity C-reactive protein
HTN	Hypertension

HU	Hounsfield Units
IMT	Intima-media thickness
IPH	Intraplaque haemorrhage
IRAS	Integrated research application system
LDL	Low density lipoprotein
LRNC	Lipid rich necrotic core
LVF	Left ventricular failure
MAP	Mean arterial pressure
MHz	Megahertz
MI	Myocardial infarction
mm	Millimetres
mMRI	Micro MRI
MRI	Magnetic resonance imaging
ms	Milliseconds
OCT	Optimal cutting temperature compound
OxPVD	Oxford Peripheral Vascular Disease Study
PACK trial	Prevention of Atherosclerotic Complications with Ketanserin
PAOD	Peripheral arterial occlusive disease
PBS	Phosphate buffered saline
PC5K9	Target for cholesterol lowering medications
PFA	Paraformaldehyde
PTFE	Polytetrafluoroethylene
REC	Research Ethics Committee
RIP	Rest in peace

SES	Socioeconomic status
STEMI	ST elevation myocardial infarction
TASC	Trans-Atlantic Inter-Society Consensus
TIA	Transient ischaemic attack
TOF	Time-of-flight
USS	Ultrasound scan
VEGF	Vascular endothelial growth factor

Abstract

Comparative analysis of atherosclerotic disease in the carotid and femoral arteries.

Introduction

Peripheral arterial occlusive disease (PAOD) is a common vascular pathology affecting up to 60% of the population over 85 years. It often presents in a triad of atherosclerotic disease, alongside myocardial ischaemia and cerebrovascular accident. Best medical therapy is used in the treatment for all three; and shows a 16% reduction in stroke events and 10-40% reduction in cardiac events. POAD has not been subject to the same level of research, and primary analysis indicates little reduction in deterioration or amputation rates for 30 years.

Methods

The study aimed to use in vivo and ex vivo analysis of carotid and femoral atherosclerotic plaque from patients enrolled in the OxPVD study. Each participant underwent pre-operative carotid and femoral (ipsilateral) MRI scans, and ex vivo plaque was analysed using micro CT, micro MRI and histological staining. Comparative analysis was completed for carotid versus femoral atherosclerosis.

Results

Results from this study have shown that despite risk factor modification, overall outcomes of patients with POAD remain unchanged. Patients do however present to the services 7 years older. Secondly, novel MRI DANTE sequences can successfully characterise femoral atherosclerotic plaque in vivo.

Using micro CT and micro MRI, we have further quantified differences in the composition of carotid and femoral disease. Smokers tend to have a higher percentage of calcium within the plaque. Femoral plaque calcium is significantly more dense than all carotid plaque calcium. Finally, histological staining has implied that the disease processes behind femoral and carotid atherosclerosis may be different; with carotid showing fat infiltration and femoral plaque containing osteoblastic/osteoclastic like cells.

Conclusion

These results highlight key compositional differences between carotid and femoral atherosclerosis; femoral atherosclerotic calcium appearing much more dense, and possibly derived from bone-like calcium progenitors. With this in mind, it may be possible to review different therapeutic targets for PAOD.

Research impact and dissemination

Publications

- Frost, R et al. Navigator-based reacquisition and estimation of motion-corrupted data: Application to multi-echo spin echo for carotid wall MRI. Magn Reson Med. 2020; 83: 2026– 2041.
- De Buck M et al. Novel 10-Channel Phased-Array Coil Design for Carotid Wall MRI at 3T. PLoS One. 2023 Aug 9;18(8)

Future Publications

- K Hurst, Choudhury R, Tzima E, Handa A. Comparative analysis of femoral and carotid atherosclerotic plaque.

Prizes

- Sol Cohen Prize Session. Vascular Society of Great Britain and Ireland
- Rising Stars. Charing Cross Vascular Conference
- Oxford-Wolfson Marriott-Patel Graduate Scholarship in Surgical Sciences
- Royal College of Surgeons Edinburgh Research Priming Grant

Presentations

- K Hurst, Choudhury R, Tzima E, Handa A. Comparative analysis of femoral and carotid atherosclerotic plaque. NDS Student Symposium, Oxford
- Hurst K, Choudhury R, Handa A. Comparative analysis of femoral and carotid atherosclerotic plaque. South West Vascular Surgeons AGM (cancelled due to covid)
- Hurst K, Choudhury R, Handa A. Comparative analysis of femoral and carotid atherosclerotic plaque. NDS Away Day, Oxford
- K Hurst, Lee R, Handa A. Fate of the Claudicant. Vascular Society ASM
- K Hurst, Lee R, Handa A. Fate of the Claudicant. Charing Cross Symposium
- K Hurst, Lee R, Handa A. Fate of the Claudicant. NDS Away Day, Oxford

Chapter 1: General Introduction

1.1 The burden of atherosclerotic disease in the 21st century

1.1.1 Epidemiology of atherosclerotic disease

Atherosclerosis is a chronic, and often progressive inflammatory disease of medium or large arteries, specifically due to an atheromatous deposition. It derives from a Greek origin meaning 'hardening or loss of elasticity of an artery'.

An artery consists of three distinct layers which lie circumferentially around a lumen (1);

- The tunica intima, as the innermost surface. This is a single layer of endothelial cells, forming an endothelial barrier, on top of an extracellular matrix and internal elastic lamina
- The tunica media, containing smooth muscle cells and the external elastic laminae
- The tunica externa (adventitia), built from connective tissue, and also the home of resident immune cells, fibroblasts and stem cell progenitors

Atherosclerosis was initially thought to be a disease of ageing until the mid 19th century, when two key hypotheses were first in debate. 'Incrustation' by von Rokitansky; suggesting that atherosclerosis was due intimal thickening from fibrin deposition (2), and a 'lipid hypothesis' by Virchow, suggesting a fat accumulation (3). However, atherogenic research in rabbit models, at the beginning of this century (Anitschkow, 1913), proved there to be a direct link between atheroma and dietary and blood cholesterol (4). The same correlation was also

observed in humans in the late 1980s within the results of the Framingham Study, and confirmed again following intervention of lipid lowering interventions in the 1990s (5). Since this time, research and understanding of the processes behind atherosclerotic deposition and progression have grown exponentially. It is now believed that atherosclerosis initiates from sub-endothelial retention of cholesterol-rich lipoproteins, and once retained, a cascade of internal responses; including lipoprotein modification, expression of cell adhesion molecules and recruitment of inflammatory cells ensues; leading to arterial damage in a previously healthy vessel. This is known as the 'response to retention' hypothesis (6, 7).

Further studies have shown that the retained lipoproteins must also contain apolipoprotein B (apoB) to be able to drive the development of atherosclerosis. ApoB on a (low density) lipoprotein (or LDL) acts as a ligand for LDL receptors within the body. Consequently, murine models overexpressing apoB have high levels of atherosclerosis (8).

The retention of apo-B lipoproteins causes an accumulation of cholesterol laden macrophages called 'foam cells' within the intima. Over time, these atherogenic areas can develop into fibrous legions with regions of lipid necrosis, connective tissue and smooth muscle cells (7).

Over decades the plaques can grow large enough to stenose or obstruct the vessel lumen; leading to peripheral vascular disease or angina, or as shown by Ambrose *et al*. (9) and Fuster *et al.* (10), vulnerable plaque can rupture; causing unstable angina, myocardial infarction or stroke. The production of connective tissue and deposition of calcium as part of the inflammatory response, are the main causes for hardening of the arteries and loss of their

elastic recoil. Figure 1 shows the initiation and progression of atherosclerosis, depicted for publication.

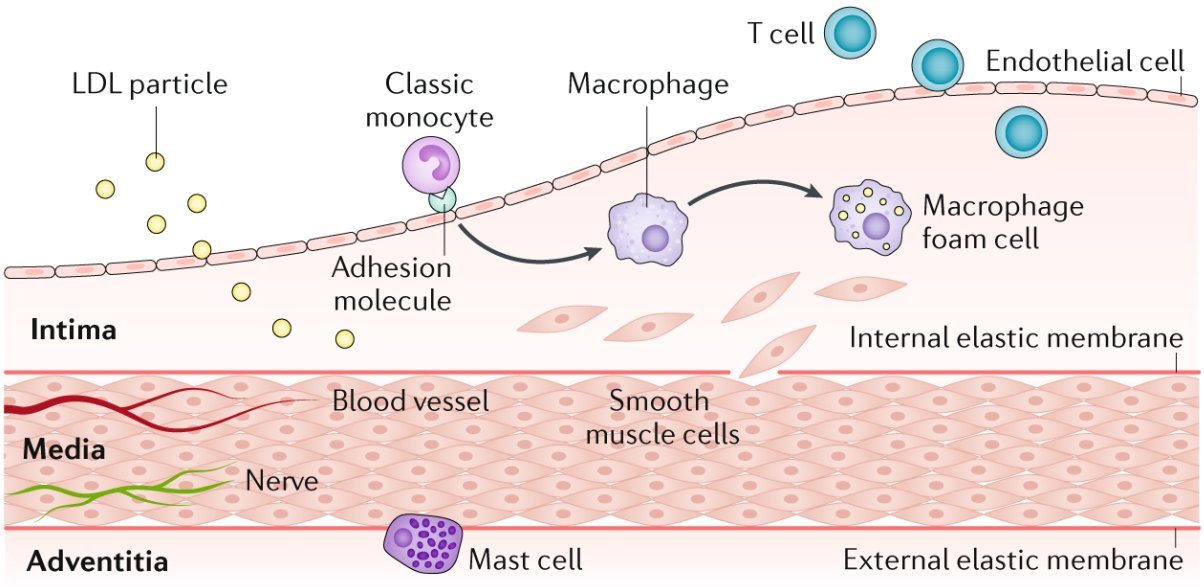


Figure 1: initiation and progression of atherosclerosis. Libby, P., Buring, J.E., Badimon, L. *et al.* Atherosclerosis. *Nat Rev Dis Primers* **5,** 56 (2019). https://doi.org/10.1038/s41572-019-0106-z (11)

Atherosclerosis remains the leading cause of vascular disease worldwide (12), and in the western world approximates to half of all deaths (7). This is despite a significant decrease in overall mortality from ischaemic heart disease and stroke since the 1950s; from 700 per 100,000 per year in the UK in 1950, to <200 in 2010 (for men), and from 450 per 100,000 in 1950 to <100 in women.

Consistent with a reduced mortality, over the last two decades the incidence and re occurrence rates of acute MI and stroke have reduced. A nearing 70% reduction in 25 years has been described in the Acute Myocardial Infarction Trends in Europe (AMITIE) study and equivocal results have been shown in the UK alone. An 18% reduction in ischaemic stroke since 1990 has also been documented (13, 14).

These are fairly staggering results, due to a combination of efforts; increasing community surveillance, advancing medical care, the introduction of primary prevention and greater patient awareness (12). This aside, atherosclerosis still remains one of the largest global heath burdens, and such results are still to be mirrored in less wealthy countries. Consequently, atherosclerosis remains an eminent topic for research.

1.1.2 Epidemiology of peripheral arterial occlusive disease

Peripheral arterial occlusive disease (PAOD) is a common vascular pathology, presenting with a range of symptomatology; asymptomatic through to critical limb ischemia. PAOD has been shown to affect up to 10% of the independent population, this figure doubling in those greater than 70 years, and in the Rotterdam study was seen in nearly 60% of those >85 years (15-17). With significant numbers of patients requiring follow up, intervention and/or amputation, PAOD has a huge associated morbidity.

The natural history of the disease shows that in patients who are asymptomatic or who experience typical claudication; approximately 70-80% remain stable over 5 years, 10-20% may

experience worsening symptoms and 5-10% will go onto show critical limb ischaemia from occlusion of the artery. Of this cohort approximately 20% will have a non-fatal cardiovascular event, and their overall mortality is 10-15% (18, 19). In comparison, those who present with critical limb ischaemia portray one year outcomes of 30% amputation and 25% mortality (18).

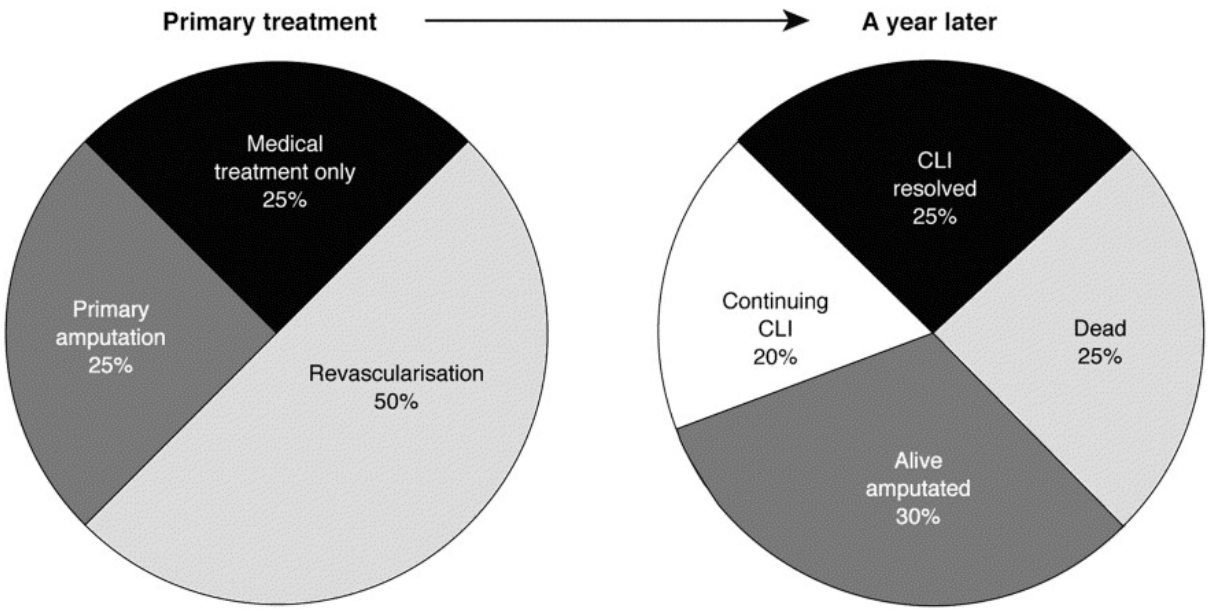


Figure 2: Fate of the patients presenting with chronic critical leg ischemia, from the Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II) (18).

In light of the above, peripheral vascular disease causes a significant burden to the National health system, headed predominantly by the functional disability of patients experiencing symptomatic disease (20).

There are also a subset of patients who present to the vascular services with occlusive vascular disease from an *embolic* cause, rather than atherosclerosis; known as acute limb ischaemia. This presents very differently to chronic limb ischaemia; with a sudden onset cold, pale, painful

and pulseless limb. These patients are also susceptible to transient ischaemic attacks or cerebral/coronary infarcts from such an embolic source.

1.1.3 Cost of atherosclerosis to the National Health Service

It is difficult to estimate the overall cost of atherosclerosis due to the vast number of presentations, varying stages of disease, and numerous interventions required. However, it is evident that not only does the associated morbidity of atherosclerosis consume significant amounts of resource, the consequent pathologies absorb an extremely large health budget. Healthcare costs in the UK for cardiovascular disease alone are in excess of £9 billion per year, with an extra £4 billion on aspects not directly associated with healthcare (21). Studies in the United States have shown cost related to atherosclerotic disease to be in excess of $1000 per person per month following diagnosis (22), and projected costs for 2015 to 2030 are expected to be up to $202 billion (17).

1.2 Risk factors for atherosclerosis

1.2.1 Common risk factors for atherosclerosis and the 'triad' of atherosclerotic disease

There are multiple genetic and environmental factors linked to atherosclerotic disease and its progression. These include; high cholesterol levels - in particular low density lipoprotein cholesterol (LDL-c), hypertension, tobacco smoking, diabetes mellitus, obesity, inactive

lifestyle and age (23). As well as lifestyle modifiable factors, there are also known genetic dispositions. Many genetic mutations in; LDL receptors, the apoB gene, PC5K9 leading to increased LDL-c levels, ABCA1 causing severe HDL deficiency, have all been discovered in recent years, but with such complex environmental and genetic interactions within the process of atherosclerosis it has not been possible to achieve absolute prevention or cure (24).

As described, atherosclerosis is characterised by endothelial dysfunction and inflammation (25). This initial intimal insult can initiate longer term plaque formation with the build-up of cholesterol, lipids, calcium and cellular debris within the intima. The American Heart Association (AHA) have histologically classified atherosclerotic legions into various legion 'types' along this time course.

Legion Type	Description
I	Initial legion with foam cells (fatty streak)
II	Fatty streak with multiple layers of foam cells
III	Pre atheroma with extracellular lipid pools
IV	Atheroma with confluent extracellular lipid core
V	Fibro atheroma
VI	Complex plaque with possible surface defect or haemorrhage or thrombus
VII	Calcified plaque
VIII	Fibrotic plaque without lipid core

Atherosclerosis is often a multisite disease, with the most commonly effected vascular arteries being the coronary arteries, cerebral arteries and peripheral arteries. Patients may present with disease in a single area, but for those who have multiple site involvement, the risk of adverse events is considerably higher (26).

Several studies have had either a primary aim to assess, or have reviewed as a secondary outcome, the prevalence of atherosclerotic disease in different end organs. Results confirm the concept of atherosclerotic deposition in varying territories, with figures of around 23% in two areas, and 3-8% in three areas (18, 27).

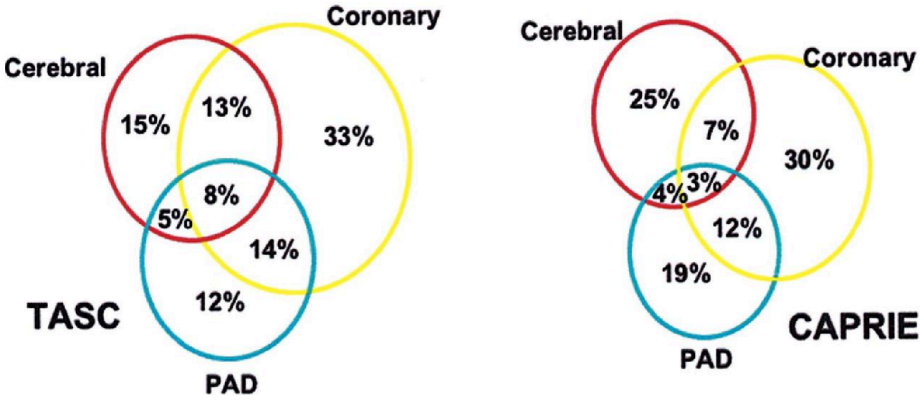


Figure 3: Clinical overlap between peripheral arterial disease (PAD), coronary artery disease (coronary), and cerebrovascular disease (cerebral) in the Trans-Atlantic Inter-Society Consensus (TASC) working group with approaching 2,000 patients (left) and in the Clopidogrel versus Aspirin in Patients at Risk of Ischemia Events (CAPRIE) trial with approximately 20,000 patients (right) (28).

Figure 3 shows the various presentations of symptomatic atherosclerotic disease within different studies; the TASC working group; reviewing outcomes of patients with peripheral vascular disease, and the CAPRIE trial; observing the clinical reduction in atherosclerotic events following clopidogrel or aspirin administration. Both of these trials were multicentre, international, large cohort projects, selecting patients with atherosclerotic disease in at least one arterial region. They however, only used patient history (confirmed by ECG, CT head, ABPI, or previous intervention/surgery) as inclusion criteria. Participants were not imaged to confirm the presence or absence of atherosclerotic plaque in other arterial beds.

That said, this still raises the question; why should atherosclerosis only present in a single area in 60-70% of patients? The pathogenesis would suggest it should display a more global distribution of symptoms.

Upon analysis of the smaller 'overlap' areas, there appears to be a greater correlation between POAD and coronary disease, in comparison to PAOD and cerebrovascular disease. Lekakis *et al*. also proved this using ultrasound and coronary angiography; showing that the presence of peripheral vascular disease was an independent criterion for the severity of coronary atherosclerosis (using a Gensini score), but the same was not observed with coronary disease (29). From this research he stated '*there are territorial differences in various arterial beds regarding their response to risk factors*'.

With such behaviours in atherosclerotic deposition being observed throughout the research field, many theories have been openly discussed;

Deposition differences may be a result of the arterial wall composition in different locations; with carotid arteries being elastic, to allow for high cardiac pressure near to the heart, and femoral arteries being more muscular to carry blood over longer distances (30).

Alternatively, it may be due to the way these different vessels behave in response to risk factors, for example;

- elastic common carotid arteries showing significant arterial stiffness in response to hypertension, which is not observed in muscular femoral arteries (31). Studies have also shown hypertension to be a major risk factor for carotid atherosclerosis and less so for femoral and coronary (32).
- carotid arteries showing a significant response to lipid lowering medications; by reducing IMT thickness, which is not observed in the common femoral arteries (33, 34).

Carotid and femoral arteries also arise from different embryological structures; the carotid developing from the third aortic arch and the proximal aortic ventral sac, and the femoral from the umbilical and axial artery. These differences, along with others (tabulated below for comparison), may be extremely relevant to the way and/or extent in which atherosclerotic disease is distributed.

Table 1: Comparison of the known structural differences between the carotid and femoral arteries

Carotid	Femoral
Elastic arterial wall; increased number of collagen and elastic filaments creating a thick tunica media	Muscular arterial wall; smooth muscle within tunica media (thin media)
Elastic arteries effected by hypertension	Muscular arteries more adaptive to hypertension
Hypertension a significant risk factor	Smoking, diabetes both significant risk factors
Atherosclerotic plaque responds to lipid lowering medications	No published response to lipid lowering medications
High basal nitric oxide production	Low basal nitric oxide production
Develops from third aortic arch	Develops from umbilical artery
High mean shear wall stress and low oscillatory shear index	Low mean shear wall stress and high oscillatory shear index
Foam cell predominant legions on autopsy (30)	Fibrous plaques on autopsy (35)
Predilects to areas of bifurcation	Predilects to areas of bifurcation

Other theories include the differences in sheer wall stresses within varying arteries (36, 37), variations in mural tensile stress (38), or flow dynamics/ changes in the vessel diameter (39). It is however already well described that atherosclerotic deposition predilects to areas of bifurcation (40).

Overall, atherosclerotic plaque has been extensively studied; in relation to disease states, histology, genetics, metabolomics, as can be seen in appendix table 1. These studies however

frequently examine only one arterial bed at a time (i.e coronary arteries or carotid arteries), and then invariably depict outcomes of atherosclerotic disease as a whole.

1.2.2 The effects of hypertension on carotid and femoral atherosclerosis

Many studies have analysed the carotid arteries over the last thirty years, with cerebrovascular and coronary disease being attractive and well-funded topics of research. Although some studies have included femoral analysis, the disease process in the peripheral arteries is much less understood (41).

Hypertension has been shown in many instances to be an independent determinant for carotid atherosclerosis (42, 43). Firstly, by observing an increase in carotid wall thickness (specifically intima-media thickness) in those patients with hypertension (44), as well as reducing the risk of hypertensive related strokes with the introduction of blood pressure reducing treatments (45). That said, intima-media thickness in recent years, has divided opinion as to whether it truly represents atherosclerosis. Studies have shown correlation between IMT and early systemic atherosclerosis; including a recent report observing 21,000 participants where increased IMT was a predictor of sudden cardiac death (46), as well as carotid IMT being linked to future risk of stroke (47).

Similar effects have simply not been observed within the femoral arteries; which may be due to differences in the patterns of atherosclerotic disease, or that peripheral vasculature has not

received the same degree of research interest. Papers have however, whilst attempting to study femoral IMT, confirmed the independent link to the presence of coronary disease (48).

1.2.3 The effects of statins on atherosclerosis

HMG-CoA reductase inhibitors, also known as statins, have widely been used over the last twenty years for primary prevention and secondary prevention of cardiovascular disease. In agreement with the 'lipid hypothesis' - suggesting that cholesterol plays an important role in atherogenesis, statins have been proven to lower levels of blood cholesterol (LDLs) and in turn reduce cardiovascular events (49).

Statins have also been shown throughout the literature to reduce both coronary (34) and carotid plaque volume (50). Authors have observed remodelling of the carotid plaque after statin therapy; with increasing calcium content and reducing areas of lipid core. These changes appear to stabilise the plaque from intraplaque haemorrhage and rupture, and consequently reduce stroke rates (51).

Again, femoral atherosclerosis has not been shown to exhibit the same effects to statin therapy; POAD outcomes unfortunately do not appear to have improved following statin engagement. Studies have identified a possible advantage to lipid lowering medications in early/low grade legions (TASC A/B); where primary patency rates following femoral stenting improved with statin use, but this was not observed for TASC C/D legions (or as a cohort overall) (51). Other than this, within PAOD research, the main advantage for statins appear to be for

coronary plaque stability and reduction in myocardial ischaemic events in PAOD patients with concurrent myocardial disease (52).

This does raise the question as to why this would be the case if atherosclerosis was truly uniform in different territories? Why should a reduction in blood cholesterol prevent and reduce myocardial events but have little to no impact on peripheral atherogenesis?

1.2.4 Arterial remodelling in the context of atherosclerosis

Atherosclerosis is associated with numerous structural changes from the start with endothelial dysfunction, retention and macrophage infiltration, through to smooth muscle cell migration. The remodelling process has been studied in coronary, carotid and femoral arteries alike and has unearthed interesting data on the processes in each area.

Coronary and carotid artery atheromatous plaques appear to be eccentric within the artery wall and grow towards the adventitia before narrowing the artery lumen, also known as ‘positive remodelling’ (53).

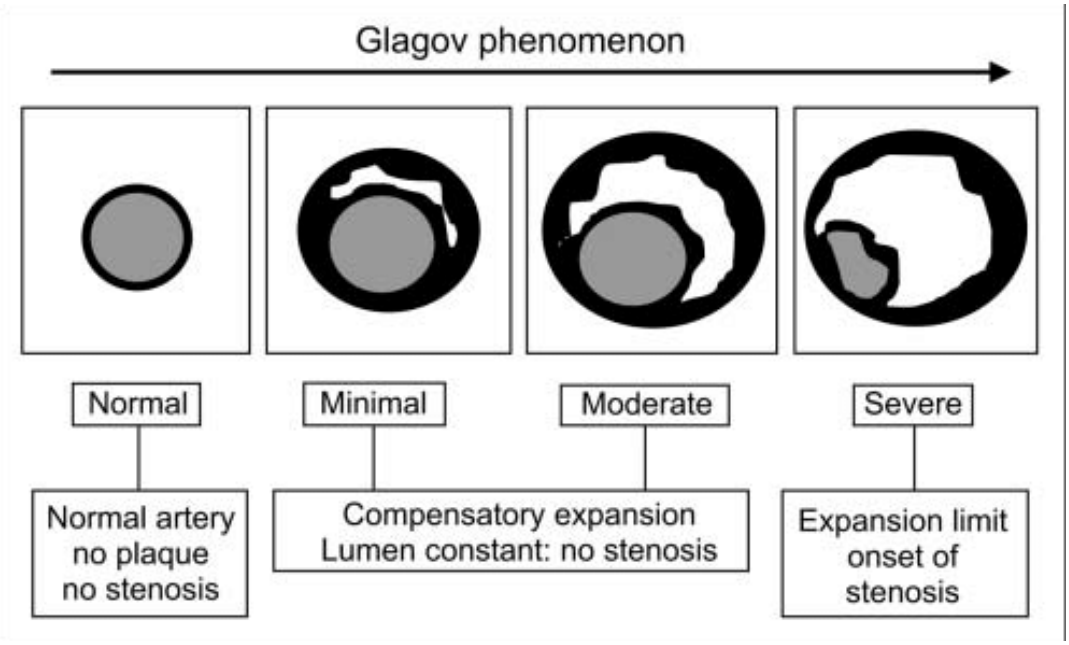


Figure 4: Diagrammatic representation of the progression of atherosclerosis in coronary arteries by 'positive' remodelling. Adapted from Glagov at al. Compensatory enlargement of atherosclerotic coronary arteries (1987) (52, 54).

Alongside eccentric plaque formation and positive remodelling of the artery, it is now widely recognised that the presence of 'intraplaque haemorrhage' and a 'lipid rich necrotic core' both play a key role in the vulnerability and progression of carotid atherosclerotic plaques (55). Femoral legions however, appear to have significantly less lipid rich necrotic core (LRNC) and intraplaque haemorrhage (IPH) in a study by Polonsky *et al.*, with only 0.3% of patients showing radiological evidence of femoral atherosclerotic IPH (56).

In further comparison, peripheral arteries (femoral) have been observed to predominantly adopt a 'negative remodelling' process by which they create shrinking of the vessel wall at the site of plaque burden (57).

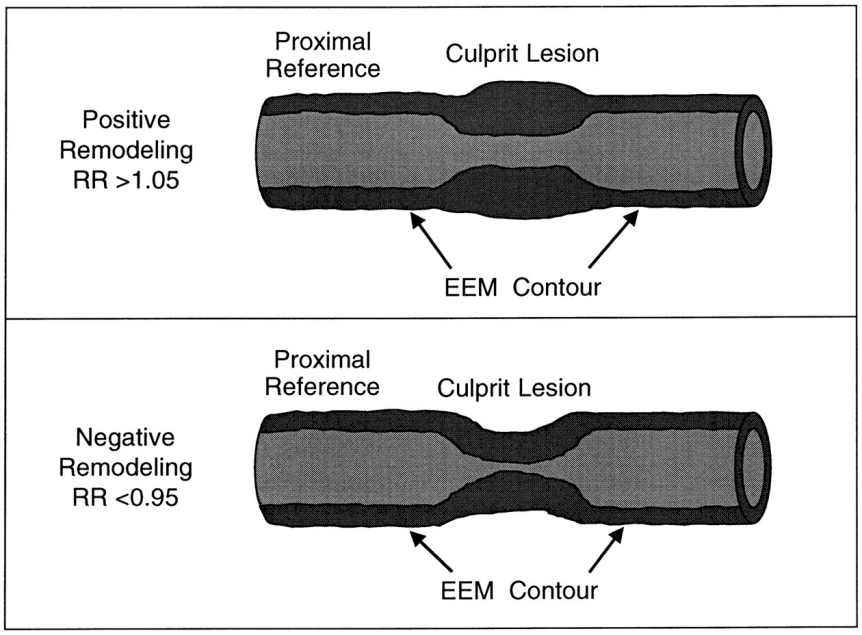


Figure 5: Depiction of negative arterial remodelling. Schoenhagen at al. (2000) Extent and Direction of Arterial Modelling in Stable versus Unstable Coronary Syndromes (58).

Interestingly, a paper by Schoenhagen *et al*. (2000), showed that in the context of coronary disease; positive remodelling was associated with unstable clinical presentations, and negative remodelling was seen in greater proportions in stable patients (58).

These important phenomena indicate that atherosclerotic plaque in various arterial beds may differ; and poses the question that plaque instability and rupture within the peripheral arterial is rare, possibly due to its ability to negatively remodel and adopt a more stable nature?

1.2.5 Risk factors for peripheral arterial occlusive disease

There are multiple risk factors relating to peripheral vascular disease; firstly in keeping with atherosclerosis - age, smoking, diabetes mellitus, dyslipidaemia, and more specific to PAOD; hypercoagulable states, hyperhomocysteinaemia and chronic renal insufficiency. Raised CRP levels also seems to predict a future risk of developing peripheral vascular disease, supporting the prediction that chronic inflammation leads to atherothrombosis (59, 60).

Of the above, factors which appear to be most significant are diabetes and smoking, followed by age and elevated CRP levels in those with asymptomatic disease (61, 62), and dyslipidaemia in patients progressing to critical limb ischaemia (63).

Several factors linked to disease progression have also been described (64), the majority following a common stem of raised inflammatory markers; high baseline values of CRP and interleukin-6 have been shown in patients with a trajectory of rapidly worsening disease (65).

Inflammatory markers have also been linked to early mortality. Examples include; increased levels of Hs-CRP correlating to short term mortality in patients with PVD (66), and higher death rates in those patients with elevated d-dimer values (67). The difficulty however lies in the overlap between peripheral vascular disease and “central” coronary arterial disease (CAD). The pathophysiological processes of atherosclerosis have common factors in both pathologies. Many studies have identified this problem and been unable to delineate biomarkers for progression of peripheral vascular disease alone (68, 69).

Apart from markers of inflammation, there are other putative markers of PVD progression. Examples of these include: markers of endothelial dysfunction (soluble cell adhesion molecules) (70, 71) von Willebrand's factor (72, 73) , flow mediated dilatation (FMD of the brachial artery), markers of oxidative stress (homocysteine, hydoxy-2-deoxy-2deoxyguanosine) (74). Angiogenesis modulators (VEGF, Hepatocyte growth factor) (75, 76), lipoproteins (oxidative LDLs, lipoprotein a) have also shown relevance in the progression of peripheral vascular disease (77).

1.2.6 Variations in presentation of patients with atherosclerotic disease; stenosis versus rupture of unstable plaque

Presentation of atherosclerosis can vary in patients; firstly in its severity, but also with acute versus chronic disease.

POAD presents in varying severities from intermittent claudication through to critical limb ischaemia. This is thought to be due to **chronic** atherosclerotic progression and greater vessel stenosis, until ultimately the vessel occludes.

For peripheral vascular disease, **acute** events are seen when;

- an embolic source blocks a peripheral artery (causing an acute limb ischaemia)
- or in patients with a background of PAOD; platelet aggregates occlude diseased vessels, causing a sudden worsening of chronic disease.

For both acute or acute on chronic conditions, it is believed the atherosclerotic plaque itself remains stable (78).

With this disease pattern, patients often present with fairly mild symptoms; and as blood supply to the lower limb becomes gradually restricted, neovascularisation takes place (79). Limb ischaemia only becomes critical, causing rest pain and ulceration, when there are two or more levels of restricted arterial flow; for example stenosis/ occlusion in both the common femoral and popliteal arteries. This, in comparison to carotid atherosclerotic presentation, is markedly different.

Cerebrovascular incident related to carotid atherosclerosis has been linked to intraplaque haemorrhage, formation of necrotic core and consequent plaque instability (80). A phenomenon also seen within coronary plaques (81). The presence of IPH and LRNC has been shown to accelerate; plaque growth, luminal narrowing, surface disruption and consequent cerebral (or coronary) events as can be seen in Figure 6.

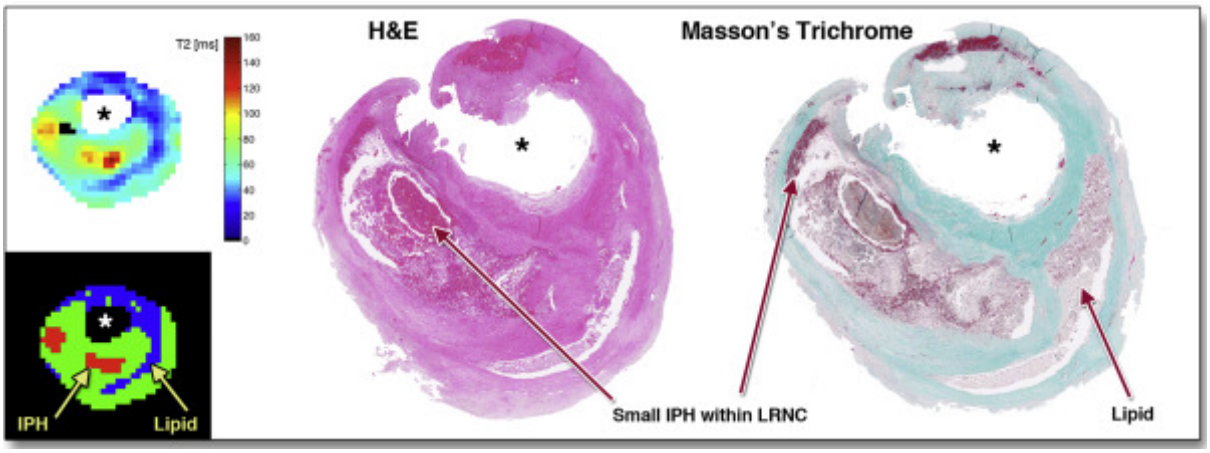


Figure 6: Intraplaque haemorrhage and necrotic core seen within symptomatic carotid endarterectomy specimens. Chai et al. Quantification of Lipid-Rich Core in Carotid Atherosclerosis Using Magnetic Resonance T_2 Mapping: Relation to Clinical Presentation (81)

Further studies have shown that atherosclerotic carotid disease accounts for approximately 25% of occlusive strokes, with a further 14% caused by atrial fibrillation. As for PAOD, emboli from sources such as atrial fibrillation are the most significant causative factor of acute events (82).

Table 2: Comparison of presentation and outcomes from carotid atherosclerosis and femoral atherosclerosis

Carotid atherosclerosis	Femoral atherosclerosis
Atherosclerosis causing acute presentation of stroke (25%)	Atherosclerosis causing chronic presentation of POAD. (Acute events thrombotic or embolic)

Acute histopathology: intraplaque haemorrhage and lipid rich necrotic core in symptomatic legions	Minimal documented histopathology: intraplaque haemorrhage seen in 0.3% on MRI.
Atherosclerosis causing 25% strokes	Atherosclerosis not known to cause acute events, only chronic events

1.3 Treatments for atherosclerosis

1.3.1 Primary and secondary prevention

Following identification of the modifiable risk factors for atherosclerotic disease progression, there has been a preferential move to prevent atherosclerosis as well as cure. Large multicentre studies and systematic reviews; such as the Cochrane review by Taylor et al. on the use of statins in primary cardiovascular prevention, have analysed early preventative measures with hugely favourable results. Raw data from this review showed that statin treatment for 5 years in those at risk of coronary disease would avoid 18 major cardiovascular events in every 1000 people (83). Lifestyle modifications and healthy lifestyle campaigns have also been shown to be an important initial step, with an 84% reduction in cardiovascular risk following lifestyle changes.

Early meta analyses on the primary prevention of hypertension has shown anti hypertensives to be effective in reducing cardiovascular risk, with no obvious lower limit, however modern equivocal studies have shown BP <140 may not have any benefit and may be harmful (84).

Regarding antiplatelet medication; there is conflicting recommendations on its use in primary prevention, but good evidence for secondary prevention of atherosclerotic complications (85).

Although medical treatment plays a significant role in prevention, patients can also help guide their own outcomes with exercise and dietary modifications. A plethora of studies have shown that nutrition (often described as 'heart healthy' eating) can decrease atherosclerotic disease progression and sequalae (86).

Secondary prevention plays a significant role in reducing further morbidity and mortality after detection/event. Outcomes from the EXPRESS study for TIA and minor stroke showed a reduction from a 10% to a 2% stroke risk following fast initiation of antiplatelet and blood pressure medications. These effects have also been seen in myocardial infarction; aspirin post MI leading to a 12% reduction in deaths, 31% reduction in re-infarction rates and data from a systematic review, for every 20 patients treated with aspirin a cardiovascular insult would be prevented (87).

As previously alluded to, similar interventions used for the secondary prevention of peripheral vascular disease, have not halted disease progression. They appear only to have effect on the reduction on cardiovascular disease within the same cohort (88). That said, smoking cessation has clearly been shown to increase the success rate and length of patency in peripheral arterial intervention (89).

1.3.2 Best medical therapy for the prevention of atherosclerotic plaque progression

With reproducible, significant results with several medical interventions, combined 'Best Medical Therapy' has become a cornerstone for the management of atherosclerotic disease. This combination treatment includes blood pressure control, lipid lowering treatments, diabetic control and antiplatelet medication (90); all of which have shown significant reductions in morbidity and mortality in the context of coronary and cerebrovascular atherosclerosis.

Regarding cerebrovascular disease; medical therapy has been the greatest contributor to the reduction in stroke rate for asymptomatic carotid stenosis; from an annual risk figure approaching 4%, down to 0.5% (91). These results have also been mirrored in cardiovascular disease, with antiplatelet therapy, statin and antihypertensive management reducing events and improving the prognosis of obstructive myocardial infarction (88). Although papers agree on this conclusion, exact values on the combined effects of medical secondary prevention have not been published.

Again, peripheral arterial disease has not observed the same benefits from either individual or combined therapy. The Heart Protection Study showed no improvement in PAOD with statin use, antiplatelets have only shown to be beneficial post-surgery/intervention for patency rates, and no study has specifically investigated hypertension in PAOD. Research has however seen a benefit from smoking cessation and exercise therapy (increasing collateral vessels) on PAOD

outcomes, with up to 70% increase on walking distance (82). This will be further discussed in 1.3.4.

1.3.3 Impact of lipid lowering therapies in carotid and coronary disease

A Cochrane review on the effects of statins for cardiovascular disease showed that all-cause mortality, as well as combined fatal and non-fatal cardiovascular and cerebrovascular were all reduced following statin administration (82). Since the early randomised controlled trails on statins in the 1990's, the benefits of both primary prevention and secondary prevention have been proven with level one evidence. In fact, systematic reviews observing the overall effects of statin therapy have reported them to be the single most effective medication in reducing atherosclerosis; showing a 42.7% reduction in death rate in asymptomatic people and 34% reduction amongst patients with cardiovascular disease (92).

Considering carotid disease only, statins have been shown not only to reduce stroke incidence up to 30% (50), but also reduce the overall thickness of the artery (specifically the intima, IMT), and change areas of the plaque known to be unstable (intraplaque haemorrhages) to areas of calcification (93). Changing the composition of vulnerable plaque may be a significant reason in why statins have such a dramatic effect on the prevention of atherosclerotic complications.

1.3.4 Treatments of peripheral arterial occlusive disease

PAOD can be treated conservatively, with medical intervention; for which some have been mentioned, or with surgery (open or endovascular). Conservative management begins with lifestyle modifications (/risk factor modifications) to reduce the impact of stenotic disease and prevent progression. As part of conservative management, increasing personal activity to promote recruitment and engagement of collateral vessels, is extremely important (18). In fact, up to three quarters of patients with intermittent claudication will improve or plateaux symptoms following combined conservative and medical management (88).

1.3.4.1 Best medical therapy for the treatment of peripheral arterial occlusive disease

Research into the benefits of best medical therapy for the treatment of PAOD have lagged behind that of symptomatic coronary disease and cerebrovascular disease. Consequently, there lacks evidence in the field as well as understanding of the risks for and against treatment (94). To date, cohorts of subjects with peripheral vascular disease have shown a reduced morbidity and mortality from coronary disease following administration of best medical therapy, but no effects have been directly observed to the peripheral arteries, or atherosclerotic plaque within. This may be due to such limited research on femoral arteries, or possibly the fact that the same phenomena are simply not observed.

1.3.4.2. Surgery for peripheral arterial occlusive disease

If conservative and medical treatment have been unsuccessful, it is possible to intervene surgically upon the atherosclerotic plaque. This can be via an endovascular arterial stretch, to improve blood flow distally, or diverting the blood flow around the plaque with a bypass graft. For arteries with more favourable access, such as the carotid and common femoral, it is possible to open the artery and directly remove the plaque and arterial intima (endarterectomy). This is a common operation for patients with carotid or common femoral stenosis causing symptoms, and therefore a point of access for this study.

1.4 Outcomes of peripheral arterial occlusive disease

1.4.1 Literature review of peripheral arterial occlusive disease outcomes

Over the last twenty-five years several studies have reviewed the outcomes of patients with peripheral arterial occlusive disease, and a few of these papers have shaped and scoped our understanding of disease progression. Results from a comprehensive literature review using 'EMBASE' and 'PUBMED' can be seen below in table 3. However, the main seminal papers will now be discussed.

In 1989, *Dormandy et al.* published a systematic review stating that 5% of men over the age of 50 experienced symptoms of intermittent claudication. This population had a three times increased risk of mortality; 50% due to myocardial ischaemia, 15% due to stroke and 25% from

non-circulatory causes (94). In 1991, he extended this work by analysing 1,969 patients with PAOD, for a minimum of a year. These were participants from the PACK trial (Prevention of Atherosclerotic Complications with Ketanserin), and those with PAOD in the placebo arm were included. Of this cohort: 1.5% went on to have a major amputation, 5% died and 7% required surgical or radiological intervention. This cohort had a mean age of 63 (74).

O'Riordain et al. (1991) published single centre outcomes after reviewing 112 patients from consecutive vascular clinics with intermittent claudication. They showed a 3% amputation rate, with 21% of their cohort deteriorating over the subsequent 5 years, and 66% remaining stable (95). *Dormandy at al*. also performed a single centre study in 1999. His results confirmed the above figures: 1-3% amputation rate and a 25% deterioration rate (96).

A systematic review of patient outcomes following a diagnosis of intermittent claudication in 2019 highlighted not only the lack of prior research in this field, but also the associated poor quality of evidence in why certain patients deteriorate from PAOD and others remain stable (97). The results of the 8 included studies correlated with previous data; 21% of 235 patients deteriorated after 1 year (98), 15.6% developed a 30% increase in stenosis after 1 year (99), and finally 4.8% of 617 patients went onto develop critical limb ischaemia (100).

1.4.2 The Fate of the Claudicant; Dormandy *et al.*

As vascular surgeons, the key seminal paper that had scoped our understanding of peripheral arterial disease is that by Dormandy et al. As described above, his results in subsequent years

have been replicated but no paper has reviewed the current peripheral arterial disease outcomes following significant medical advance and greater initiation of primary prevention (101). Having highlighted this, the results within chapter 3 will not only address current PAOD outcomes, but also compare to the results 25 years ago.

1.4.3 Comparison of outcomes for atherosclerosis within different arterial beds

Although atherosclerosis is a systemic disease, plaque burden within different vessel beds does seem to vary from one person to another. Patients also, although at risk of plaque in multiple sites, do not always present with multifocal disease, and when scanned often do not have disease in another bed at all. Large studies such as the Rotterdam Study have tried to review and compare calcification in major vessel beds, but conclusions (other than aortic calcification being a significant concern for increased mortality) are yet to be seen (34).

Regarding outcomes; meta analyses can confirm a significant reduction in morbidity and mortality from cardiovascular disease and cerebrovascular disease. With figures nearing a 30% reduction in deaths from 2007 to 2017 for coronary and a 14% reduction during the same years for cerebral. However, with limited research in the field, changes in overall patient outcomes of patients with PAOD are not documented in the literature, and we hope to address within this thesis.

1.5 Imaging atherosclerosis

Multiple modes of imaging have been used to visualise atherosclerosis; from ultrasound, computerised tomography to magnetic resonance imaging. All have been shown to successfully assess plaque burden, and in some cases also observe effects to treatment also.

1.5.2 Use of ultrasound and IMT in imaging atherosclerotic plaque and intima thickness

B mode ultrasonography imaging is a well-studied and utilised method to visualise the arterial wall, atherosclerotic plaque and blood flow within a vessel. With regard to carotid artery disease, one of the first structural changes instigated by atherosclerosis is thickening of the intima. This early development can be visualised on ultrasound technology, and consequently may identify high risk patients (102). Studies have found sensitivity of this method to be 78% and specificity 75% (103), and also predictive of ischaemic stroke; where over 13,000 stroke-free subjects were studied for an average of 8 years, and carotid artery legions found on ultrasound were predictive of ischaemia cerebrovascular insult (29).

Colour doppler ultrasound has been shown to be an excellent method for assessing degree of stenosis, with peak systolic velocity the most reproducible and commonly used reading (93). This remains the gold standard for assessing carotid artery stenosis within the UK National Health Service.

Again, there is a paucity of data for the use of ultrasonography in assessing atherosclerotic changes in the femoral arteries. USS with doppler is frequently used to measure flow and consequent stenosis of an artery, but there are very few studies on intima media thickness within the peripheral vessels (104).

1.5.3 Quantification of plaque burden using magnetic resonance imaging and T2 mapping

Following from ultrasonography, modern techniques using magnetic resonance imaging have distinguished not only atherosclerotic plaque but have also enabled quantification of plaque components. With a key component of atherosclerosis being lipid (as described earlier with the lipid hypothesis); for both disease initiation with the retention of apolioprotein B lipoproteins, and symptomatic plaque containing larger lipid rich necrotic core, it has been an important need to characterise lipid content and extent.

Studies have shown with T1 weighted imaging, that increased lipid volume within carotid atherosclerosis is a future predictor of cardiovascular risk (105). More recently the development of T2 mapping techniques has allowed for further quantification without the need for gadolinium contrast. The results have objectively quantified carotid lipid volume on a voxel by voxel basis (Figure 7), and identified segments of the plaque such as the lipid rich necrotic core, in vivo (81, 106).

The published techniques have led to further patient studies; including plaque analysis following statin initiation. In a study analysing carotid atherosclerotic plaque following 3 months statin use, a significant reduction of carotid lipid percentage was observed (34).

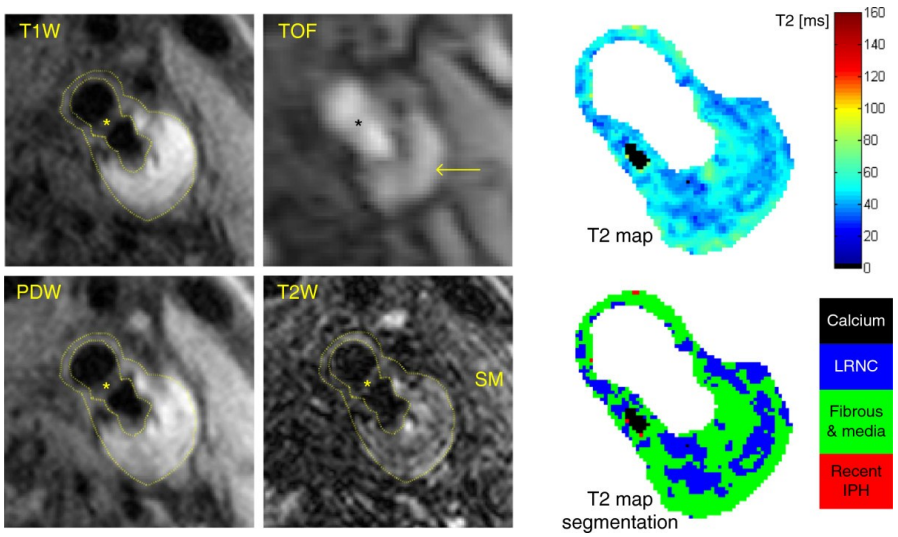


Figure 7: In vivo quantitative T2 mapping and segmentation of carotid plaque. Biasiolli, L., Lindsay, A.C., Chai, J.T. et al. In-vivo quantitative T_2 mapping of carotid arteries in atherosclerotic patients: segmentation and T_2 measurement of plaque components. J Cardiovasc Magn Reson ***15,*** *69 (2013)(106).*

These methods have again solely targeted the carotid artery, and consequently imaging of the femoral vessels in similar settings has not been achieved.

Other groups have used MRI to image the femoral artery and carotid artery together, however often results for the two vascular territories have been combined. For example, in a paper by Fuster and Fayad, it clearly states that they 'chose to study carotid and femoral arteries

together since the pathophysiology of atherosclerosis is thought to be similar in both arterial beds (107)'.

1.5.4 The role of micro CT and micro MRI in visualising atherosclerotic plaque

The importance and value of micro CT has been known for some time, and many experiments have used its potential to see and evaluate atherosclerotic samples, from mice hearts through to carotid arteries, in three dimensions. Its ability to scan small objects with significantly increased resolution has been extremely advantageous (108). With regard to carotid imaging, scans have been compared to equivalent histology with excellent results, particularly for highlighting features such as large lipid cores and haemorrhages (109). These imaging techniques have yet to be used to quantify and assess femoral plaque.

Micro MRI has rarely been used for atherosclerotic research. To date, published studies in this area have only been in animal experiments and embryonic research (110).

1.6 Summary

Common risk factors of age, gender, cholesterol and hypertension are all linked to atherosclerotic disease progression, and current treatment for atherosclerosis is largely uniform. However, patients continuously present with disease in different arterial beds, and overall patient outcomes do not neatly correlate with the extent of disease. Why do certain

patients experience a stroke but never an amputation? Why is treatment more effective for certain atherosclerotic plaques than others?

Throughout this introduction we have discussed obvious differences between carotid and femoral atherosclerotic plaque, but what these main differences are and why, is still not known. This uncertainty leads us to our research question; Using comparative analysis what are the differences between of carotid and femoral atherosclerotic plaques?

1.7 The Question

What is already known

1. As part of this study we have reviewed current outcomes for patients with PAOD, as seen in chapter 3. Overall, they remained unchanged for over 25 years. In 1991; 1.5% major amputation, 5% mortality, mean cohort age of 63 (74). In 2018; amputation rate 1.4%, mortality 6.6%, and mean age 69.9. Patients with worsening PAOD (i.e requiring repeat intervention), also had a lower prevalence of cerebrovascular disease (9% cf. 17%).

2. Primary prevention (anti-hypertensives, statins and anti-platelets) have made a marked difference to stroke and MI outcomes, but this significant effect in halting or reducing atherosclerotic progression has not been observed in POAD (111, 112).

3. Statins have been shown to cause rapid regression of carotid and aortic atherosclerotic plaque. Structural changes associated with improvements in arterial stiffness and endothelial function (34, 113).

4. Hypertension has been shown to be a more significant risk factor for carotid disease than femoral disease (29). Smoking is a much greater risk factor for femoral atherosclerosis than carotid atherosclerosis, and smoking cessation has shown to reduce in femoral disease progression.

5. Femoral IMT is a predictor of coronary calcium/calcification and consequently coronary occlusion. Carotid calcification however does not predict coronary calcification/occlusion (114).

6. Symptomatic carotid plaque contains intraplaque haemorrhage, necrotic core, and is less calcified. This can be visualised on USS, CT and MRI (81). This is not seen or has not been studied within femoral arteries.

7. Carotid plaque area is more predictive of disease extent than IMT (115), but carotid IMT is predictive of MI (116).

8. Macrophages from lipid-rich carotid plaque are involved in the activation of inflammatory pathways and overall plaque destabilisation (117), and by using T2 CMR imaging it is possible to identify patients with lipid rich plaques in vivo (118).

9. Acute PAOD events present differently to acute cerebrovascular events, where a tight stable stenosis occludes with a thrombus/embolus cf. rupture of an unstable plaque.

10. Bisphosphonates given to mice have shown to reduce arterial calcification. This has not been trialled in human models, but could this reduced calcified atherosclerosis (119).

What is unknown

1. Why does atherosclerotic disease predilect to different arterial beds in particular people? To date assumptions have been made that it is a global disease burden and consequently coronary disease, cerebrovascular disease and peripheral arterial disease have all been treated in parallel.

2. What are the effects of statins on femoral disease? Statins have been shown to stabilise carotid and coronary plaque on USS and MRI imaging, and to reduce numbers of MI's and strokes. There is no literature on the effect of statins on femoral disease.

3. Statins reduce mortality in patients with peripheral vascular disease, but this is due to a reduction in early death from coronary disease. Why do statins not have any effect on peripheral atherosclerosis?

Why is this unknown

1. Carotid and coronary disease have been the mainstay of atherosclerotic research

2. Femoral disease has not been as extensively researched, and in those studies where femoral plaque has been included, it has been historically difficult to section and analyse.

1.8 Hypothesis

'Is atherosclerosis a global arterial burden?'

Peripheral arterial occlusive disease is a significant health burden, affecting up to 10% of the population; and leads to reduction in mobility, chronic pain, limb amputation and ultimately death. PAOD progression is poorly understood and overall outcomes have not changed in parallel to other atherosclerotic diseases (stroke, cardiac disease) in over 25 years. This study aims to analyse atherosclerotic plaque in both the neck and lower limbs, to establish whether they are indeed the same disease process.

Null hypothesis; Atherosclerotic plaque is the same in all arterial beds

1.9 Experimental aims

This study aims to identify the histological and radiological differences between atherosclerotic plaque of the carotid artery and that of the femoral artery. We will be using gross histology; to identify the cells that make up the plaque, including fibrous tissue, lipid and calcium. Alongside this; ex vivo micro CT and micro MRI scans to assist in further plaque characterisation. Secondly, T2 weighted MR imaging to provide in vivo visualisation of plaque composition and distribution within the artery.

If significant differences in plaque morphology are observed, this information may aid in future treatment targets for patients with peripheral arterial occlusive disease as well as help to improve overall outcomes for patients with PAOD.

Secondly, direct in vivo and ex vivo comparison of atherosclerotic plaques will allow for MR sequence validation and the potential for future scanning to predict patient outcomes.

Chapter 2: Methodology

2.1 Research idea

To assess the differences within carotid and femoral plaques, it was agreed that ex vivo plaque analysis would be the most preferential method. Patients are regularly admitted to the Vascular Surgery Ward for removal of atherosclerotic plaque by a carotid endarterectomy or femoral endarterectomy. To allow for comparison of carotid and femoral plaque these patient groups were selected. For appreciation as to why patients usually undergo an endarterectomy on either the carotid or femoral artery in their lifetime, it was also decided that each participant would have MRI on both vessels (carotid and femoral) prior to any intervention. In this way, each patient would act as their own control.

To allow for; enrolment of patients into the study, an extra non clinical MRI scan, and for access to plaque which is normally disposed of, an ethics application was composed, submitted and accepted.

2.2 Ethics

As part of an academic fellowship and doctorate, a full IRAS ethics application, alongside Oxford University Hospitals NHS Foundation Trust approval documentation was written, amended, submitted and granted.

Multi-modal Assessment of Patients with Peripheral Vascular Disease.

Oxford Peripheral Vascular Disease Study – OxPVD 15/NW/0972

University of Oxford 158717

North West - Haydock Research Ethics Committee

Proportionate Review Sub-committee of the North West - Haydock Research Ethics Committee

Ethics approval documentation can be found within appendix C.

2.3 Study population

Upon completion of ethical approval, preliminary plaques were accrued to try to optimise plaque analysis techniques. This was completed ahead of recruitment of our study population.

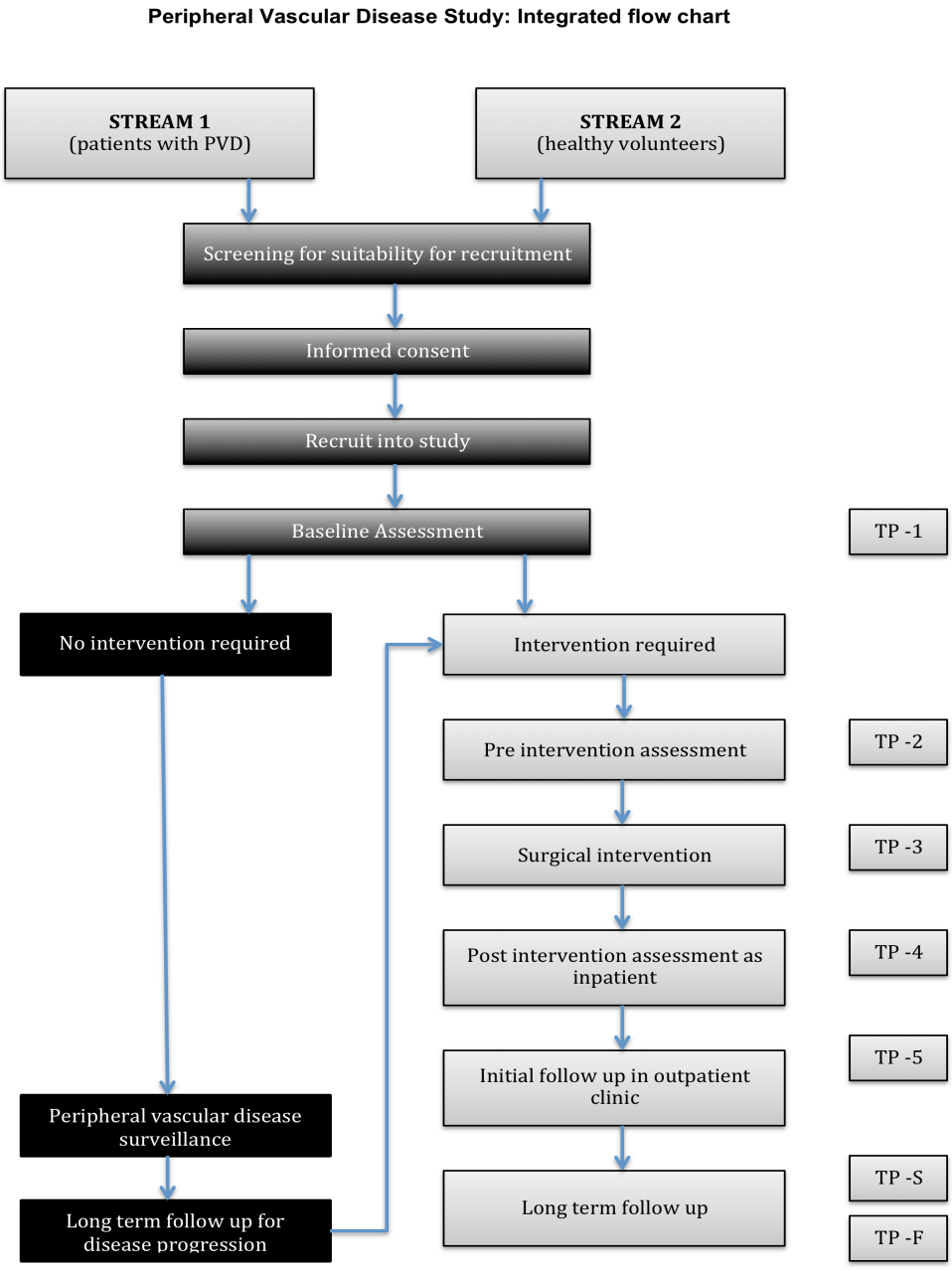
Patients presenting between February 2019 to March 2020; to the Oxford Regional Vascular Unit with either lower limb peripheral arterial occlusive disease or carotid artery stenosis, (and requiring either carotid or femoral endarterectomy), were invited into the study. Further recruitment from March 2020 was unable to take place due to the closure of Universities and termination of research Nationally in response to COVID-19. At this time all elective vascular surgery was paused for 18 months, to allow for resource pooling to required areas within the National Health Service.

Patients were given the option of enrolling in the study OxPVD prior to their procedure. Consent was purely on a voluntary basis following a discussion with myself. All volunteers were

given a patient information leaflet and time to reflect and ask questions. They also had the choice to withdraw at any time.

The REC approved consent form, patient information leaflet, and clinical record form can all be found in appendix B.

Figure 8: Integrated flow chart of OxPVD patient timeline



All patients had the option to voluntarily give access to their clinical records, scans and outcomes, following discussion and signature on the study consent form. Each participant was also be asked to complete a patient clinical record form (CRF) on agreement of participation. This collated all important and relevant information on medical history, medications and patient demographics.

2.3.2 Inclusion criteria

Inclusion criteria

Participants may enter the study if:

- Male or female with a confirmed diagnosis of peripheral arterial occlusive disease, those with carotid disease and healthy volunteers
- Age >16 years

Exclusion criteria

Participants may not enter the study if:

- there is any clinical concern or risk to their safety, or
- they have a sufficient needle phobia to prevent venous access.

All other patients will be included to allow for accurate patient demographics.

Additional criteria for participants undergoing CMR:

For those participants undergoing MRI specifically as a research assessment, these exclusion criteria apply:

- Claustrophobia which limits/prevents participants from remaining in the CMR scanner
- Participants who cannot lie flat on the scan table
- Participants with metallic implants, pacemakers, implantable defibrillators etc
- Patient who are unwell or require pre-operative monitoring/ intervention

Study withdrawal

Each participant has the right to withdraw from the study at any time.

2.4 Data collection for outcomes of patients presenting with peripheral arterial occlusive or carotid disease from 2016 to 2018.

As described, there is limited data on the recent outcomes of patients with peripheral arterial occlusive disease. To obtain a modern data set on patients presenting with PAOD, a review of our own patient outcomes within Oxford University Hospitals NHS Foundation Trust was detailed and executed.

All new patient referrals to the Oxford University Hospitals Vascular Surgical Unit were included and reviewed. These spanned all patients seen in vascular specific clinics across two surgical centres and 5 consultant surgeons. Daily clinic appointment schedules identified all the vascular surgical patients and from this every initial clinic letter was read to confirm the

diagnosis. After selecting those with leg pain, patients with confirmed PAOD (reduced or absent pulses on examination and peripheral arterial stenosis shown on duplex/CTA/MRA) were included.

Data analysis was over a 12 month period from July 2015 to June 2016. Following inclusion of those with confirmed PAOD, patients were followed up via consecutive clinic letters, discharge summaries, clinical records and relevant scan results. All outcomes were recorded and tabulated. Results were analysed and compared.

2.5 MRI protocol

Each study patient underwent cardiovascular T2 weighted MRI of the carotid and femoral arteries prior to any surgical procedure. The protocol for carotid imaging has been published by Chai *et al*. Femoral imaging sequences were optimised as part of this study, as they had not been described in the literature, and are detailed below.

The MRI scan facilitated visualisation of the arterial wall and any calcium, fatty lipid core, fibrous cap and intraplaque haemorrhage present. Each patient also acted as their own control; allowing for femoral arteries and carotid arteries to be directly compared.

Each scan was reviewed by a specialist in medical imaging (L.B), to firstly assess the quality of the scan and then it's distinguishable data. Each carotid and femoral scan was given a grading 0 to 3 (0 – unable to use for analysis, 1- poor, 2- adequate, 3 – good). Following this it was

possible to review each patients MRI images and by using the AHA classification of atherosclerotic disease, score the level of disease seen within the artery.

2.3.2 Carotid atherosclerosis and T2 mapping image analysis

The methods for visualising carotid vessels using MRI have been well documented and have been a significant part of the research from my collaborating research group. For this study, patients were imaged on a Siemens Verio - 3T wide bore scanner - running VB17 software, and to enhance signal a Machnet bilateral 4 channel phased array coil was placed around the participants neck, in the carotid region. Bright-blood time-of-flight (TOF) angiography was used to detect the bifurcation of the carotid and any lumen stenosis. For the carotid artery, the bifurcation was used as the midpoint of the scan, allowing for visualisation of the common carotid and into the internal carotid artery i.e the entire plaque.

Once location of the scan was confirmed, each patient underwent a T1 and T2 weighted MRI scan followed by a two-dimensional carotid T_2 mapping; echo time of 9 to 127 ms and repetition time of 2,000 ms; using a modified excitation multiecho spin-echo sequence that uses a nonselective DANTE pulse train. DANTE acquired 10 slices of 2 mm thickness each, covering 2 cm of the target carotid artery, in 8 min.

2.5.3 Femoral atherosclerosis and T2 mapping image analysis

As a new method of imaging the femoral arteries and potential pathology, MRI sequencing and optimisation had to first be achieved. For this a participant (myself) was invited into the OxPVD study but was not included in the main analysis. During this time an Invivo 32 channel phased array coil was used and placed over the participant's pelvis. The scanning sequence used for the carotid arteries was replicated for the femorals. With this, it was possible to visualise the femoral wall with plentiful clarity to assess any associated pathology.

To ensure enough signal through to the femoral arteries, which lie deeper than the carotid vessels within the body cavity, it was not possible to scan both common femoral vessels at the same time. With a study scan length already approaching one hour for carotid and femoral combined, it was decided to only scan the femoral proceeding to operation, or the ipsilateral femoral artery to the carotid operation. This was a decision made with patient comfort, delay to surgery, costings for going into a second hour of scanning time, and value of what a second femoral scan result would bring, all in mind.

2.5.4 Control patients presenting with coronary disease

As part of discussions, following preliminary results of the first 6 recruited participants to OxPVD, we felt it would be beneficial to the study to invite cardiac patients with significant coronary disease (i.e triple vessel disease) to the study. In this way we would be able to review

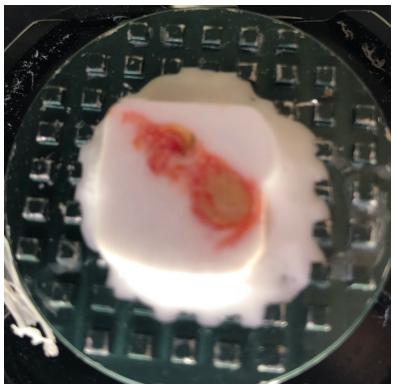
the atherosclerotic burden within the carotid and femoral vessels in a patient known to have life-limiting atherosclerosis.

2.5 Histological analysis

During carotid endarterectomy or femoral endarterectomy, atherosclerotic plaque from the common/ internal carotid or common femoral artery was isolated via a standard surgical technique. Each artery was surgically opened and plaque removed along with its associated intima. At this time the specimen was placed into phosphate buffered saline (PBS) and taken on ice to the laboratory where is could be washed. Each specimen received a minimum of 3 washes in PBS, or this was repeated until washings were clear and no longer blood stained.

The tissue was fixed using 4% paraformaldehyde (PFA) in PBS for 24h (up to 32h), before transfer to a falcon containing PBS for storage prior to embedding.

During preliminary research for this study, femoral plaque was also trialled for frozen sectioning using optimal cutting temperature compound (OCT) to embed the plaque. Previous studies used this method and have shown successful sectioning of carotid tissue in this manner (120), unfortunately however, this early work on the femoral plaque proved this technique not be possible. It was tried on multiple different days, with minor alterations in technique, mounting slides, and air temperature. During the process, areas shown as beige/light brown in colour in picture 1, tended to completely crumble and disintegrate on sectioning at 4 degrees Celsius. At that time it was believed these areas were possibly of calcification.



Picture 1: OCT embedded femoral plaque for frozen section

In light of this it was decided that frozen sectioning femoral plaque would not be part of the study, and although a longer process to achieve, paraffin embedding and sectioning should be used for both the carotid and femoral tissue to ensure adequate and reproducible results.

2.6.2 Femoral calcification

There is very little published literature on the histological analysis of femoral plaque; which may be due to the difficulties in sectioning, or lack of research on femoral disease. Either way, during preliminary work, it became obvious that sectioning and staining were going to be challenging. As described, frozen section was removed from the planned protocol prior to the start of the study, as it was felt that maintaining consistency throughout specimens would be extremely difficult. As the project evolved (and more femoral plaques were enrolled and sectioned), it also became apparent that not only was it impossible to orientate the plaque due

to its fragmented nature, the solid elements within its structure made all sectioning an extremely difficult process.

To overcome this, it was decided to try to decalcify the femoral specimens in an attempt to section and stain slices of the plaque.

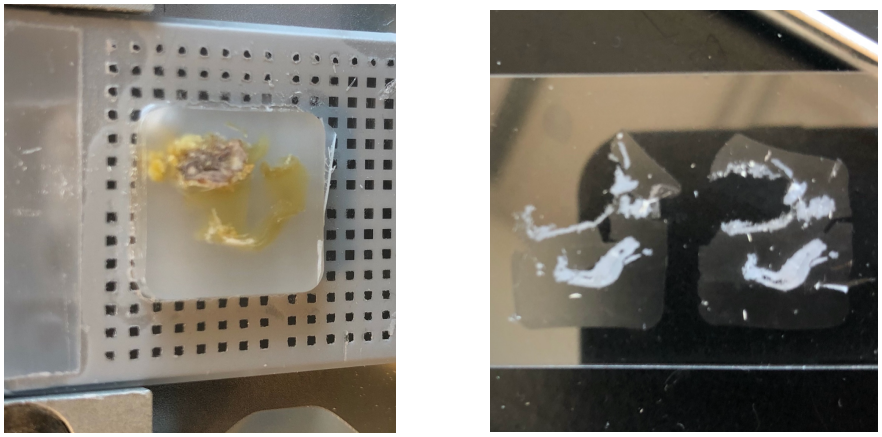
As per many standardised protocols on tissue decalcification, the femoral plaque was placed into 10% formic acid for 24 hours, prior to fixation with PFA as stated above (see protocol in appendix B).

2.6.3 Paraffin embedding and sectioning

Upon availability of the embedding machine, samples were cut into 5mm segments and put in tissue cassette with the caudal (proximal) end facing down. Each segment was then labelled to ensure a caudal to cranial order (where possible), before being transferred to a bath of 70% EtOH. For the majority of the femoral tissue, it was not possible to distinguish orientation as the plaque was removed in multiple small sections at the time of operation. Overnight the tissue moved through multiple dehydration steps, and onto wax embedding (see protocol in appendix c). The following day specimens were transferred to a tissue embedder where warm wax was poured onto the tissue to enable production of a paraffin block for sectioning.

Each block was placed onto ice prior to sectioning, to ensure the paraffin remained hard and strong enough for adequate samples.

Using a microtome, the paraffin blocks were sectioned 5-6um thickness and transferred to a floatation bath to allow for application to a glass slide, ready for staining.



Picture 2: a) femoral plaque embedded in paraffin, within microtome for sectioning, b) effects of solid sections within the plaque causing fragmentation of the paraffin slice.

2.6.4 Staining

Having obtained paraffin sections of 5µm, at regular 0.5cm intervals throughout the plaque, it was possible to histologically examine the tissue using;

- Haematoxylin and eosin (Sigma-Aldrich, United Kingdom),
- Masson's trichrome (Merck-Millipore, United Kingdom), and
- Von Kossa (Merck-Millipore, United Kingdom).

These stains were able to identify the microscopic structure of the plaques as well as key plaque components. Masson's can identify collagen in the fibrous tissue as green, as well

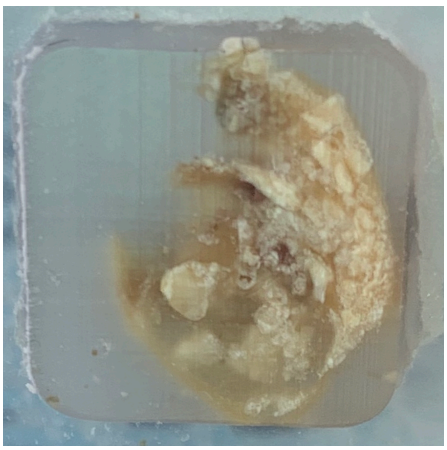
as fibrin and erythrocytes as bright red. Lipid remains transparent and unstained in both H&E and Masson's stain. Stained slides were photographed and reviewed. For this review process I was assisted and was able to learn from a consultant histopathologist (E.F), within the Oxford University Hospitals NHS Foundation Trust.

2.6.5 Complications and study expansion

Other than the expected learning curve from fixing, embedding, sectioning and staining; as all these processes were personally new, there were a few complications within the above which needed to be overcome. The largest of these; how to successfully section the femoral plaque.

As can be seen in picture 2; the solid matter present within the femoral plaque caused repeated fragmentation of the paraffin section. This not only effected the composition and shape of the section on the slide, it also meant it was extremely difficult to adhere the specimen to the glass slide. The effect of this was pronounced following multiple washes during the staining process. By the end of the necessary protocols, many of the plaque sections had simply come away from the slide.

Different slides, such as charged glass were used with a slightly improved effect, however for certain samples (as seen below in picture 3); it was extremely difficult to achieve adequate slide adherence.



Picture 3: Femoral plaque embedded in paraffin for sectioning

Although the fact that femoral plaque could not be sectioned in the same way as carotid plaque may have given indication that the two plaques could be different, it was still pertinent to ensure we found ways to prove this scientifically, and identify what the differences could be.

Using the preliminary data from our study participants' in vivo MRI scans, it was apparent that many of the femoral plaques were being classified as AHA 7; indicating calcified plaque. Even with the additional process of decalcification as previously described, marginal improvements could be elicited from femoral sections. Decalcifying also inevitably changed plaque composition, and meant that carotid and femoral sections were treated differently; neither of which were ideal in a comparative study.

It was decided to look for alternative methods of plaque analysis, especially when it became evident that some of the issues related to femoral sectioning could not be easily overcome. Ways of reviewing the plaque as an entire entity was key. Two of the options researched and felt appropriate for plaque analysis were the use of micro CT and micro MRI.

2.7 Micro Computerised Tomography

Micro computerised tomography (micro CT) is a non-destructive imaging tool using serial two dimensional x-rays to produce a three dimensional image (figure 9). It has been widely used within the biomedical field to visualise bone and cartilage as well as cardiovascular structures (micro CT angiography) (121).

With generous support of the Kennedy Trust for Rheumatology Research, we were able to utilise the imaging facilities available and scan carotid and femoral plaques through high resolution micro CT (100-200 seconds per scan).

Images were transferred to FIJI Image J for visualisation and analysis.

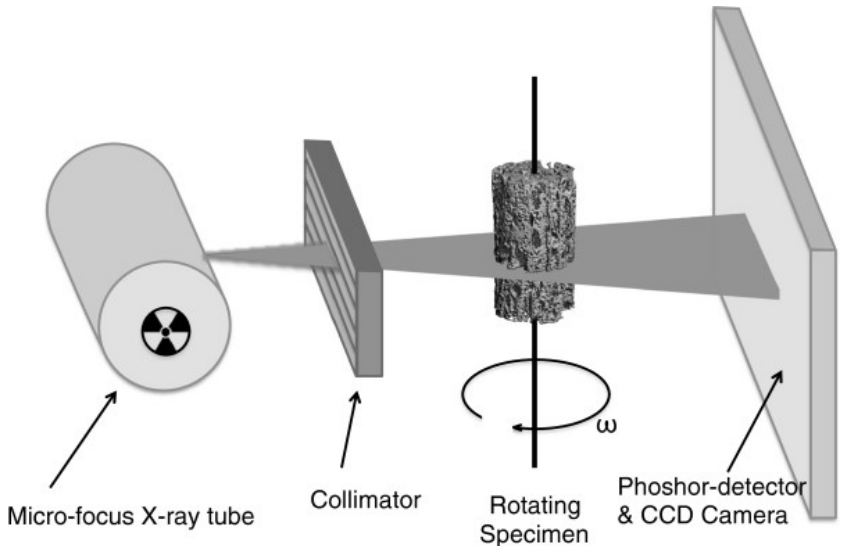


Figure 9: Principal components of a microcomputed tomography scanner. Boerckel JD, Mason DE, McDermott AM, Alsberg E. Microcomputed tomography: approaches and applications in bioengineering. Stem Cell Res Ther. 2014 Dec 29;5(6):144 (122).

For detailed Micro CT analysis 22 volumetric images were considered. From the scanned 2D Dicom slices, the 3D image volume was generated using a script written in MATLAB R2014a; and the voxel values were converted into Hounsfield Units (HU) using the 'Rescale Slope' and 'Rescale Intercept' information provided in the Dicom headers.

In order to minimise the noise in image volumes, a 3D median filtering was applied. Since the 3D volumes contained artefacts, as well as the falcon tube and minimal drops of PBS (post depletion from the specimen storage solution), they were identified and removed using an automated approach. The background noise/air voxels were automatically removed using a thresholding-based approach. In most cases, this threshold was estimated within HU of -300 to -250.

From the foreground voxels, the image regions were segmented based on their HU values. We used the standard HU scores to identify;

- Calcium with HU > 300,
- Grey Area with HU between 45-300,
- Blood with HU between 30-45,
- Fat with HU between -100 to 0, and
- Soft Tissue with HU between -300 to -100.

From the segmented regions, the scores were generated as the ratio of voxels in each region with the total number of voxels in the volume (after removing the artefacts and background voxels).

Results from these images were then transferred into Prism software to assess differences between values presented for carotid and femoral atherosclerotic plaques.

To allow for further image analysis and specific quantification of the plaque, FIJI software was utilised. Images were scanned through and measured to find the area of greatest stenosis. For carotid this was fairly easy orientate, whereas for femoral; the biggest fragment of plaque was use for further analysis. Using freehand drawing around the circumference of the most stenotic section of plaque, pixel density was recorded.

Graphical representation and raw data figures of pixel density were used in comparative analysis between femoral and carotid plaque.

2.8 Micro Magnetic Resonance Imaging

Micro MRI has been widely used in the biomedical field for both functional and anatomical imaging, although most often in small animals. It is particularly suitable for soft tissue, and therefore often preferred for scans of the brain, nerves and spinal cord.

Following a literature review, it became apparent that mMRI could also be a viable option to image atherosclerotic plaque, although again these papers were based on animal plaque rather than human. With this as another form of comparative imaging, it was decided to proceed with scanning our own samples; in the hope plaque composition/architecture may seen.

Excised samples were embedded in gadolinium-doped agar within a PTFE supporting plastic matrix, and were stored at 4ºC prior to scanning. As previously described in the context of the imaging of embryonic mice (123), the use of gadolinium embedding in a water-based medium permits the detection of high-signal-to-noise ratio of tissue samples, by significantly reducing their T1 and providing the opportunity for 'negative contrast' images to be acquired, i.e. those in which the gel medium is bright, and other structures are darker. Furthermore, embedding is necessary for the long-scan time micro-MRI process to avoid mechanical vibrations or movements, and to provide a physical support for the samples.

Following embedding, individual samples were bundled together within a borosilicate quartz NMR tube (45 mm OD), and placed in a 45 mm quadrature birdcage 1H 500 MHz probe attached to a preclinical 12 T MRI scanner (magnet: Magnex Scientific, Oxford; Console: Modified Varian DDR2 / OpenVnmrJ).

Samples were maintained at 21±0.5 ºC via combination of an air-conditioned ambient environment and a Bruker BVT3000 variable temperature unit (running an open-source reimplementation of its control software (124)). Scout gradient-echo images were then obtained, and the sample shimmed using a third-order shimset to a ~150 Hz linewidth, reflecting the large degree of magnetic field inhomogeneity ultimately caused by air-tissue interfaces and the interface of hard and soft tissue types, following the interface conditions that follow from Maxwell's equations.

To enable the determination of the Ernst angle, (the flip angle that yields optimal signal per unit time), whole-sample T1 and T2 was measured via inversion recovery and multi-echo spin

echo and determined to be approximately 50 ms and 20 ms respectively. Accordingly, a high-resolution partial Fourier scan was initiated, with an acquisition matrix size of 1280x1280x1280 in a FOV of 35.2x45.2x45.2 mm^3; 100 kHz readout bandwidth, 50º flip angle with an adiabatic RF pulse; TR 16.752 ms; TE 4.544 ms; 9 averages; and 70% partial Fourier scheme. Reflecting the partial Fourier nature of the acquisition, reconstruction of the imputed half of k-space was performed using BART (on a dual Xeon Gold 6252 workstation computer running Ubuntu 20.04 with 768 GiB ram) giving a final matrix size of 1280x1280x1830 and thus a final reconstructed resolution of 27.5 µm x 27.5 µm x 24.6 $µm^3$. After reconstruction, images were manually analysed in FIJI (120, 125).

As described previously, further plaque quantification could be achieved by drawing around the atherosclerotic plaque. As the plaque was embedded within agar and placed into a tube, it was possible to compare sections throughout the plaque. Pixel density through the sections of plaque were identified and recorded; as both raw data and graphically. With this quantification, it was again possible to compare carotid and femoral slices and therefore their composition.

2.9 Immunofluorescence

As part of a collaboration with a second research group; leading pioneering work on the role of endothelial cells in atherosclerotic progression; carotid and femoral sections underwent immunofluorescent staining with DAPI, PECAM-1, Plexin D1 (PLXND1).

DAPI is a basic agent used to stain all nuclei (DNA), PECAM-1 is an endothelial marker, and PLXND1 is a mechanosensor within endothelial cells that regulates vascular function in the presence of atherosclerosis.

Carotid sections were utilised by our collaborating group to demonstrate their lab based results within human models. This provided an opportunity to compare femoral endothelial results, and see if PLXND1 acts in a similar way in femoral atherosclerosis. The shared protocol can be seen in appendix B and further background and results within chapter 7.

2.10 Statistical analysis

Due to reduced OxPVD recruitment, the total numbers of participants in many of the subgroups created for data analysis were often too small to allow for statistical review. That said, it was possible within certain groups to show if results were statistically significant ($p<0.05$).

For those values documented throughout the thesis, raw data was imputed into Prism (www.graphpad.com) and unpaired t-testing completed – showing these specific results were not by chance.

Prism was also used to create appropriate graphs as can be seen throughout the write up.

As part of my academic clinical fellowship, I completed a postgraduate diploma (PGDip) in Health Research. A module of this degree was dedicated to research statistics, and consequently I was able to draw on this prior knowledge to aid in my basic analysis.

3. Chapter 3: The Fate of the Claudicant 25 years after Dormandy

3.1 Abstract

Introduction

In 1989, Dormandy et al. showed that 5% of men over the age of 50 develop symptoms of intermittent claudication. This population had a three times increased risk of mortality. In 1991, he subsequently followed 1969 patients with peripheral arterial occlusive disease (PAOD); 1.5% had major amputation, 5% died and 7% required surgical or radiological intervention.

Methods

This study aimed to review all 'new patient' referrals to the vascular services during a consecutive twelve month period, from July 2015 to June 2016. All vascular clinics within a single centre were included. Following identification of those with confirmed PAOD; patient demographics, disease severity and outcomes were recorded and analysed. This was then compared to a cohort from 25 years prior.

Results

361 patients had confirmed PAOD, of which 88 (24%) were re referrals to the vascular services. Of the 273 new referrals; 163 (58%) were managed with best medical therapy, 90 (33%) with

angioplasty and 16 (6%) with operative management. Overall amputation rate was 1%, and one year mortality 7%.

Conclusion

In 1991 one year mortality rate was 5% (7% in 2017). Amputation rate for the Dormandy cohort was 1-2% (ours 1%). Finally, 25% of the patients referred in early 90's deteriorated, now a 24% re referral rate. 25 years on from the Dormandy seminal paper 'Fate of the Claudicant'; despite more aggressive risk factor modification, the only significant change seen is that patients typically present 7 years older. Outcomes however remains disappointingly unchanged.

3.2 Aims

The aim of this initial study was to review the outcomes of patients with peripheral vascular disease in current times.

From background research for this doctorate, it was clear that peripheral arterial occlusive disease has been an under researched topic. Coronary and cerebrovascular atherosclerotic events have been the forefront of clinical studies due to their mortality rates, and consequently modern outcomes of POAD remain unknown. With this in mind, it was decided to try to discover this data. With an overarching question of 'why do some patients have a carotid endarterectomy and others a femoral endarterectomy, but not both?', we felt it important to understand how primary prevention, improved detection, increased intervention had changed

or not changed the natural history of POAD. Particularly as the same data is available for the other main atherosclerotic diseases; coronary and cerebrovascular.

This study aimed to not only act as a comparative exercise between the various types of atherosclerotic disease, but also inform vascular surgeons on the current outcomes of PAOD.

3.3 Methods

For background to this study, an initial literature search was performed using EMBASE and MEDLINE: keywords 'intermittent claudication', 'peripheral vascular disease', 'peripheral arterial (occlusive) disease', 'outcomes'. From this search we were able to extract the relevant studies focusing on population based outcomes of those with peripheral arterial occlusive disease, two decades prior to the cohort being studied.

To obtain our modern day comparator, **all** new patient referrals to the Oxford University Hospitals Vascular Surgical Unit were included. These spanned all patients seen in vascular specific clinics across two surgical centres and 5 consultant surgeons. Daily clinic appointment schedules were collated and used to identify all the vascular surgical patients and from this every initial clinic letter was read to confirm why the patient has been referred and their diagnosis. After selecting those with leg pain, patients with confirmed PAOD (reduced or absent pulses on examination and peripheral arterial stenosis shown on duplex/CTA/MRA) were included. Leg pain referrals were peripheral arterial occlusive disease was not the cause of their pain were excluded.

We analysed data over a 12 month period from July 2015 to June 2016, split into two six month periods to assess reproducibility. The first six month period selected was from 01 Jan 2016 to 30 June 2016.

Following inclusion of those with confirmed PAOD, patients were reviewed via consecutive clinic letters, discharge summaries, clinical records and relevant scan results from our electronic patient records system. All outcomes were recorded and tabulated. Results were analysed and compared; 1) in relation to the results of the published literature, 2) as representations versus primary presentations to clinic.

Cause of death was confirmed by details on the patients' death certificate, or in the few cases where this was not available, from the bereavement office, coroner or general practitioner.

This process was repeated for a second six month period; (01 July 2015 – 30 Dec 2015) to validate the results. An earlier timeframe was used to ensure adequate follow up time. The same methods were utilised in patient identification and selection, and all those with confirmed PAOD were included in the overall analysis

3.4 Literature review

As described above, an important part of this project was to review the past literature on peripheral arterial occlusive disease. It was apparent from an initial search that recent studies or reviews on this topic were not available. Consequently, this led to an in depth search of what

had been published within the last twenty to thirty years. Results of this, and the relevant outcomes have been tabulated in table 3. From these studies, it has been possible to draw conclusions on the outcomes of patients presenting with PAOD in the recent decades, as part of a comparator dataset.

Table 3: Literature review of patients with claudication

	Number of patients	Mortality	Follow up	Amputation	Intervention	Outcomes (1)	Outcomes (2)	Outcomes (3)
The fate of the claudicant--a prospective study of 1969 claudicants. Dormandy JA, Murray GD. Eur J Vasc Surg. 1991 Apr;5(2):131-3.	1969	4.3% (1.8% MI, 1.3% stroke)	1 year	1.6%				
Outcome events in patients with claudication: a 15-year study in 2777 patients. Muluk SC et al. J Vasc Surg. 2001 Feb;33(2):251-7; discussion 257-8.	2777	12% overall (42% at 5 years and 65% at 10 years) 66% cardiac	15 years	<10% (10 year cumulative)	18% (10 year cumulative)			Death predictors: older age, low ABPI, medicated DM and stroke
The natural history of claudication: risk to life and limb. Dormandy J, Heeck L, Vig S.	Review	30% at 5 years, 50% at 10 years, 70% at 15 years)	5 years	1-3% (11x greater in smokers)		50% became symptoms free during follow up	¼ deteriorate (6-9% in first year of diagnosis, 2-3% thereafter)	2-4% non fatal cardiovascular event each year

Semin Vasc Surg. 1999 Jun;12(2):123-37.								
Intermittent claudication in 8343 men and 21-year specific mortality follow-up. Bowlin SJ, Medalie JH, Flocke SA, Zyzanski SJ, Yaari S, Goldbourt U.	8343 (360 with IC, 7983 symptom free)	44% IC death, 29% symptom free death	21 years				IC 3rd highest predictor of stroke mortality	Higher risk factor than coronary heart disease
Realistic expectations for the patient with intermittent claudication. O'Riordain DS, O'Donnell JA. Br J Surg. 1991 Jul;78(7):861-3.	113	33% (44% MI, 29% CVA) 8% at 2 years, 23% at 5 years, 40% at 8 years If ABPI <50%: 20% at 2 years, 50% at 5 years, 69% at 7 years	5 years (min)	3% amputation rate 3% critical ischaemia		Survivors: 66% stable of improved symptoms 21% shortened claudication symptoms	57% smokers continued to smoke	

Risk factors affecting the natural history of intermittent claudication. Rosenbloom M et al. Arch Surg. 1988 Jul;123(7):867-70	195 (310 claudicating limbs)	18.9% (36% after 5 year, 50% after 8 years) (23% MI, 9% CVA, 17% Ca)	Up to 8 years	2.6% amputation rate	11%		25% of limbs developed critical ischaemia at 5 years, 41% at 8 years	
A six year prospective study of fibrinogen and other risk factors associated with mortality in stable claudicants. Banerjee AK, Pearson J, Gilliland EL, Goss D, Lewis JD, Stirling Y, Meade TW. Thromb Haemost. 1992 Sep 7;68(3):261-3.	333	34% (78% CV death)	6 years					Fibrinogen increase nearly 2 fold in those with probability of death in 6 years
Vascular morbidity and mortality during long-term follow-up in claudicants selected for peripheral bypass	155 (selected for bypass)		8.6 years		39% required intervention (17% in first year and	38% major vascular events (increase risk with diabetes,	Annual risk increase 3.5% of major vascular event	

surgery. Dawson I, Sie RB, van der Wall EE, Brand R, van Bockel JH. Eur J Vasc Endovasc Surg. 1998 Oct;16(4):292-300.					2.8% thereafter)	cardiac disease, HTN)		
Haemostatic factors and prediction of ischaemic heart disease and stroke in claudicants. Smith FB, Rumley A, Lee AJ, Leng GC, Fowkes FG, Lowe GD. Br J Haematol. 1998 Mar;100(4):758-63.	607 patients	34.6% (42% from IHD, 13.8% stroke, 29.5% non CV)	6 years			33.4% no new vascular events or deterioration	Fibrinogen and D dimer raise associated with total coronary events. Stroke rates associated with t-PA antigen.	15.3% non fatal MI
Fate in intermittent claudication: outcome and risk factors. Jelnes R, Gaardsting O, Hougaard Jensen K, Baekgaard N, Tønnesen KH, Schroeder T.	257 patients	44% (twice mortality in men than women, 4 x mortality in	6.5 years			Disease progression 7.5% to critical ischemia in first year, 2.2% thereafter.	Ankle systolic below 70mmHg, toe systolic below 40mmHg associated	

Br Med J (Clin Res Ed). 1986 Nov 1;293(6555):1137-40.		under 60 years)					with progression.	
Relationship between intermittent claudication, inflammation, thrombosis, and recurrent cardiac events among survivors of myocardial infarction. Narins CR, Zareba W, Moss AJ, Marder VJ, Ridker PM, Krone RJ, Lichstein E. Arch Intern Med. 2004 Feb 23;164(4):440-6.	966 without claudication , 78 with claudication .	Claudication independent risk factor for 2 fold increase in death/non fatal cardiac event, 5 fold increase cardiac mortality.				Patients with IC had enhanced fibrinogen, D dimer, CRP and amyloid A concentrations	Those with IC were less likely to receive beta blockers after index infarction	
Incidence, natural history and cardiovascular events in symptomatic and asymptomatic peripheral arterial disease in the general population. Leng GC, Lee AJ, Fowkes FG, Whiteman M, Dunbar J, Housley E, Ruckley	160	Relative risk of death same for symptomatic and asymptomatic claudicants	5 years		8.2% vascular intervention (including amputation)	28.8% had pain after 5 year	1.4% leg ulceration	

CV. Int J Epidemiol. 1996 Dec;25(6):1172-81.								
Patients with coronary, cerebrovascular or peripheral arterial obstructive disease differ in risk for new vascular events and mortality: the SMART study. Achterberg S, Cramer MJ, Kappelle LJ, de Borst GJ, Visseren FL, van der Graaf Y, Algra A; SMART study Group. Eur J Cardiovasc Prev Rehabil. 2010 Aug;17(4):424-30.	3563					POAD had highest rate of smoking, hypertension and hyperlipidaemia	Rate of any vascular event per year 2.5%	PAOD patients increase risk of coronary events over coronary artery disease (HR = 1.6)

3.5 Outcomes of patients with PAOD following referral to a tertiary vascular centre

From our initial cohort (01 Jan 2016 – 30 June 2016); 168 patients presented to the vascular services and were diagnosed with peripheral arterial occlusive disease. This excluded those patients referred with 'leg pain' where the cause was deemed to be neurogenic or otherwise.

Forty of the 168 (24%) were re-referrals to the vascular service. Of this cohort: 2 had undergone a prior major amputation, 19 had an angioplasty, 15 a previous bypass/endarterectomy and 3 were managed conservatively at their prior/initial appointment. Of the same group at representation, two (5%) went onto have an above knee amputation, 15 (38%) had further angioplasty, five (13%) had a surgical procedure, 18 (45%) were managed conservatively and three patients subsequently died. These were not included in further analysis.

Taking the 128 new referrals (figure 1A), 71 (55%) were managed with best medical therapy, 46 (36%) with angioplasty, 8 (6%) with operative management and three died before duplex investigation or intervention. There were 4 deaths post intervention.
Patients who presented during this period had an overall amputation rate of 1/128 (0.8%), and mortality of 7/128 (5%).

Analysis of our second (validation) cohort (01 July 2015- 30 Dec 2015) revealed the following results; 193 patients were referred into the services with confirmed POAD. Of these, 48 were

re-referrals (25%). Upon analysis of this cohort: 22 patients had had prior angioplasty, 14 had undergone surgical intervention and 12 patients were managed conservatively.

Following review, 22 were offered and underwent angioplasty (46%), 21 were managed conservatively (44%), 6 patients had limb salvage surgery (3%) and one patient underwent a major limb amputation.

Of the 145 new referrals (figure 1B), 44 were managed with angioplasty (30%), 92 conservatively (63%), 8 operatively (6%), and one patient was lost to follow up. For this cohort the overall amputation rate was 1/145 (0.7%), and mortality was 7/145 (4.8%).

Figure 5a: Outcomes of patients from January 2016 to June 2016

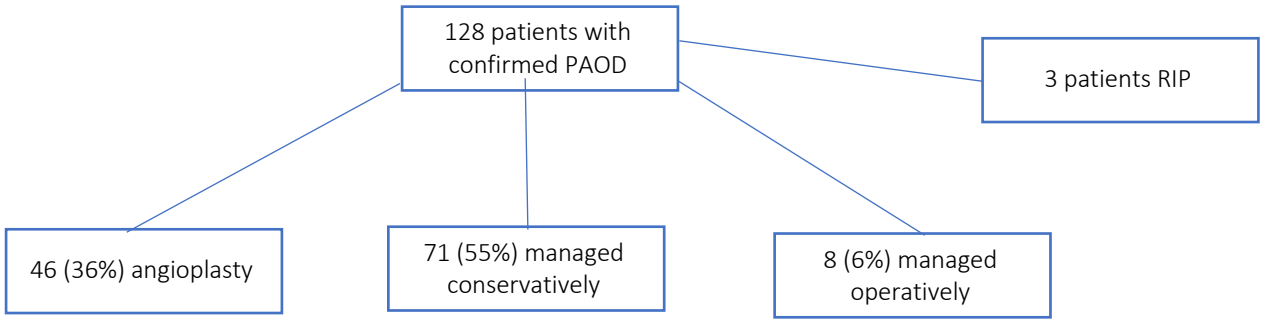
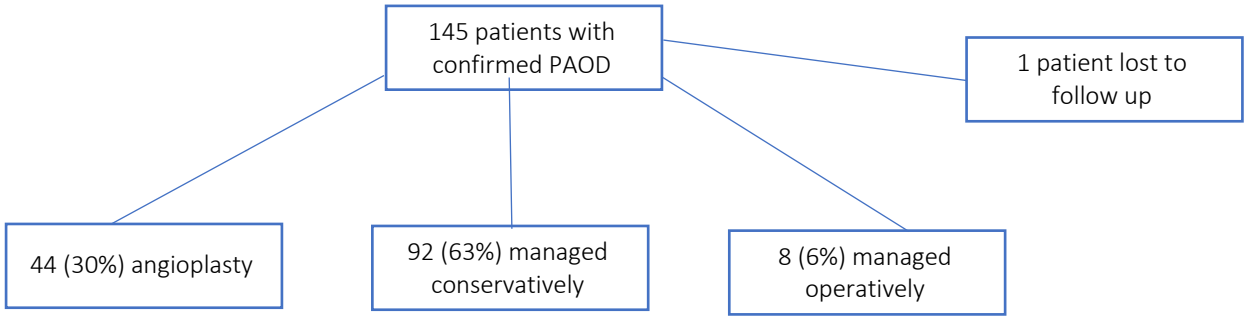


Figure 5b: Outcomes of patients from July 2015 to December 2015



3.6 Variations in PAOD patient characteristics; 1992 to 2018

In total 361 patients presented to the services with a diagnosis of peripheral arterial occlusive disease over a twelve month period. Interestingly 88/361 (24%) were re-referrals to the services. Of the new referrals (273 patients), 163/273 (58%) were managed with best medical therapy, 90/273 (33%) managed with angioplasty and 16/273 (6%) managed operatively. The overall amputation rate was 5/361 = 1.4% and the overall mortality was 24/361 = 6.6% with a mean age of 70 years.

It is possible with these results, to compare and contrast outcomes from our cohort to that within the literature review. Secondary analysis of the characteristics of our cohort alongside our comparator (patients presenting 25 years ago) can also be conducted. Results shown in Table 4.

Table 4: Characteristics of patients with known PAOD

Characteristics	Dormandy Study. (1999)	Oxford Study. (2017) n=361
Age	63.2	69.9
Sex, male	80%	71%
MAP >120	14%	3%
Hypertension	57%	68%
Race, Caucasian	98%	99%
Diabetes	15%	35%
Coronary disease	27%	32%
Cerebrovascular disease	9%	15%
Haemoglobin	14.8	13.4
Arterial reconstruction	40%	18% - 39%
Anti-anginals	21%	11%
Anticoagulants	9%	17%
Anti-diabetics	10%	24%
Diuretics	23%	22%
Anti-hypertensives	13%	65%
Anti-platelets	Not recorded	67%
Statin	Not recorded	75%

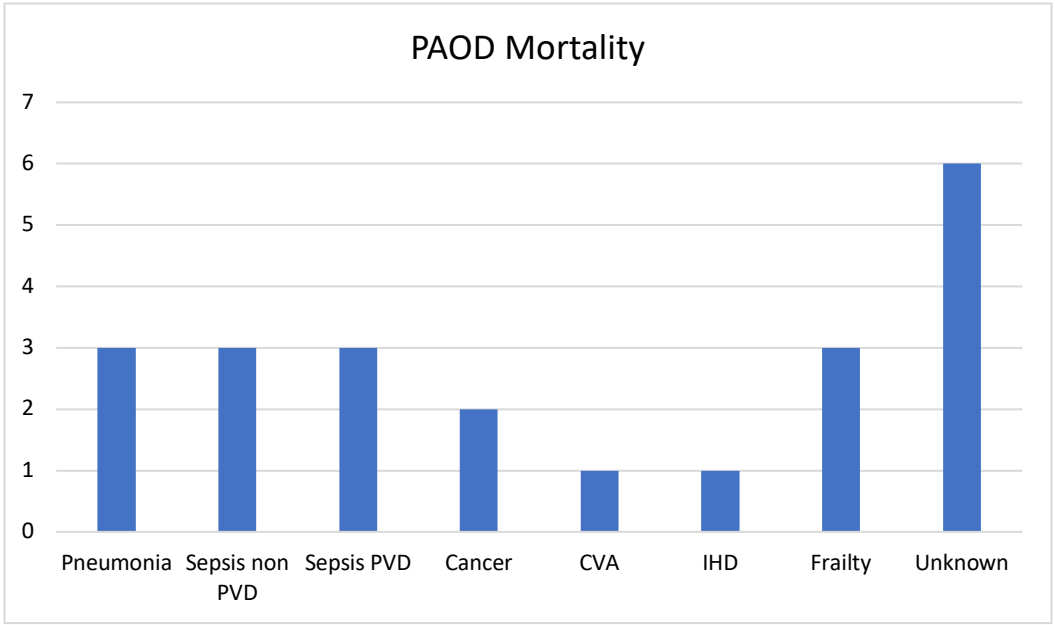
The average age of referral in 2018 was nearing 70, compared to 63 in 1999. There was a 9% increase in female presentations ($p < 0.01$). An 11% increase in the number of patients presenting with a diagnosis hypertension ($p < 0.01$), and of these patients only 3% had uncontrolled hypertension with a mean arterial pressure >120 (cf. 14%) ($p < 0.01$). A 20% increase in the numbers of patients with diabetes ($p < 0.01$) was also observed, and a moderate

6% increase in CVA/TIA (p <0.01). Similar numbers of patients presented in both groups with coronary disease (approximately 30%). In light of the greater number of diabetes and hypertension diagnoses, more patients presented in our cohort on oral treatments for these conditions.

Two thirds of our patients were taking anti-platelet medications, 75% statins, and nearly a fifth oral anticoagulation (c.f 9%).

All-cause mortality was also reviewed as part of the study (Figure 9). Of those with a known cause of death 18% were due to PAOD related sepsis, and over 30% due to cardiovascular disease.

Figure 9: Causes of mortality in patients with PAOD



3.7 Comparative analysis of patients following initial diagnosis of POAD to those re-presenting with worsening disease

A final comparative analysis was within our own cohort; comparing those patients with newly diagnosed POAD (primary presentation) to those representing to the services (i.e had previously been discharged from vascular follow up.)

The average age of patients re-presenting was 71 years, compared to first time referrals of 61 years. There were similar numbers of females in each group, with slightly more with diabetes (+8%), hypertension (+12%) ($p<0.05$) and patients with known coronary artery disease (+10%) in the re-presentation group. Interestingly the stroke/TIA prevalence halved (9% cf. 17%) in the representation group.

All studied characteristics were considerably higher within the PAOD cohort compared to the general population as can be seen in Table 5.

Table 5: Characteristics of PAOD patients; new referrals compared to patients who represented.

	PAOD Cohort		Representation cohort		UK Population	
Age	60.5		71.4			
	Female	Male	Female	Male	Female	Male
Sex	27%	73%	31%	69%	50%	50%
Hypertension	66%		78%		29%	
Diabetes	33%		41%		6%	
Coronary Disease	29%		39%		11%	
Cerebrovascular disease	17%		9%		2%	

3.8 Discussion

This study of 361 patients presenting to vascular surgical clinics provides an up to date analysis on the current outcomes of peripheral arterial occlusive disease; an area which is profoundly under evaluated amidst the greater numbers of studies focusing on peripheral atherosclerosis in the context of coronary or cerebrovascular pathology.

The principal findings in this study are that; despite increased availability and productivity of endovascular procedures, significant medical advances, and the introduction of best medical

therapy, the overall numbers of patients who deteriorate from POAD (24% cf, 24%), and the percentage who require amputation (1-3% cf 1.4%) has remained unchanged for over 25 years. This is in stark contrast to the complete reduction in number of strokes and myocardial infarction following primary and secondary prevention with statins, antihypertensives and antiplatelet medications (126).

A noted difference however is a 7 year age increase at primary presentation; with initial presentation being 63 for Dormandy at al., and 70 years for our cohort. The reasons for this may include decreased smoking (127), effective primary prevention strategies (128), changes to indications for surgical intervention, or possibly may reflect demographic variations between the current (Oxford) and previous (London-based) populations, but it is likely that the introduction of *best medical therapy* has played a significant role here. Although seven years is a vast improvement for POAD patients, it does not follow the same trends in increasing age at presentation for cardiac disease (129).

Upon direct comparison of the two cohorts it can be seen that there is a statistically significant increase in the number of patients with diagnosed hypertension and diabetes in 2018, and consistent with this - significantly greater numbers of patients taking antihypertensive and antidiabetic medications. In fact a MAP <120 mmHg was only present in 3% of our cohort (cf.14%).

An interesting parallel is the worrying static mortality rate of patients with PAOD. Dormandy et al. reported a mortality rate of 5%, and our modern day cohort of patients showed to have a mortality rate of 6.6%.

Although overall percentages remain similar, the cause of death appears to have varied; for example 13% from myocardial infarction in 1991 compared to 5% in 2018. This change does mimic trends seen in the published literature; that best medical therapy has reduced coronary events and deaths. It also suggests that patients who were previously dying from coronary events are now being protected from this but dying from alternative causes.

Our second analysis; comparing primary presentation versus patients re-presenting to the services also highlighted some interesting results. The number of females were similar in each group and, as expected, the prevalence of hypertension, diabetes and coronary artery disease were higher in the patients who had re-presented with recurrent/more severe PAOD. Interestingly however, the number of patients with worsening PAOD had half the prevalence of cerebrovascular disease compared to those who were presenting for the first time.

In light of the above results it certainly raises the question as to whether atherosclerotic plaque in the peripheral vessels is indeed similar to atherosclerotic plaque in alternative arterial beds. Best medical therapy (primary and secondary prevention) and advances in intervention have made a significant difference in outcomes for cerebrovascular and coronary disease, but these do not seem to have been replicated to the same degree for PAOD over the last 25 years. In addition, those presenting with recurrent or severe PAOD do not appear to have the same overall disease burden in other atherosclerotic vessels – particularly the cerebrovascular circulation.

3.9 Conclusions

25 years following the seminal paper by Dormandy et al. on 'the fate of the claudicant'; mortality rates remain at approximately 5%, the rate of amputation at 1-3% and around one quarter of patients continue to deteriorate from their disease. The age of presentation to vascular services however is around 7 years later than previously seen in 1999.

Patients who re-represented to the services after initial review +/- intervention were more likely to have diabetes, hypertension and coronary disease than new presentation patients but, interestingly, their prevalence of prior cerebrovascular accident was significantly less.

Although there are well established risk factors for atherosclerotic disease (i.e tobacco smoking, elevated cholesterol, hypertension, diabetes mellitus and family history), not all atherosclerotic beds tend to be affected to the same degree, if at all. In light of this, we propose that further research into the plaque morphology in PAOD is warranted, and that particularly peripheral arterial occlusive disease may benefit from more extensive biological review and likely alternative pathways of management.



4. Chapter 4: OxPVD study patient recruitment cohort analysis

4.1 Background

Common risk factors of age, cholesterol and hypertension are all linked to atherosclerotic disease progression, and current treatments for atherosclerosis are largely uniform. However, patients continuously present to health services with symptomatic disease in different arterial beds, and overall patient outcomes do not neatly correlate with the extent of disease.

Over recent decades, the majority of research has focused around myocardial infarction and stroke prevention; with significant interest in understanding the cause of atherosclerotic deposition. Much of this work has been upon coronary and carotid vessels, with little investigation into peripheral vascular atherosclerosis (7, 11).

With the understanding that atherosclerosis is due to; apolipoprotein B associated lipoprotein retention (lipid hypothesis) and consequent inflammatory cascade and plaque formation (8), as well as further studies showing significant statin effects on plaque regression, and hypertension being proven a major risk factor; best medical therapy has been adopted as a primary treatment measure (82).

Despite the introduction of best medical therapy, the outcomes for patients with PAOD have remained unchanged for over 25 years. Primary prevention has however made a marked difference to the incidence of both stroke and MI. Why are treatments more effective for certain types of atherosclerotic plaque than others? Why do certain patients experience a stroke but never an amputation?

To address this question, we felt it pertinent to look at the femoral arteries and their associated atherosclerotic plaque in greater detail, and compare this to carotid atherosclerosis. Patients presenting to a vascular surgical team are regularly investigated for either carotid or femoral stenosis, and if deemed appropriate, will undergo carotid or femoral endarterectomy respectively. These patients therefore provide an appropriate cohort to analyse both carotid and femoral atherosclerosis, in vivo and ex vivo.

Following ethics approval for said research, it was possible to initiate and commence the Oxford Peripheral Vascular Disease Study (OxPVD); with the aim of assessing femoral atherosclerotic plaque, and its potential similarities and differences to carotid atherosclerosis.

OxPVD successfully achieved Oxford University Hospitals NHS Foundation Trust approval. Alongside this, agreement from the Research Ethics Committee (REC) following submission via Integrated Research Application System (IRAS) was also granted. Patients with either significant carotid disease and recent stroke, or PAOD effecting their quality of life, (and requiring surgery), were considered for enrolment into the study (OxPVD).

4.2 Aims

The study aimed to address the overarching question; is atherosclerotic plaque the same in all arterial beds? Specific analysis was focused on the comparison between carotid and femoral atherosclerotic disease.

4.3 Methods

With the data and understanding as detailed in previous chapters, it was decided the best way to meet our aims, and provide comparative analysis of atherosclerotic regions, was to review the carotid and femoral plaques both in in-vivo and ex-vivo formats. This type of analysis within the femoral region has not been well described in the literature previously, providing the opportunity for new and innovative research, as well as the ability to answer our study questions.

For in vivo analysis; collaboration with a group leading pioneering work in the field of atherosclerotic imaging enabled our plaques to be visualised using MRI, prior to surgical removal. In vivo imaging also allowed for every patient to act as their own control; as it was possible (and part of our protocol) to scan both the femoral and carotid areas for each study participant.

The detailed methods for the OxPVD study including;

- ethics

- patient enrolment
- in vivo MRI and histology
- further plaque analysis

are all detailed within the methods section of this thesis.

To allow for alterations and adaptions to the study, following any early analysis and/or complications; OxPVD sort ethics approval with the potential of a variety of assessments for patients with peripheral atherosclerotic disease (appendix B). However, early confirmed study methods were for pre-operative in vivo MRI, followed by ex vivo analysis of atherosclerotic plaque; limiting the patient contact required for the study and increasing patient recruitment.

With detailed and careful review on the study structure, our initial protocol planned for ex vivo histological analysis (a method which had worked well for carotid atherosclerosis). Unfortunately, following issues as detailed in the methods section regarding the structure of the femoral plaque, it was decided to review other means of assessing atherosclerotic structure without the need for sectioning and/or altering the components of the plaque. This would allow for not only information on plaque composition, but also on the three dimensional structure of the plaque.

Two key methods discussed were micro computerised tomography and micro magnetic resonance imaging; both allowing for plaque visualisation and probable characterisation, but without the need for sectioning. These techniques have limited but recognised use in atherosclerotic research, but unfortunately most are predominantly in animal models. Within

the University of Oxford both scanners (micro CT and micro MRI) were sourced, and a trial plaque in each of the modes produced images with enough quality for analysis (108, 110).

4.3.2 Issues in patient recruitment

Aside from exclusion criteria for scanning, there were also some obstacles during the recruitment process which lead to a steady patient recruitment rate.

The main complication to overcome was the National Health Service system. Patients admitted for femoral or carotid endarterectomies were often only required to attend the hospital at 7.30am on the morning of said operation. In many of these cases a pre-operative assessment had been completed well in advance to the given operation date, and consequently unless the patient was willing to attend a busy tertiary referral hospital for an MRI scan (without re-imbursement), it was not possible to see the patient prior to the day of theatre.

To ensure the patients had adequate time to understand the study, and process the information on what OxPVD would involve, I personally called each patient 1-2 days in advance of their procedure; to give them time to think if they would like to be a participant. Following this, on the day of the operation I re-iterated the information and each patient was handed a hard copy of the study aims and consent. After time for reading, they could again ask any questions, before signing a consent form if in agreement.

In light of the above, patients willing to be participants could not be first on the operating list for that day, as this did not allow enough time for the consent process and completion of MRI scan before the start of the operation. As a voluntary action, the research element to this study was not to interrupt any part of the NHS clinical process. The MRI scan taking one hour plus 30 minutes minimum to transfer and consent, meant that if the patient was expected to be taken to theatre before 10.30am on the morning of admission, the MRI scan (and therefore study participation) could not go ahead.

If patients were admitted to a ward the night before the operation, it was often much easier to complete the consent process and facilitate the MRI scan prior to the operation start time.

The above process was reviewed and agreed within my transfer viva. One of the main points raised within the viva was that some patients may wish to take part in the study and contribute to the results, but not want/be able to in complete the MRI scan. It was advised to not eliminate participants on this basis; hence why some study numbers have plaque enrolled but not an in vivo MRI scan. Within the study cohort there were also patients who had to stop the MRI part of the way through the scan. Consequently, for some data sets, femoral and carotid imaging for the same participant could not be achieved.

One of the biggest issues to patient recruitment however was experienced just shy of a year into the study recruitment process. At the beginning of 2020 all patient recruitment ceased secondary to the coronavirus world pandemic. At this time, I was personally taken out of my research environment and redeployed to clinical duties, and all vascular non urgent operations (which included **all** elective femoral endarterectomies) were stopped. As femoral

endarterectomies were labelled a category three, they were not restarted within the department for a further 18 months.

This was discussed within my confirmation viva, as a significant proportion of my final DPhil year was disrupted. It was agreed that further patient recruitment 2+ years down the line would not be the most beneficial use of time, and that other comparative work should be considered as an alternative. This can be found in later chapters.

If recruitment had continued throughout the final year of research, it was hoped that approximately 20 femoral endarterectomy specimens would have been collected, with similar numbers of carotid specimens within the comparator group.

4.4 OxPVD recruitment

4.4.1 Analysis of patient recruitment characteristics

Table 6: OxPVD patient recruitment

Number	Carotid/Femoral	Sex	DOB	Date	Age	Caucasian	Smoker	DM	HTN	In vivo Scan
OXPVD-00001	femoral	M	28/04/1932	20/02/2019	86	y	ex	n	y	y
OXPVD-00002	carotid	m	29/07/1952	20/02/2019	66	y	ex	y	y	y
OXPVD-00003	femoral	m	18/01/1941	20/02/2019	78	y	ex	y	y	y
OXPVD-00004	femoral	m	19/04/1940	20/02/2019	78	y	non	n	n	y
OXPVD-00005	carotid	f	10/01/1937	21/02/2019	82	y	ex	n	n	y
OXPVD-00006	femoral	f	26/05/1963	06/03/2019	55	y	yes	y	n	y
OXPVD-00007	carotid	m	21/11/1947	08/03/2019	71	y	ex	y	y	y
OXPVD-00008	carotid	m	19/03/1942	01/04/2019	77	y	yes	n	y	y
OXPVD-00009	femoral	m	13/01/1939	02/04/2019	80	y	ex	n	n	y
OXPVD-00010	carotid	m	04/02/1947	03/04/2019	72	n	non	n	y	y
OXPVD-00011	carotid	m	18/10/1938	04/04/2019	80	y	ex	n	y	y
OXPVD-00012	carotid	m	27/06/1949	10/04/2019	69	y	ex	n	y	y
OXPVD-00013	carotid	f	14/06/1961	24/04/2019	57	y	non	n	n	y
OXPVD-00014	cardiac	m	31/03/1946	24/04/2019	73	y	non	n	y	y
OXPVD-00015	cardiac	m	05/09/1952	25/04/2019	66	y	non	n	y	y
OXPVD-00016	femoral	m	14/07/1944	29/04/2019	74	y	ex	y	y	n
OXPVD-00017	carotid	m	30/09/1942	01/05/2019	76	y	ex	n	y	y
OXPVD-00018	femoral	f	19/08/1935	02/05/2019	83	y	yes	y	y	n
OXPVD-00019	femoral	f	27/04/1934	02/05/2019	85	y	non	y	y	n
OXPVD-00020	carotid	f	02/05/1942	24/06/2019	77	n	non	n	y	n
OXPVD-00021	carotid	f	23/04/1943	10/07/2019	76	y	ex	n	y	y
OXPVD-00022	carotid	m	02/07/1941	27/08/2019	78	y	ex	n	y	y
OXPVD-00023	femoral	m	19/11/1956	26/09/2019	62	y	ex	n	y	y
OXPVD-00024	carotid	m	20/04/1955	21/09/2019	64	y	yes	n	y	y
OXPVD-00025	asymptomatic carotid	-	-	15/01/2024	-	-	-	-	-	n
OXPVD-00026	asymptomatic carotid	-	-	25/01/2024	-	-	-	-	-	n
OXPVD-00027	asymptomatic carotid	-	-	30/01/2024	-	-	-	-	-	n

As can be seen in table 6; 27 participants were enrolled into the study to compare the atherosclerotic plaque of carotid and femoral arteries. 9 femoral endarterectomy patients, 13 symptomatic carotid endarterectomy patients, 3 asymptomatic carotid endarterectomy and 2 control patients awaiting a triple heart bypass operation.

The first 24 patients were full participants, with active consent and engagement in pre-operative imaging, and post-operative plaque analysis (where possible).

All asymptomatic carotid plaques were collected at a later date following the advice of confirmation viva assessors. It was felt that a comparison of both asymptomatic and symptomatic carotid atherosclerosis; to femoral plaque would give a more complete picture and allow for direct comparison of the results. (With symptomatic carotid atherosclerosis equating to a stroke by an acute embolic event, asymptomatic carotid atherosclerosis remaining as stable plaque, and femoral atherosclerosis causing disease from both thrombotic and embolic natures).

Due to post pandemic hospital research restrictions, the asymptomatic carotid specimens were collected with no identifiable links to the patient. They remained under the same ethics protocol. Consequently, only specimen analysis will be found for these plaques, no patient data or in vivo scans.

The average age of a symptomatic patient presenting for operation was 74 years (73 years for carotid pathology and 75 years for femoral). 7/24 (approaching 30%) were diabetic, 19/24

(97%) hypertensive and 17/24 (71%) a smoker or ex-smoker. Carotid vs femoral epidemiology can be seen in table 7.

Although numbers for the cohort are fairly small, there is a statistically significant difference ($p=<0.05$) between the percentage of patients with diabetes mellitus and femoral disease, compared to DM and carotid disease. Also observed but not significant, is a greater percentage of patients with hypertension and significant carotid disease 85% cf. 67% in femoral disease.

Table 7: Demographics of carotid vs femoral patients enrolled in OxPVD

	Carotid	Femoral
Age (average years)	73	75
% DM	15%	56%
% HTN	85%	67%
% Smoker/Ex	77%	78%

As previously described, 2 patients awaiting a coronary artery bypass graft for triple vessel disease were also scanned. This was a decision from a laboratory meeting, to assess if such patients, randomly selected, had atherosclerosis in other arterial beds. If greater time for recruitment had been an option, more of these control patients may have been invited into the study. With such limited numbers, it is unlikely there is any significance to the results, however the information on whether the coronary/carotid/femoral arteries were all affected provided further evidence as to why this study or future studies are pertinent.

4.4.2 OxPVD Data collection

Table 4 lists the OxPVD participants enrolled in the study, and demonstrates a couple of the issues relating to patient recruitment. Of the 24 people who consented to participate in OxPVD, 2 patients could not complete the MRI scan once they had entered the scanner. For these two patients no MRI data could be obtained.

For two further patients, carotid imaging could not be obtained due to patient claustrophobia. In the majority of cases, femoral scans were completed first to try to relax the patient, but for a small number of participants entering into the scanner head first caused too much distress, consequently a carotid scan was not possible.

Finally, 3 further participants agreed for plaque analysis only, and did not want to partake in an MRI scan; due to either personal experience or not wanting any 'extra' scans to regular clinical management. As mentioned previously, outcomes from my transfer viva encouraged inclusion of these patients, to ensure as many plaques were analysed as possible.

For the first 6 patients enrolled in the study; OxPVD 00001 – OxPVD 00006, the study protocol included in vivo MRI scan (both carotid and femoral arteries for each participant), followed by histological analysis of explanted atherosclerotic plaque. As described within chapter 2, femoral plaque sectioning was extremely difficult to achieve, despite various adaptions to the protocol, including decalcification of the plaque. It was decided that plaque would therefore undergo micro CT scanning. In light of this there is no quantitative micro CT data for the first 6 specimens which were already embedded in paraffin prior to this decision.

Following successful imaging using micro CT, a second additional scanning method was researched and used for specimens 16, 18, 19, 20, 21, 22. This included 3 carotid and 3 femoral scans. Results of the micro MRI data are detailed in chapter 6. All specimens prior to OxPVD 00016 were embedded in paraffin, sectioned and stained for histological analysis. Samples OxPVD 00016 - onwards were stored in PBS following fixation, and scanned with both micro CT and micro MRI.

Due to the closure of the laboratory in March 2020 in response to the COVID-19 pandemic; plaques which had been subject to micro CT and then micro MRI could not be embedded in paraffin and sectioned. They were stored in PBS and refrigerated at 3 degrees during the entirety of the 'lockdown', before undergoing sectioning and staining where possible in late 2021/2022.

Further plaque could also not be visualised using micro MRI methods due to closure of the scanners and prohibited patient recruitment during 2020-2022.

Table 8: OxPVD patients; AHA classification of disease and smoking history

No	Carotid AHA R	Carotid AHA L	Femoral AHA	Carotid Ca %	Femoral Ca %	PMH risk factors	Smoking stopped (y/o)	pack year
1	3	6	7			ex smoker	30	45
2	5	6	0			DM/HTN/ex smoker	30	15
3	6	5	7			DM/HTN/ ex smoker	4	93
4	5	4	7			non smoker		0
5	4	6	7			ex smoker	30	13
6	5	4				smoker		11
7			7	14		HTN/ ex smoker	35	15
8	5	6	0	42		smoker/HTN		55
9			6		43	ex smoker	4	50
10	6	5	5	5		HTN		0
11	6	6	5	14		ex smoker	20	30
12	7	7	7	25		ex smoker/HTN	4 months	75
13	3	5	3			non smoker		0
14				CARDIAC	CARDIAC	non smoker		0
15	3	5	0	CARDIAC	CARDIAC	non smoker		0
16	WITHDRAW				46	smoker	20	33
17	5	6	7	10		ex smoker	35	5
18	NO SCAN				6	DM/ smoker		62
19	WITHDRAW				9	DM		0
20	NO SCAN			0		HTN		0
21	5	6	0	27		ex smoker/DM	10	20
22	NO SCAN			18		ex smoker	15	80
23			3		1	DM	30	30
24	6	5	6					

4.5 Plaque removal following surgical procedure; macroscopic tissue analysis

Prior to imaging and quantitative analysis of the plaque, the first obvious differences between the carotid and femoral atherosclerotic specimens were on gross visualisation. As can be seen in picture 4, carotid plaque was able to be extracted in its entirety, removing a thickened intimal layer, taking the shape of the common (and often) internal carotid artery. Gross touch of the specimen revealed a rubbery consistency with areas of hardening. The inside of the artery was in most cases smooth, with artefact deep to the surface; seen clearly in the lower tile of picture 4.

In contrast, femoral plaque could not be surgically removed en-bloc, instead was offered to the study in multiple smaller sections. The intima could be visualised, but hardened sections of tissue appeared to sit on the inner surface (luminal side) of the specimen; seen within the right hand tiles of picture 4. The overall feel of the specimen was often more like rock, or hardened coral (out of water), much more solid than the carotid atherosclerotic plaque.

This initial finding assisted in our belief that carotid and femoral plaques may be different in architecture. If such was the case, it could potentially justify why femoral disease does not present in the same way as carotid disease, and why femoral disease does not respond to the same medical treatments.

Picture 4: macroscopic analysis of carotid and femoral atherosclerosis ex vivo, prior to embedding.

CAROTID FEMORAL



Further analysis of these plaques with in vivo MRI, ex vivo; histology, Micro CT, Micro MRI will all be discussed in the subsequent results chapters.

4.6 Outcomes of OxPVD patients

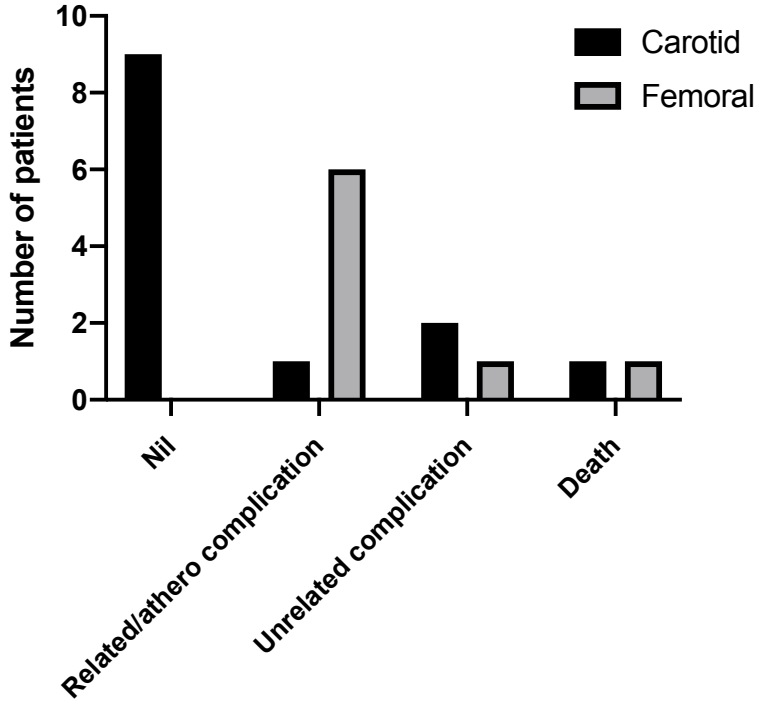
Towards the end of this study, it was possible to follow up the enrolled patients and depict the cohort outcomes within 2 years of surgery. Results of this can be seen in Table 8, and graphically in Graph 1.

Table 8: Outcomes of patients enrolled in OxPVD (12-24 month follow up).

Study number	Operation	Outcome
00001	Left CFEA	Angioplasty, bypass, amputation
00002	Left CEA	Nil
00003	Right CFEA	Iatrogenic complication
00004	Left CFEA	LVF, COPD, (RIP)
00005	Left CEA	Bladder Ca
00006	Left CFEA	Angioplasty
00007	Right CEA	Nil
00008	Left CEA	Pancreatic Ca
00009	Left CFEA	MI
00010	Left CEA	Nil

00011	Left CEA	Seizures (RIP)
00012	Left CEA	Nil
00013	Left CEA	Nil
00016	Bilateral CFEA (right)	CAD
00017	Right CEA	Nil
00018	Right CFEA	Lung Ca
00019	Right CFEA	Reoccluded – angioplasty, STEMI
00020	Left CEA	Nil
00021	Left CEA	Nil
00022	Right CEA	Lung Ca
00023	Left CFEA	Worsening symptoms
00024	Right CEA	Nil

Graph 1: Outcomes of Ox PVD Patients



As shown above, 70% of patients following a carotid endarterectomy had no further complications or admissions to hospital in the 12 -18 months following surgery, compared to 75% femoral endarterectomy patients who has atherosclerotic complications within the same time period. Both cohorts had one non PVD related death.

Of the peripheral vascular disease cohort, two patients (25%) went onto have coronary events.

5. Chapter 5: Magnetic resonance imaging of carotid and femoral arteries

5.1 Introduction

Magnetic resonance imaging is used frequently in clinical settings to visualise soft tissue; (particularly joints and ligaments), brain/spinal cord or abscesses/tumours. As described, variations in pulse sequence have also been used to depict arterial vessel walls and their associated atherosclerotic plaques. T2 mapping has further quantified atherosclerotic plaque composition.

As a published method for carotid artery visualisation, we have used this knowledge as a base for in vivo analysis of OxPVD atherosclerotic plaques. It is believed with this, along with other methods of plaque comparison, it may be possible to answer the research question; are carotid and femoral atherosclerotic plaque the same?

5.2 Aims

The aims of this section were to image the carotid and femoral arteries in the same patients (i.e each patient act as their own control), and with this assess to what extent atherosclerotic disease had burdened each vascular bed. The images obtained during this time could also be used to quantify differences between the plaques, as well as observe atherosclerotic distribution within the artery.

5.3 In vivo analysis of atherosclerotic plaque; carotid and femoral arteries

5.3.1 Methods for in vivo scanning and analysis

Details on the methodology for enrolling patients in the study and completing magnetic resonance imaging on the carotid and femoral arteries can be found in the methods section of this thesis (chapter 2).

1) Each scan was reviewed using *Horus* and *Image J* platforms. As principle investigator and new to vascular imaging, the scans were analysed by myself, as well as a specialist in the field; who was blinded to all patient information including the participants reason for hospital admission, as well as all other study results.

   Initial review of the imaging was to assess clarity and quality of the scan. A scale of 0 to 3 was used (0 – poor (reject), 1- major issues, 2- minor issues, 3 good).

2) Secondary analysis included stage of atherosclerotic disease, using the American Heart Association (AHA) classification. This was conducted for both carotid vessels, and the pathological femoral vessel OR ipsilateral femoral vessel to the pathological carotid artery.

Modified AHA Lesion Classification for MRI

- Type I–II: near-normal wall thickness, no calcification
- Type III: diffuse wall thickening or small eccentric plaque with no calcification
- Type IV–V: plaque with a lipid or necrotic core surrounded by fibrous tissue with possible calcification
- Type VI: complex plaque with possible surface defect, haemorrhage, or thrombus
- Type VII: calcified plaque
- Type VIII: fibrotic plaque without lipid core and with possible small calcifications

The information was also compared to patient demographics to see if any associations or links were significant. All statistical analyses were completed using *Prism.*

3) In vivo MRI scans were used to assess the distribution of the plaque within the artery, to see if variations in atherosclerotic deposition could be seen.

5.3.2 Results of in vivo MRI analysis

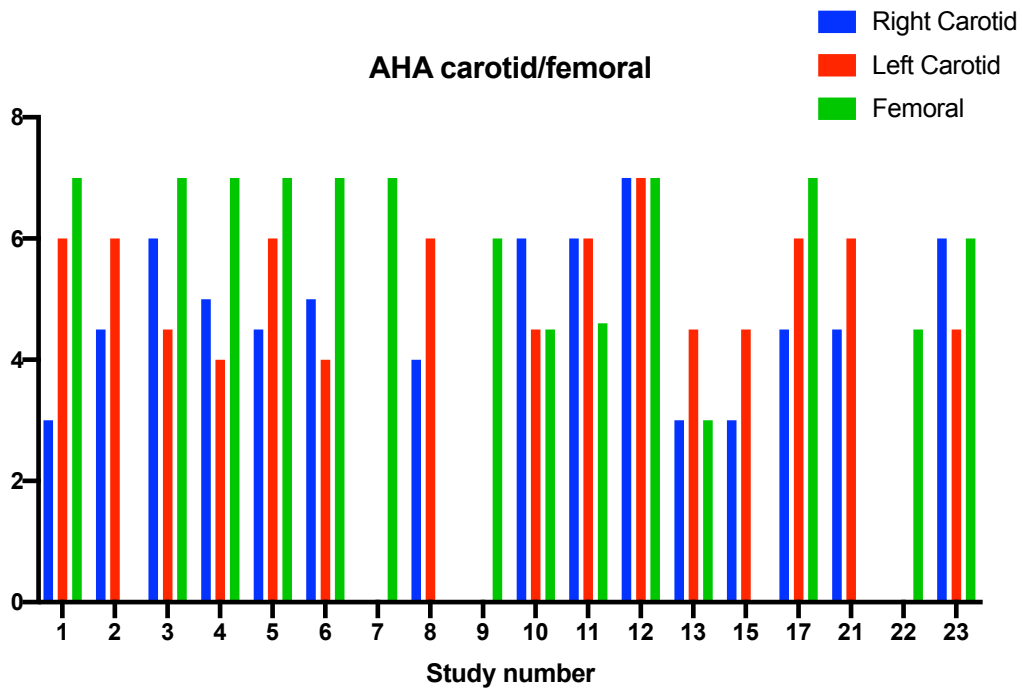
Analysis of the atherosclerotic plaque in vivo yielded some interesting results. Table 9 and Graph 2 show the stage of atherosclerotic disease within each artery according to the AHA

classification of atherosclerosis. Alongside this in brackets is the rating of the quality of the scan as detailed above.

Table 9: In vivo MRI scan for study patient; level of atherosclerotic disease visualised with the carotid and femoral arteries for each patient. Each patient used as their own control.

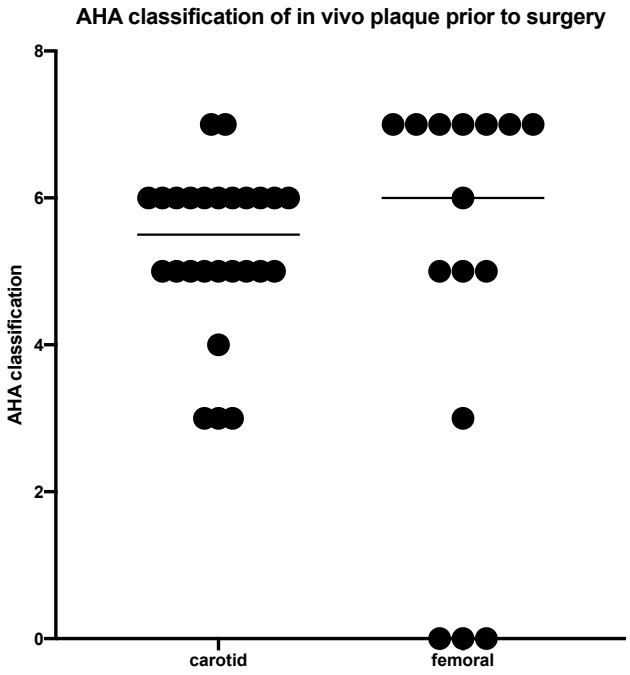
MRI SCAN NUMBER (AC19_001_)	STUDY NUMBER (OXPVD-CF-)	right carotid	left carotid	femoral
1081	**00001**	III (2)	VI (2)	VII (3)
1085	**00002**	IV-V (3)	VI (3)	0 (3)
1086	**00003**	VI (3)	IV-V (3)	VII (3)
1090	**00005**	IV-V (3)	VI (2)	VII (2)
1124	**00006**	n/e	n/e	n/a
1127	**00007**	n/a	n/a	VII (2)
1177	**00008**	IV-V (2)	VI (1)	0 (1)
1180	**00009**	n/a	n/a	VI (2)
1183	**00010**	VI (1)	IV-V (1)	IV-V (1)
1196	**00011**	VI (2)	VI (2)	IV-V (2)
1203	**00012**	VII (2)	VII (2)	VII (3)
1246	**00013**	III (2)	IV-V (2)	III (2)
1260	**00015**	III (2)	IV-V (1)	0 (2)
1270	**00017**	IV-V (3)	VI (2)	VII (2)
1409	**00021**	IV-V (2)	VI (1)	0 (2)
1487	**00023**	n/a	n/a	III (2)
1492	**00022**	n/e	n/e	IV-V (2)
1552	**00024**	VI (2)	IV-V (2)	VI (2)

Graph 2: Stage of carotid / femoral atherosclerotic disease (AHA) for each study patient



Using the above data it was possible to directly compare the AHA stage/major components of presenting disease between the carotid and femoral scans. Results of this can be more clearly compared below in graph 3.

Graph 3: AHA classification from in vivo MRI, comparing carotid atherosclerosis and femoral atherosclerosis

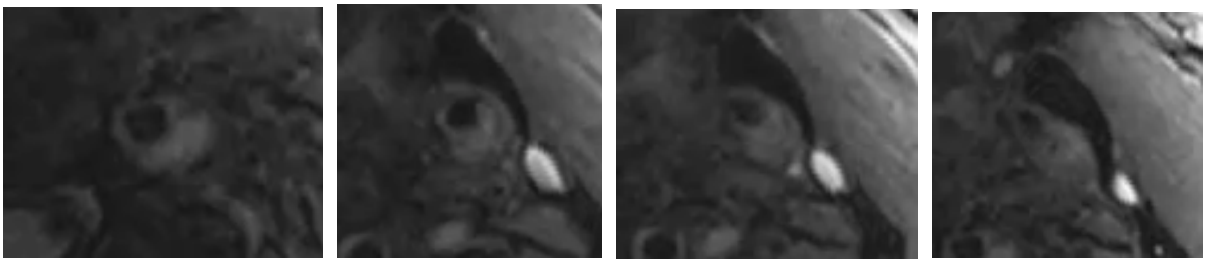


As seen in the table 9 and graphs 2&3, the majority of carotid atherosclerosis tended to fall into AHA categories IV to VI, whereas the femoral atherosclerosis was predominantly AHA VII. Type IV and V being plaque with a lipid or necrotic core surrounded by fibrous tissue, type VI complex plaque with possible surface defect/haemorrhage/thrombus and type VII being calcified plaque.

Interestingly, a significant number (11 participants) were found to have asymptomatic disease in the 'other' scanned region; with most patients presenting for femoral endarterectomy also showing low grade carotid disease, but not all carotid patients showing signs of femoral atherosclerosis on MRI.

5.4 Carotid atherosclerosis

Picture 5: Carotid artery MRI (T2 weighted)



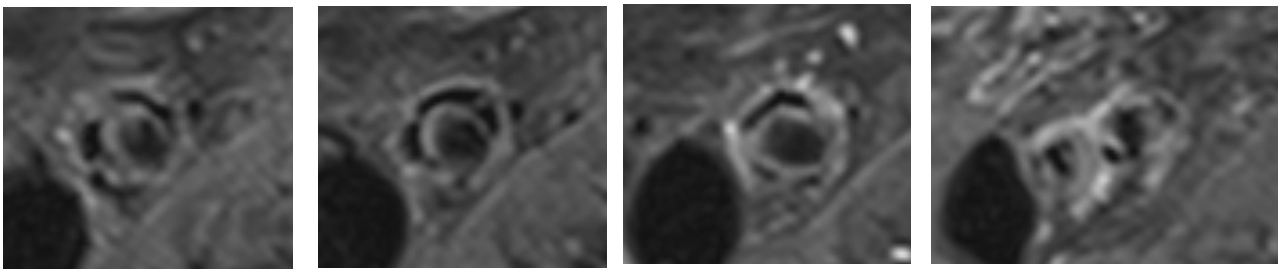
As shown in picture 5, we imaged and visualised the common carotid artery and atherosclerotic plaque using a both a T1 and T2 weighted MRI protocol (T2 pictured). In line with previous publications, the bifurcation tended to be the area of maximal disease burden; consequently, the carotid bifurcation remained the centre point for all carotid scans to ensure the full plaque length was covered.

Consistent with other carotid plaques depicted in the literature (106), the atherosclerotic plaques appeared to thicken the arterial wall, and often on a single side, rather than in a concentric pattern (see above).

Published from our collaborating group, and shown in figure 6, this area of 'light grey' between the vessel wall and the artery is fibrous tissue, fat and intra plaque haemorrhage (105). It has also been shown that this area is more likely to be symptomatic with a high lipid content (or lipid index) (105).

5.5 Femoral atherosclerosis

Picture 6: Femoral artery MRI through serial images



With limited literature, and no prior experience on imaging the femoral arteries for atherosclerosis (without contrast), this method was initially trialled on a volunteer (KH) to ensure adequate pictures of femoral arteries could be attained during the scanning process.

As can be seen from our results, using an In vivo 32 channel phased array coil over the participants pelvis, and methodology as described in the methods section; the femoral vessels and associated plaques were clearly visualised.

Interestingly however, as shown in picture 6, femoral artery atherosclerosis did not depict in the same way as carotid disease. Plaque within the carotid arteries tended to be light grey as described above, whereas in the majority of the femoral scans there appeared to be a black area between two rings of 'white', visually like a double halo.

5.6 Control data from patients with triple vessel coronary disease

Atherosclerosis is learned as a triad of disease; coronary, cerebrovascular and peripheral. Although the main purpose of this study was to compare carotid and femoral disease, we felt it pertinent to also include some coronary patients. Although patient recruitment was halted in 2020, we were still able to recruit two patients. One of which had signs of femoral disease (AHA IV), and significant carotid disease (AHA VI). The second had no obvious femoral disease and mild carotid disease (AHA III).

It is clear from this and the data above, that patients do not build atherosclerotic depositions uniformly in different arterial beds. Why are certain areas chosen for some patients and not others?

5.7 Comparative analysis of atherosclerotic plaque burden; carotid endarterectomy patients vs femoral endarterectomy patients

This part of the study has clearly shown that it has been possible to visualise atherosclerotic plaque within the femoral vessels with enough clarity to distinguish the extent of disease and classify using the AHA atherosclerosis classification. Our collaborating group have previously imaged carotid plaque, but this study has allowed for replication of this within the femoral vessels and consequent comparison between participants carotid and femoral vessels.

Two clear result sets are available from this data; the extent of disease (either carotid or femoral) from symptomatic patients, and extent of disease in other vascular beds with each participant acting as their own control.

All carotid imaging scans were also used by our collaborating group to review ways of reducing motion artefact during magnetic resonance scanning. These results have been published in the following papers;

Frost, R et al. Navigator-based reacquisition and estimation of motion-corrupted data: Application to multi-echo spin echo for carotid wall MRI. Magn Reson Med. 2020; 83: 2026– 2041.

De Buck M et al. Novel 10-Channel Phased-Array Coil Design for Carotid Wall MRI at 3T. PLoS One. 2023 Aug 9;18(8)

5.8 Discussion

The results of this chapter show obvious differences between the classification of atherosclerosis found within the carotid and femoral vessels prior to endarterectomy. As seen in Graph 2 and 3, the majority of atherosclerotic plaque is AHA IV, V or VI; results which are replicated within the literature for both symptomatic and asymptomatic carotid disease.

Interestingly, the majority of femoral disease is seen as AHA VII. Also known as calcified, or more stable plaque.

Graph 2 shows the spread of atherosclerosis throughout different arterial beds. Those participants with atherosclerotic deposition in one area (carotid or femoral) tended to show disease in the other area. However, again, disease within the carotid was majority AHA III – VI and the majority of femoral disease V – VII.

As depicted in picture 5 and 6, the presentation and distribution of the plaque on MRI also differed between carotid and femoral images. The carotid plaque (picture 5) appeared to show a narrowed lumen with surrounding grey/white matter, often more plaque on one side of the artery. With the femoral plaque (picture 6) there was more of a concentric narrowing with two distinct white circles, and area of black in between them.

With what appears to be constitutional differences, as well as depositional variation between femoral and carotid plaque, further comparative analysis was continued.

5.9 Conclusions

It is evident from the results in this chapter that although participants with atherosclerotic predisposition did indeed have disease (most often) in more than one area, the type of atherosclerotic disease present within the femoral arteries was more often calcified and

deposited in a concentric manor. Disease of the carotid artery was complex, predominantly lipid based, with haemorrhage, and showed fairly significant one sided arterial narrowing.

6. Chapter 6: Histological and radiological analysis of carotid and femoral atherosclerotic plaque

6.1 Introduction

Histology is a longstanding traditional technique used to review biological tissue; and consequently, an important part of this study.

Atherosclerotic deposition from the carotid artery is fairly easily attained and its architecture has been the primary study area for many groups. As this work is based on the comparison of carotid and femoral atherosclerosis, all methods were carried out on both sets of plaque (carotid and femoral), with histological analysis being one area of assessment.

Although the primary issues encountered with femoral sectioning and staining have been detailed previously, the histological results that were achievable are important and warrant discussion. In light of femoral sectioning issues, other methods of plaque evaluation were sought; to confirm and extend our histological analysis.

Following a detailed literature search, Micro CT and micro MRI, although not regularly used for human atherosclerotic analysis, were felt to be the most appropriate methods to assess plaque components and three dimensional structure.

6.2 Aims

The aims of this chapter were to provide microscopic analysis of both femoral and carotid atherosclerotic plaque, to aid in overall comparison of the two specimens.

A secondary aim was to evaluate the use of micro CT and micro MRI in atherosclerotic plaque quantification, and with results provide quantitative comparisons between carotid and femoral atherosclerosis.

6.3 Histological analysis of atherosclerotic plaque

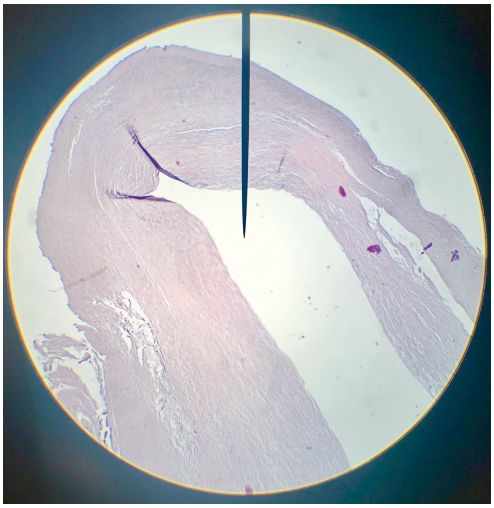
Histological analysis was initially completed on the first 13 specimens enrolled in the study. Plaque was embedded, sectioned and stained as detailed in our methods. Participants 14 and 15 were cardiology patients undergoing coronary artery bypass grafts. These patients were enrolled for in vivo assessment of their carotid and femoral arteries, no plaque was removed.

As detailed shortly, all plaque from participant 6 onwards were subject to micro CT. Following this, the specimens were sectioned and stained.

Upon review of the preliminary results and research into other methods of whole specimen analysis; plaques 16 onwards were kept in storage for imaging with micro MRI. Taking into account availability of the scanner and optimisation of the micro MRI protocol, MRI imaging

was only able to be performed as the coronavirus lockdown was commencing. Therefore following MRI imaging, plaques were placed in storage for the entire of the national lockdown. Only after this date could they be sectioned and stained.

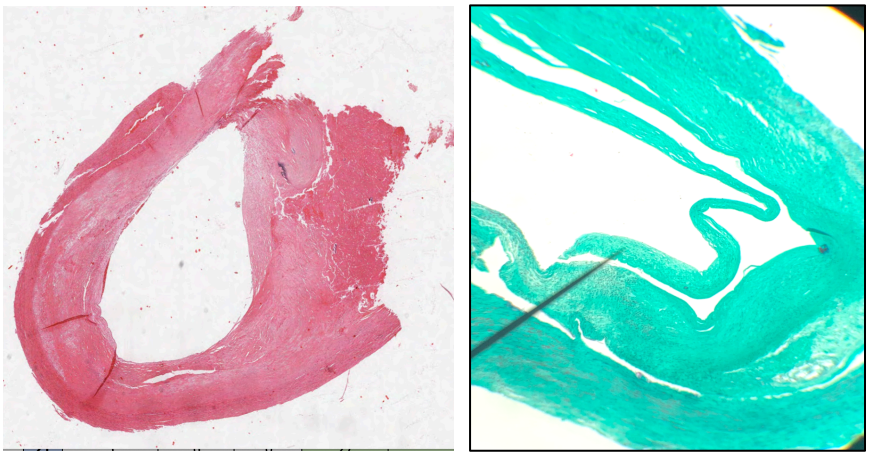
Sections of both carotid and femoral atherosclerotic plaque were used for analysis. Carotid specimens were cut at regular 5mm intervals throughout to achieve ordered paraffin blocks. Femoral plaque was removed from the patient in small sections, and each fragment fixed individually into paraffin. All tissue sections were cut at 4-µm thicknesses, mounted on Superfrost+ glass slides and stained with hematoxylin and eosin, massons trichrome and von kossa stains.



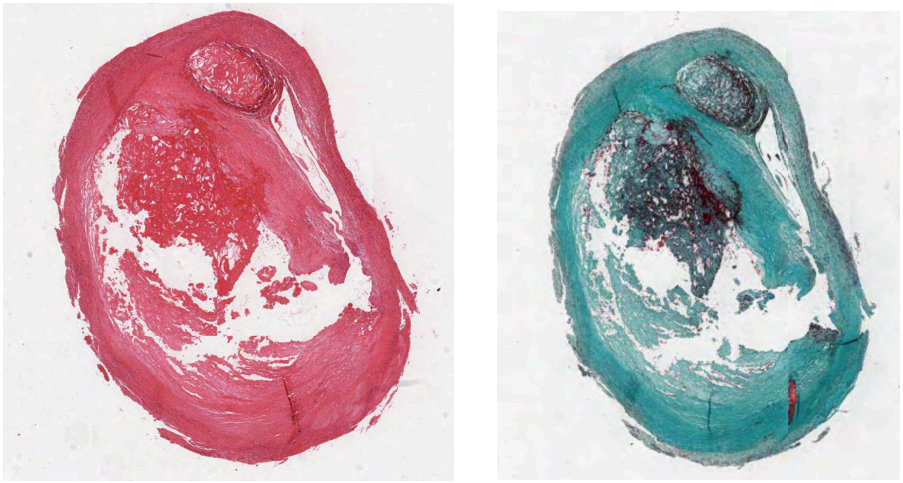
Picture 7: Haematoxolin and eosin (H&E) staining of carotid atherosclerosis

Picture 7 shows a cross sectional H&E stain of the carotid intima, taken at carotid endarterectomy, showing clear signs of atherosclerosis as detailed in the literature;

pathological intima thickening, lipid pools and reduction in intimal nuclei. These histological sections were attained and replicated throughout the carotid specimens within the study.



A B

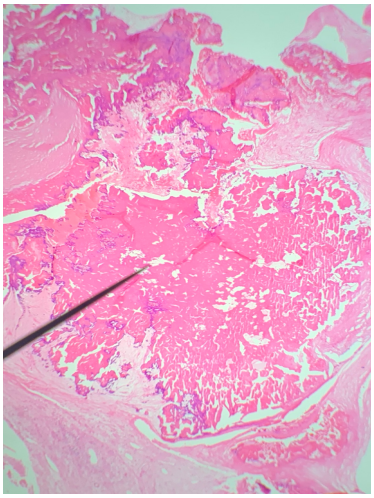


C D

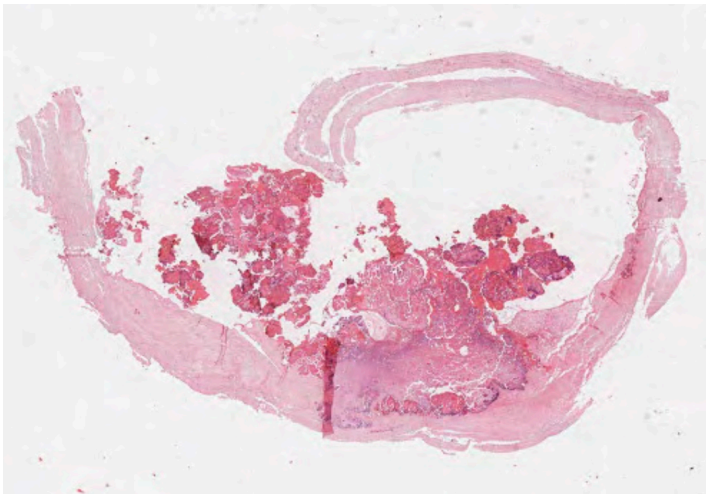
Picture 8: Histological staining of carotid atherosclerotic plaque (A: H&E staining showing intimal thickening with an area of fibrocalcification, B: Masson trichrome of section A, C: H&E staining showing area of intraplaque haemorrhage, E: Masson trichrome of section C),

As depicted in picture 8, the carotid sections successfully mimic the literature with both H&E and masons trichrome; even including the variations described with different AHA classifications of plaque; intraplaque haemorrhage, necrotic core, lipid pools (seen in C and D). These results were extremely reassuring and provided encouragement for further histological work.

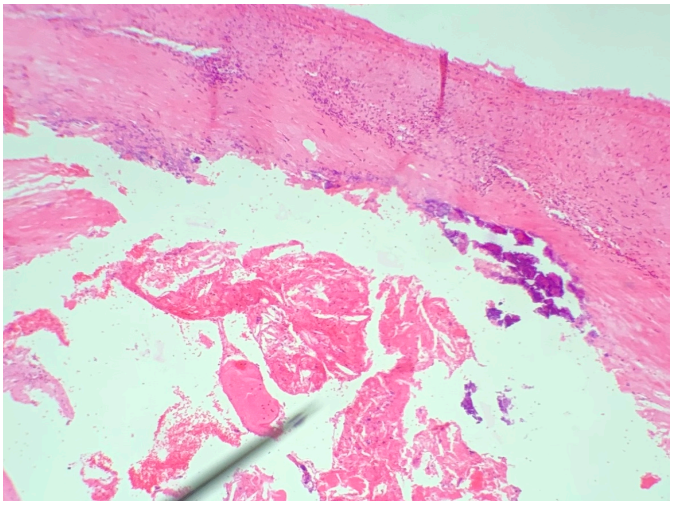
Picture 9: H&E staining of sections through femoral atherosclerotic plaque



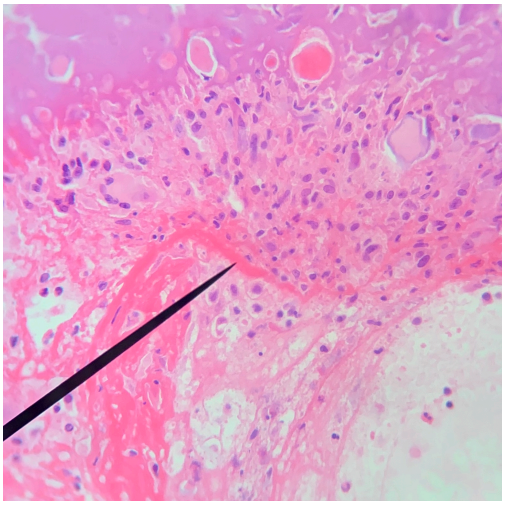
A



B



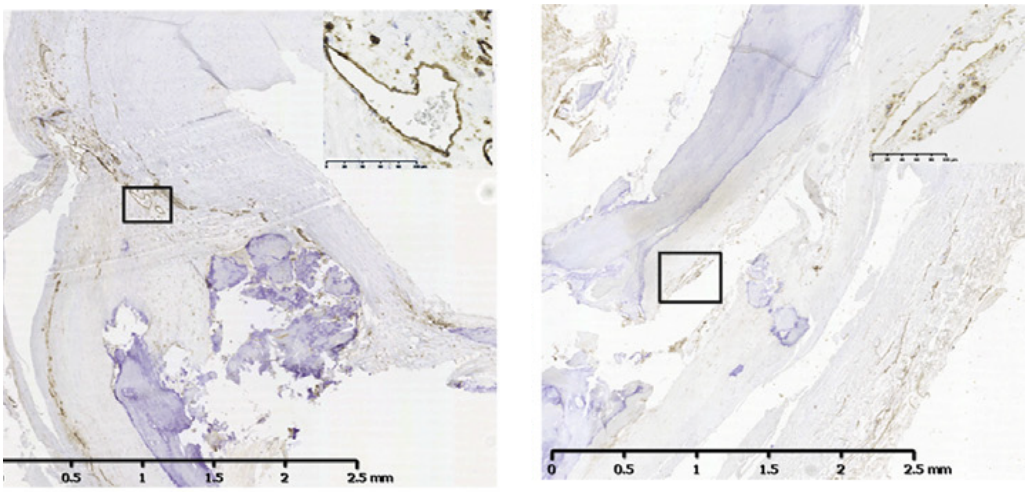
C



D

Picture 9 shows H&E staining of transverse sections of femoral atherosclerotic plaque. Each tile representing a different specimen enrolled into the study. Slides appear to show inter-luminal deposition of material, alongside standard atherosclerotic intimal changes of the intima.

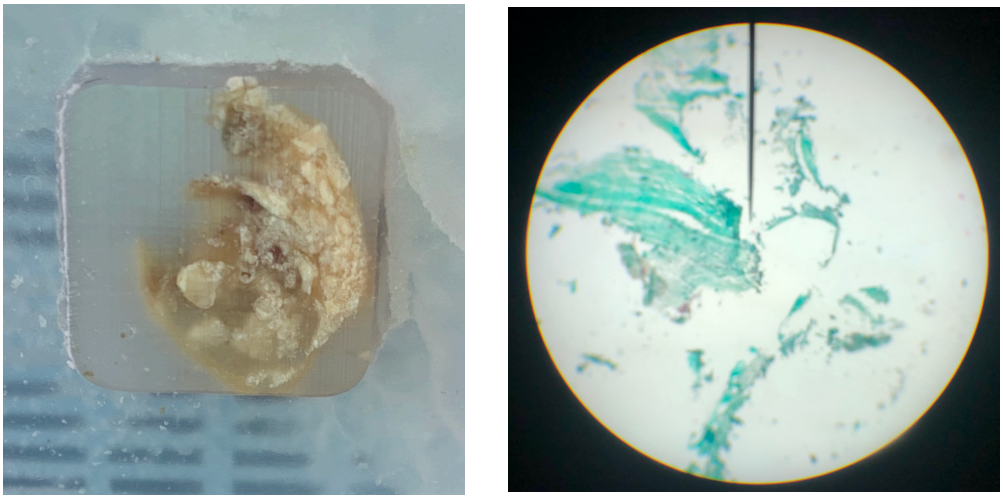
These images also correlate to those (few) seen in the literature, highlighting that we have managed to reproduce published results.



Histopathology images of femoral artery atherosclerosis taken from M. Davaine et al. Bone Like Arterial Calcification in Femoral Atherosclerotic Lesions: Prevalence and Role of Osteoprotegerin and Pericytes. European Journal of Vascular and Endovascular Surgery, Volume 51, Issue 2, 2016, Pages 259-267 (130).

As previously discussed, it was extremely challenging to gain repeated sections of femoral plaque due to its composition and fragility when passing through the microtome. Picture 10A

shows the macroscopic composition of the plaque mounted in paraffin, and 10B details the fractured, unusable slides attained during the sectioning process of some specimens.

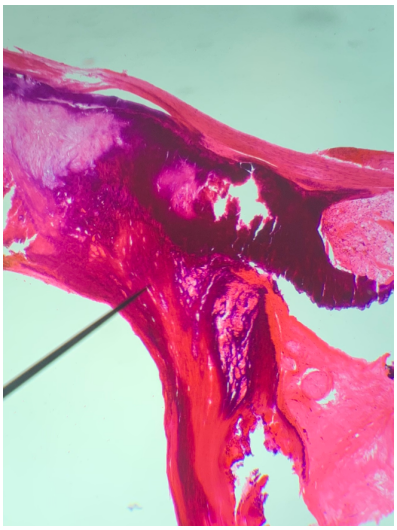


A B

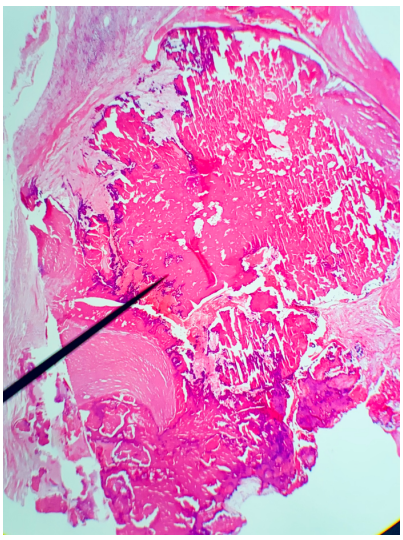
Picture 10: A - Femoral plaque set up on microtome, mounted in paraffin.

B – masons stain of femoral plaque section, fragmented and not sticking to slide

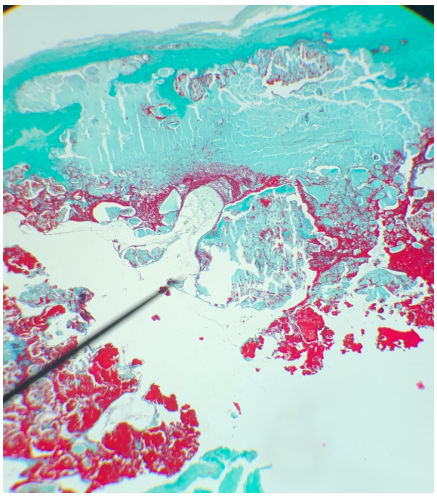
From the femoral sections attained, there were some important results to mention. Pictures 11A and 11B highlight successful H&E staining with areas of calcium (darker/purple stain) and fibrin deposition can be seen.



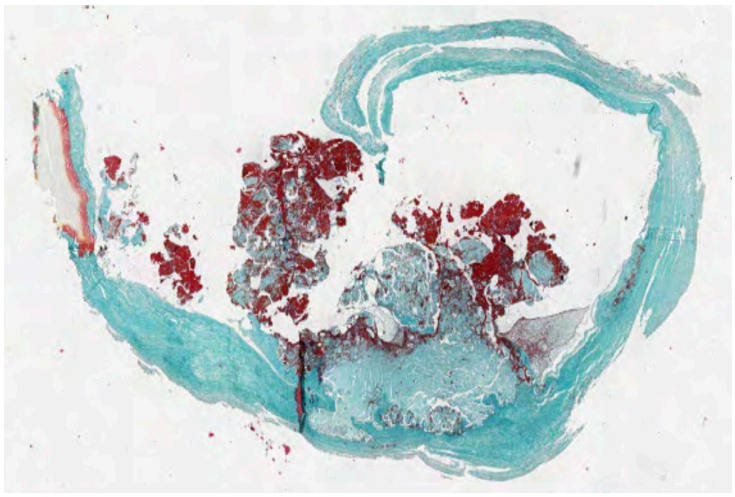
A



B



C



D

Picture 11: Histopathology specimens from femoral atherosclerotic plaques;

A and B - H&E, C and D – Massons trichrome.

Tiles C and D show femoral atherosclerosis with masons trichrome; highlighting areas of fibrin deposition in red and calcium white. Confirmation of these results can be seen below in the literature (figure 10).

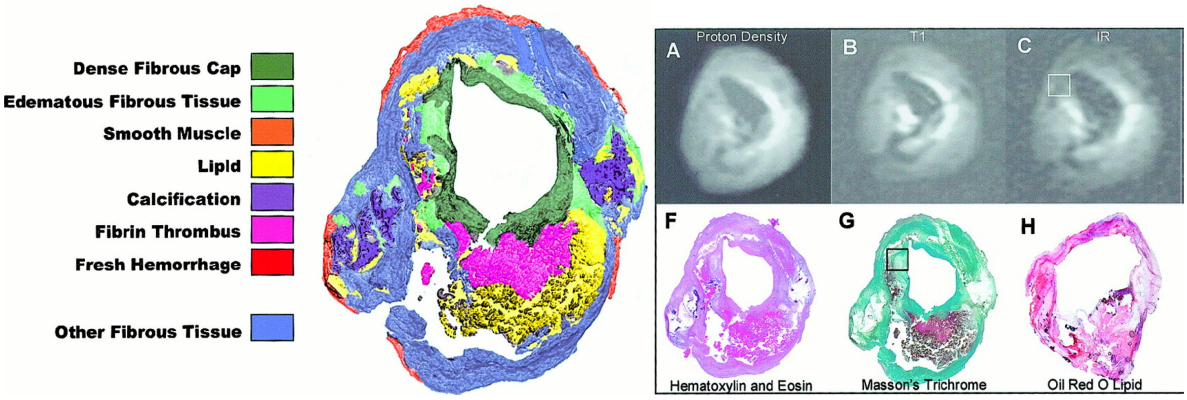
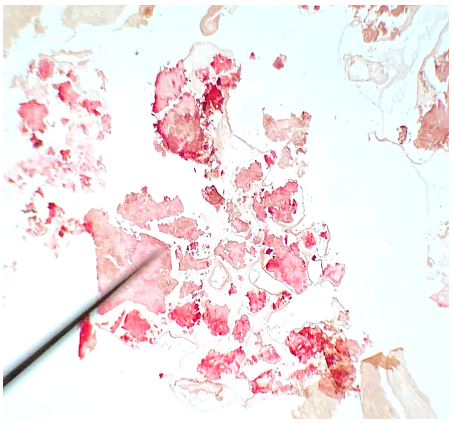
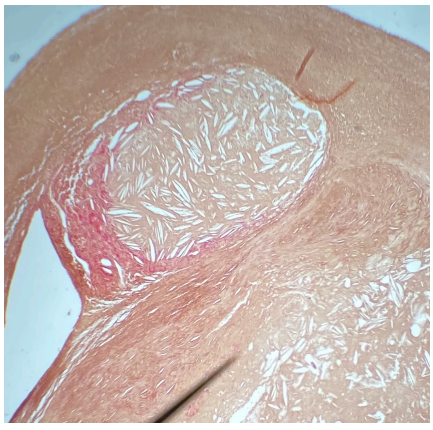


Figure 10: A color-coded digital parametric image was created by mapping the various histological components of the atherosclerotic endarterectomy cross section. Rogers W, et al. Arteriosclerosis, Thrombosis, and Vascular Biology. 2000;20:1824–1830

Although the process of staining with Von Kossa was slightly more challenging and intensive than H&E and Massons shown above, we were able to achieve some images (picture 12) with calcium labelled in red.



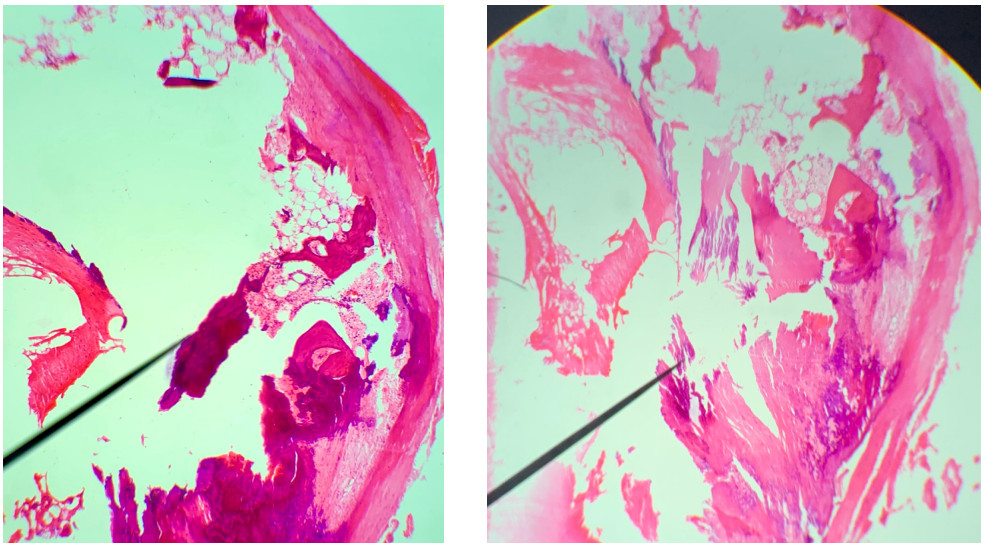
A



B

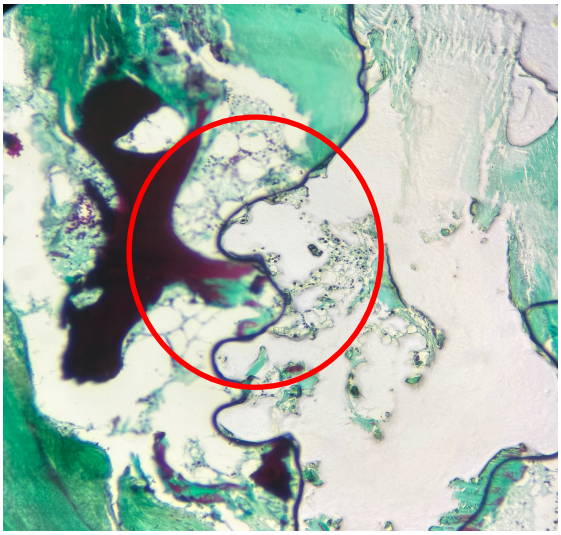
Picture 12: Von kossa staining of femoral (a) and carotid (b) plaque

Although these results provide interesting detail into the components and architecture of femoral atherosclerotic plaque, the following pictures are arguably of the greatest importance.

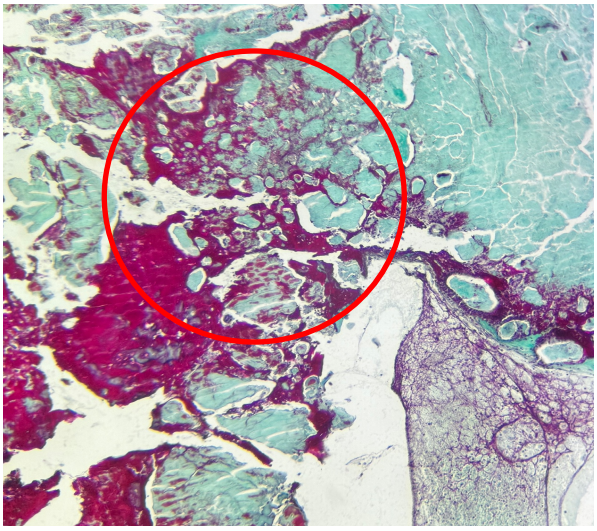


Picture 13: osteoblastic activity within femoral plaque

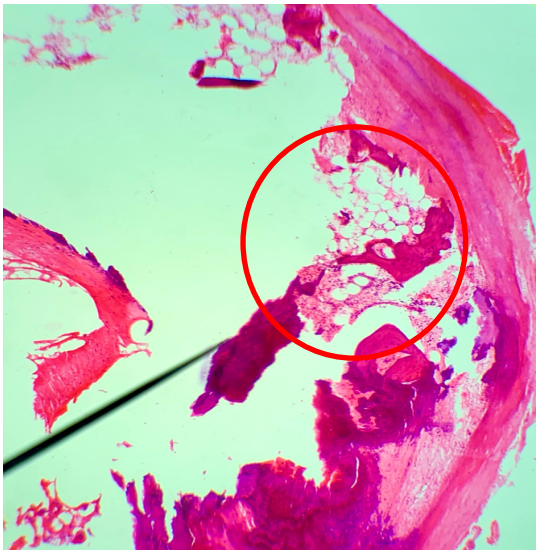
All histopathology slides were reviewed alongside a consultant histopathologist (E.F), to provide learning and give greater experiential knowledge to the results.



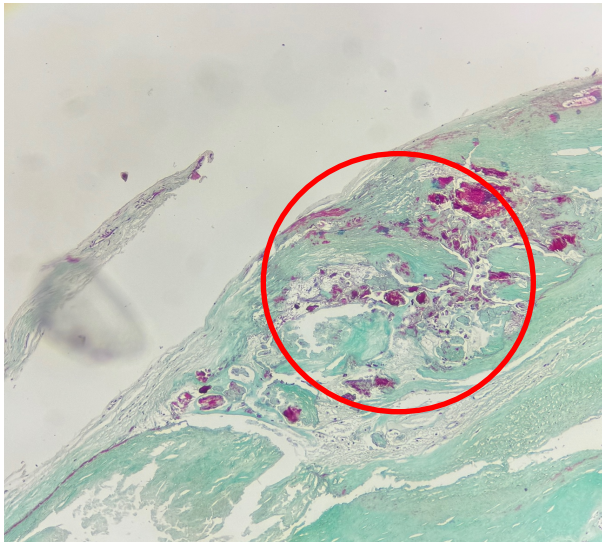
A



B



C

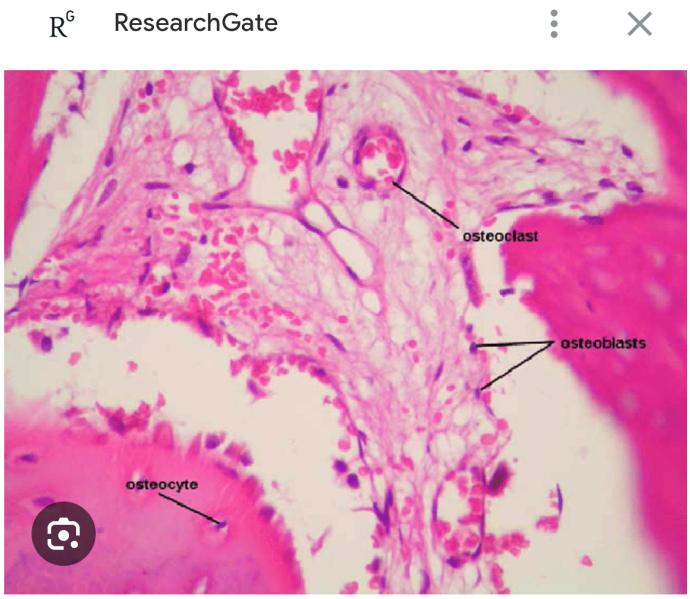


D

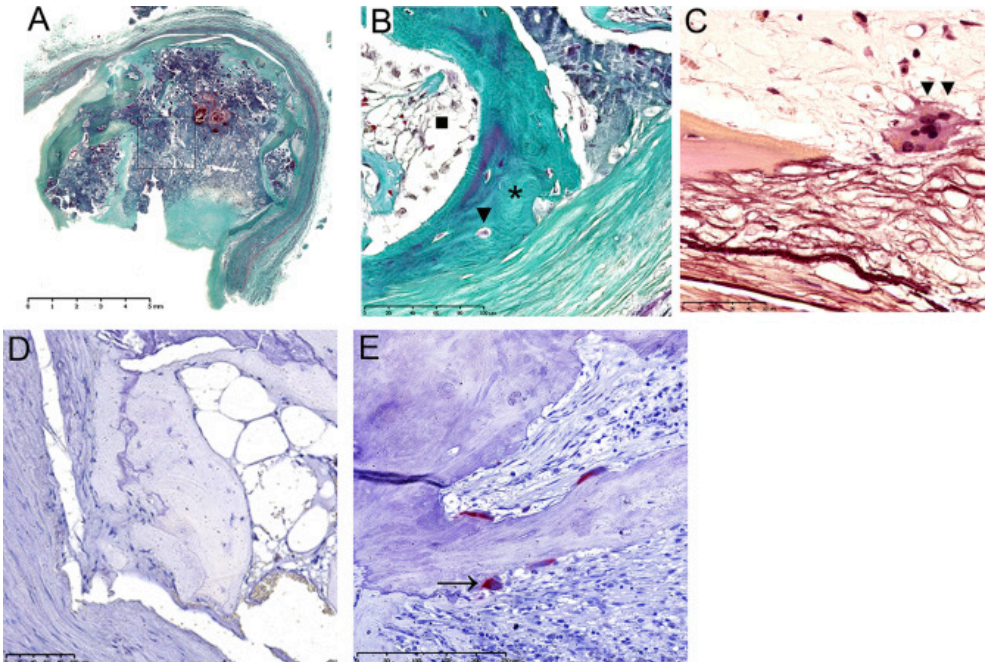
Picture 14: Different femoral specimens with areas of possible osteoblastic/ostoclastic activity.

It could be seen in four of the six sectioned and stained femoral specimens (Pictures 13 and 14), that small rounded 'bubble' like features were apparent. These were confirmed by E.F as osteoblastic/osteoclastic type cells; usually seen in bone sections.

For reference, please see bone ‘osteogenesis’ visualised with a light microscope.



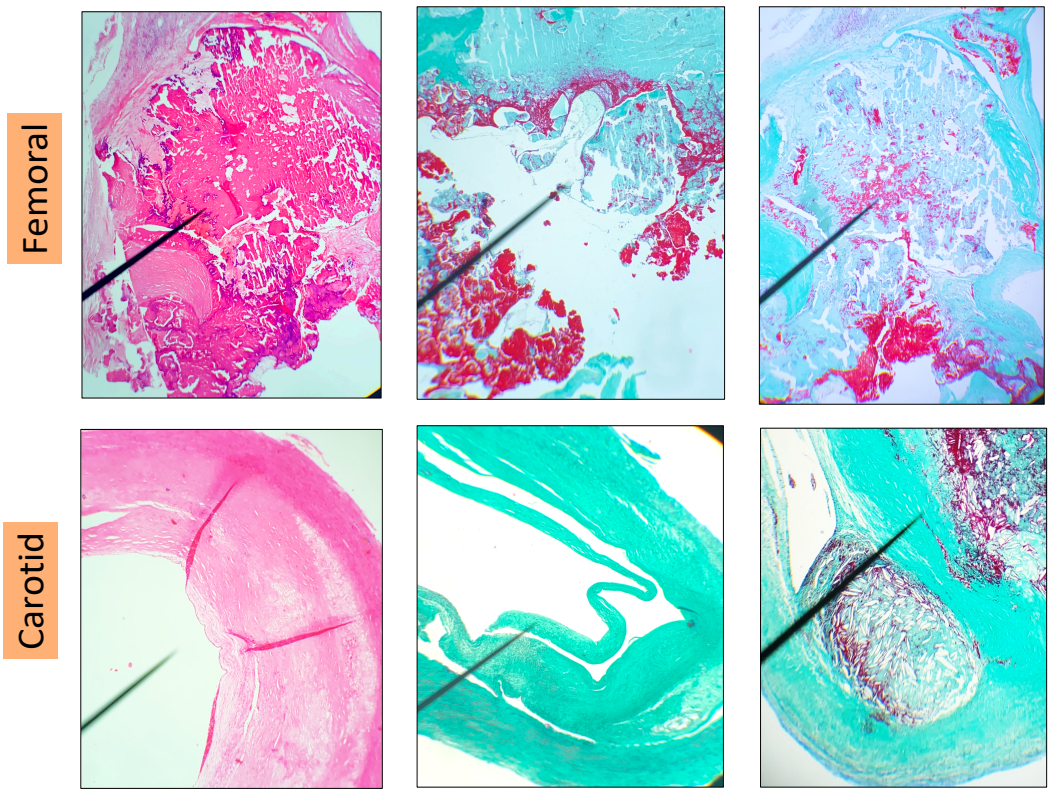
These results have been seen in one other paper in the literature; highlighting ‘bone like calcification in femoral atherosclerotic lesions. Results from Davaine at al. are shown below.



(A) Representative section of a calcified femoral artery lesion. (B) Magnification of the area of osteoid metaplasia (OM) with a dense extracellular matrix (*), osteocytes (▾), and bone marrow (▪) (Masson's trichrome). (C) Magnification of a lesion featuring an osteoclast like giant multi-nucleated cell (▼▼), in the vicinity of OM (*) (HE staining). (D) Representative section of femoral lesions with OM showing the absence of tartrate resistant acid phosphatase (TRAP)+ staining. (E) Representative section of positive TRAP staining (→).

Davaine et al. Bone Like Arterial Calcification in Femoral Atherosclerotic Lesions: Prevalence and Role of Osteoprotegerin and Pericytes. European Journal of Vascular and Endovascular Surgery, Volume 51, Issue 2, 2016, Pages 259-267.(130)

6.3.2 Comparative analysis of histological staining (carotid and femoral atherosclerotic plaque)



Picture 15: Comparative histological analysis of carotid and femoral plaque

As detailed there appears to be some obvious differences between the histological specimens of carotid endarterectomy and femoral endarterectomy. Picture 15: femoral slides highlight a highly disorganised, complex structure. There is marked fibrin deposition and areas of dense and defined calcium deposits. Within the carotid slides there is obvious intimal thickening, fatty deposition and intraplaque haemorrhage.

Despite many attempts to gain repeated and reliable histopathology results, there remained issues with consistent femoral sectioning and staining for all specimens. As comparative

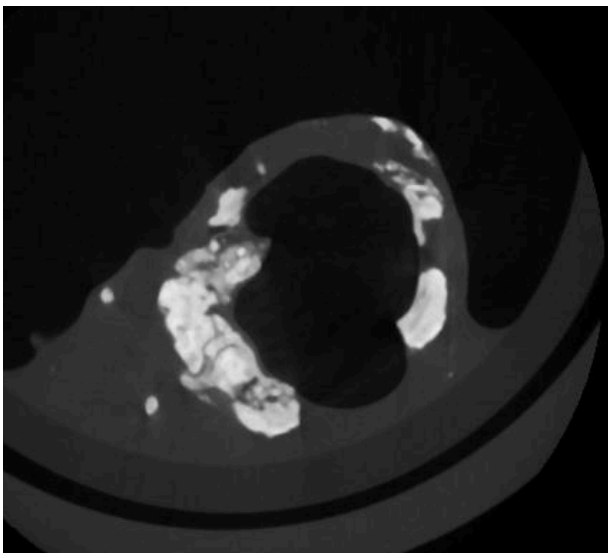
differences of carotid and femoral atherosclerotic plaque were evident even at a macroscopic level, further imaging methods were used to review and quantify differences between atherosclerosis deposited in different areas.

6.4 Ex vivo analysis of atherosclerotic plaque using (micro) computerised tomography

Micro computerised tomography (micro CT) is a non-destructive imaging tool using serial two dimensional x-rays to produce a three dimensional image. It has been widely used within the biomedical field to visualise bone and cartilage as well as cardiovascular structures. It was felt that imaging the specimens in this way would detail both plaque components and architecture.

Specimens were removed from each patient and taken to the laboratory on ice. Following washings, specimens were placed in PBS and transported to the CT scanner. Prior to scanning all PBS solution was removed, and the specimen scanned in an empty falcon.

As seen below, the images produced by the scanner were clear and resourceful. Using FIJI Image J it was possible to scan through the entire plaque in sequence, and review areas of interest.



Picture 16: Micro CT of carotid specimen

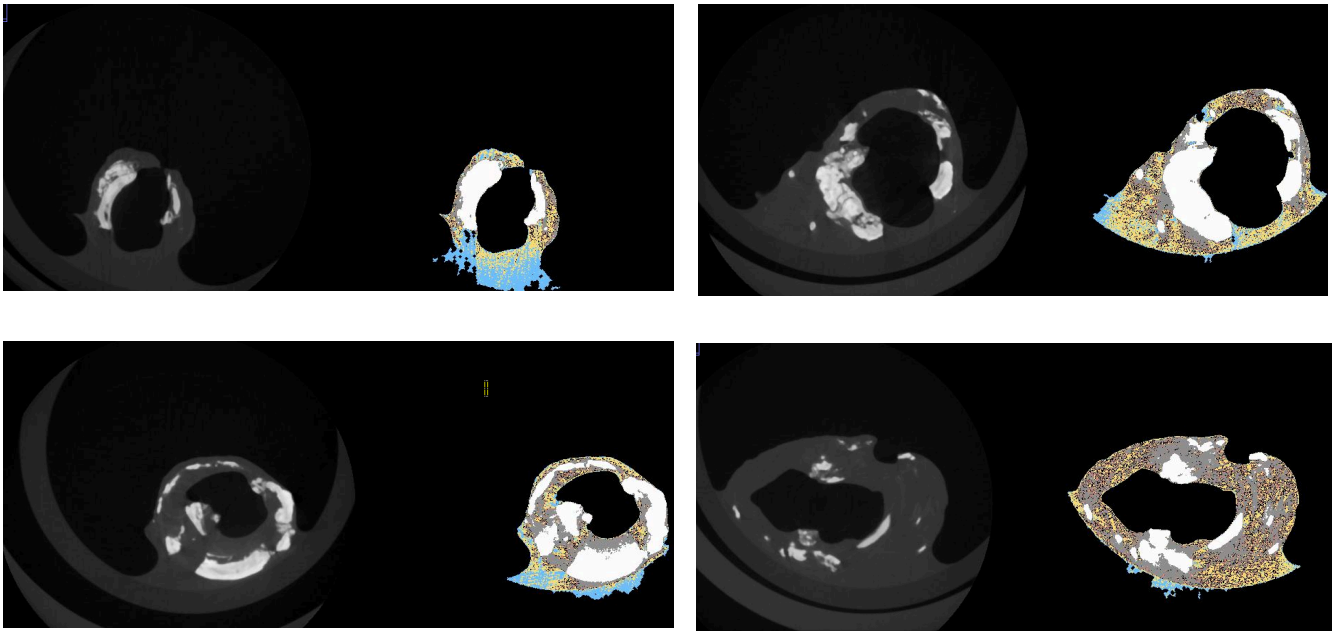
With the help of a colleague with expertise in computer coding, it was possible to use the hounsfield units (HU) supported by the literature (below) to segment each of the main constituents of the plaque, and colour code each region for visual discrimination. This coding also allowed for subtraction of the falcon tube and any residual PBS droplets from the image prior to analysis.

- Calcium with HU > 300
- Grey Area with HU between 45-300
- Blood with HU between 30-45
- Fat with HU between -100 to 0
- Soft Tissue with HU between -300 to -100.

As can be seen in the following subsections, key constituents of the plaque were identified, and with the data, percentage compositions and comparisons were achieved.

6.4.2 Carotid atherosclerosis

Picture 17: Cross-sectional micro CT imaging of individual carotid plaques enrolled in the OxPVD study



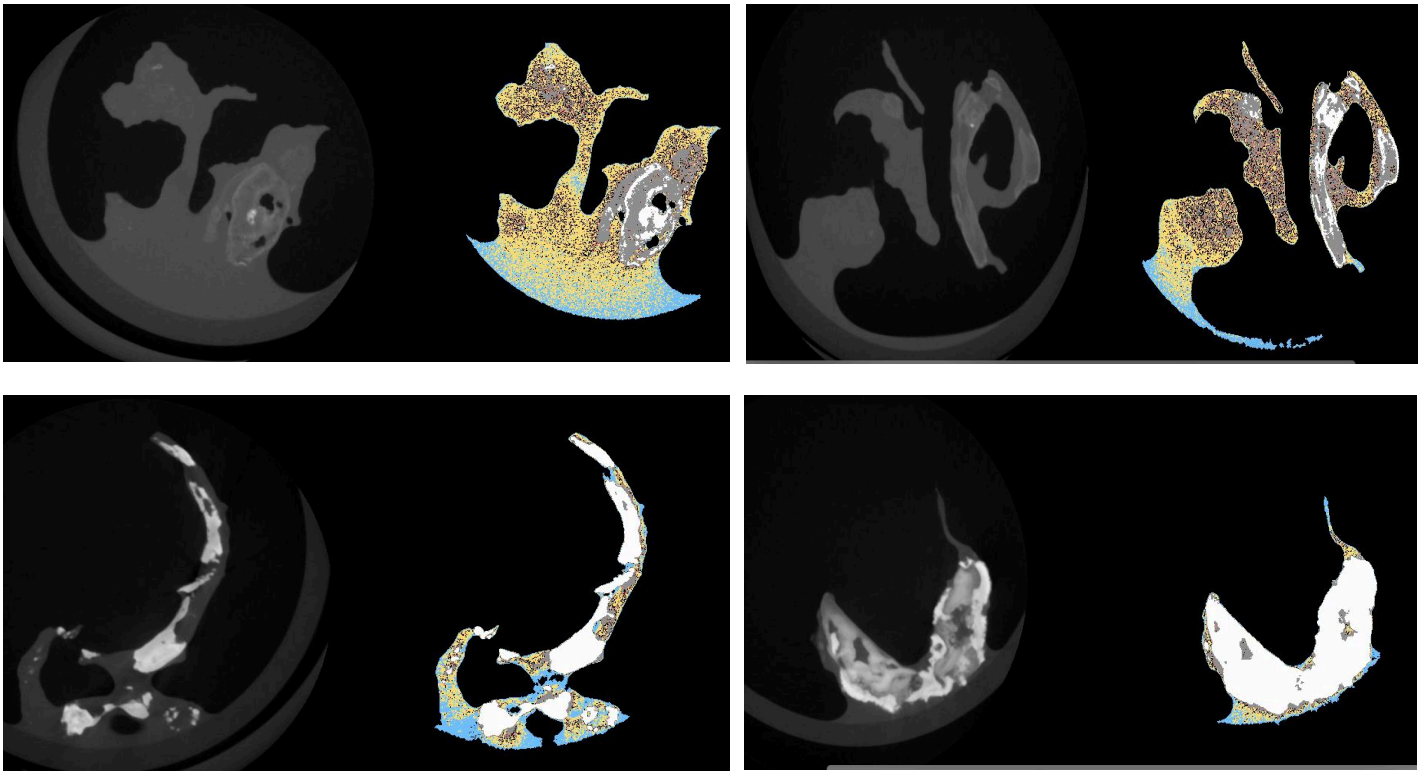
Each carotid specimen was scanned in its entirety. Using the hounsfield units listed above, atherosclerotic plaque components were identified.

Using the total number of voxels for each plaque as the denominator, it was possible to work out the percentage of each component within each plaque.

6.4.3 Femoral atherosclerosis

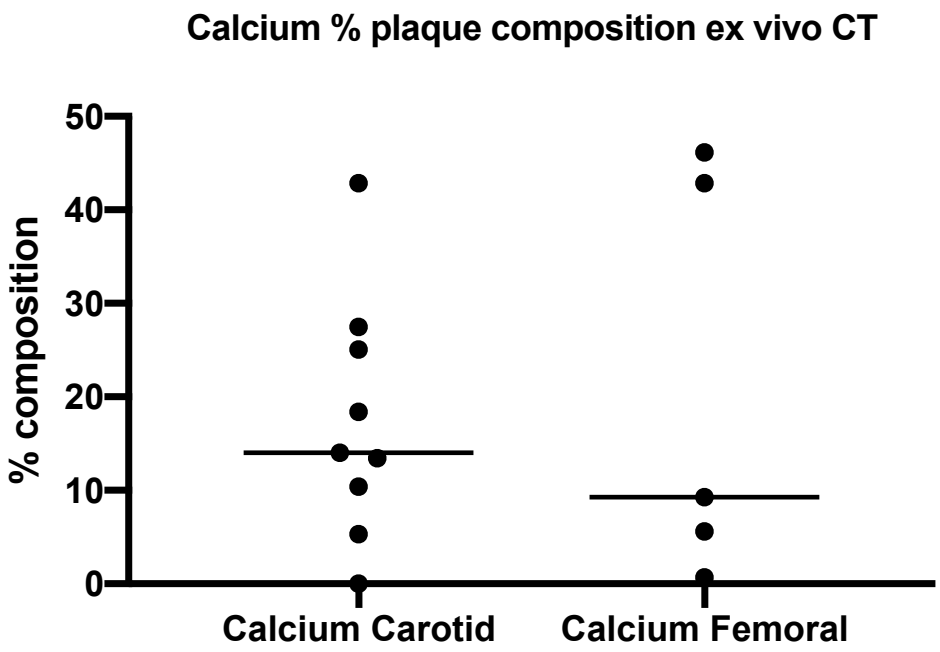
This process was repeated for all femoral plaques. Images shown in Picture 18.

Picture 18: Cross-sectional micro CT imaging of individual femoral plaques enrolled in the OxPVD study



Using the data achieved, it was possible to perform detailed analysis on the plaque components, the percentage composition and give three dimensional assessment to the area of stenosis.

Graph 4: % of calcium within carotid and femoral plaque

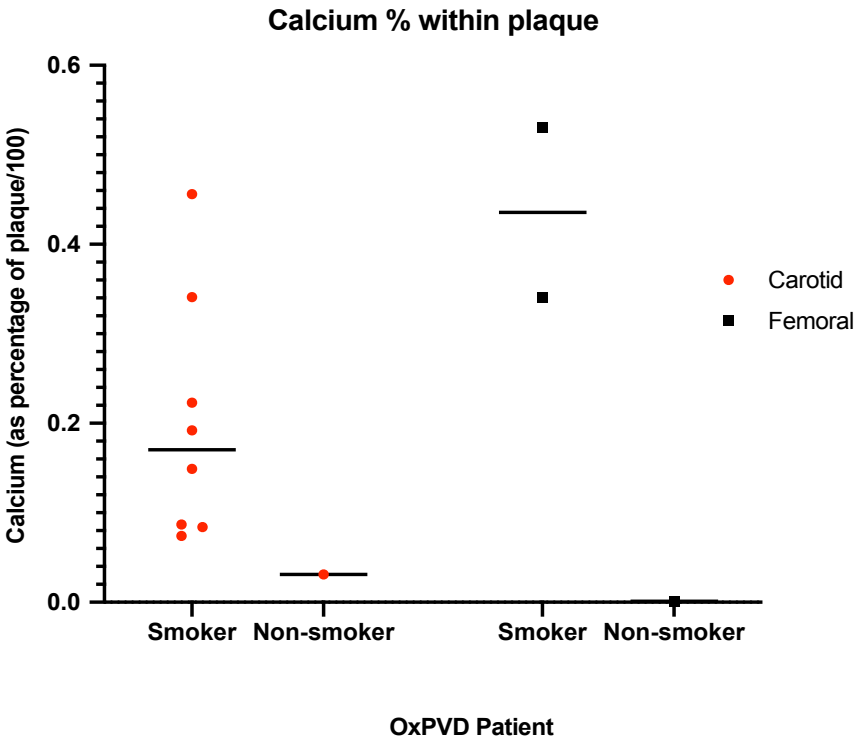


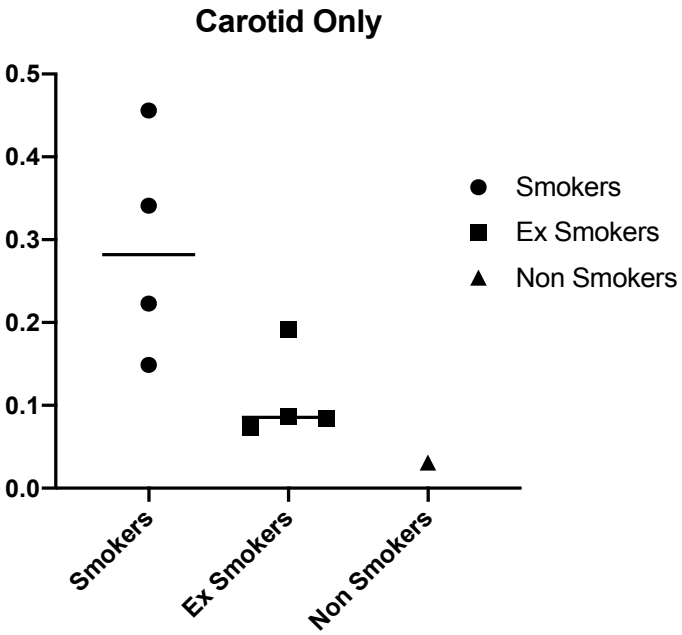
One of the first quantitative measures reviewed was percentage of calcium within each of the plaques. As we had experienced difficulty in sectioning femoral plaque we had expected the amount of calcium (highlighted with von kossa staining) within femoral atherosclerotic plaque to be significantly higher. This in fact was not the case.

There was a range from minimal amounts to around 40% calcium within the carotid plaque, and quite a split between the amount of calcium within the femoral plaque; 10% for some and approximately 50% in others. These results can be seen in graph 4.

With this in mind, we reviewed patient demographics, e.g age, smoking status, risk factors for each of the calcium percentages to see if there was any correlation.

Graph 5 A&B: % of calcium in relation to smoking habits





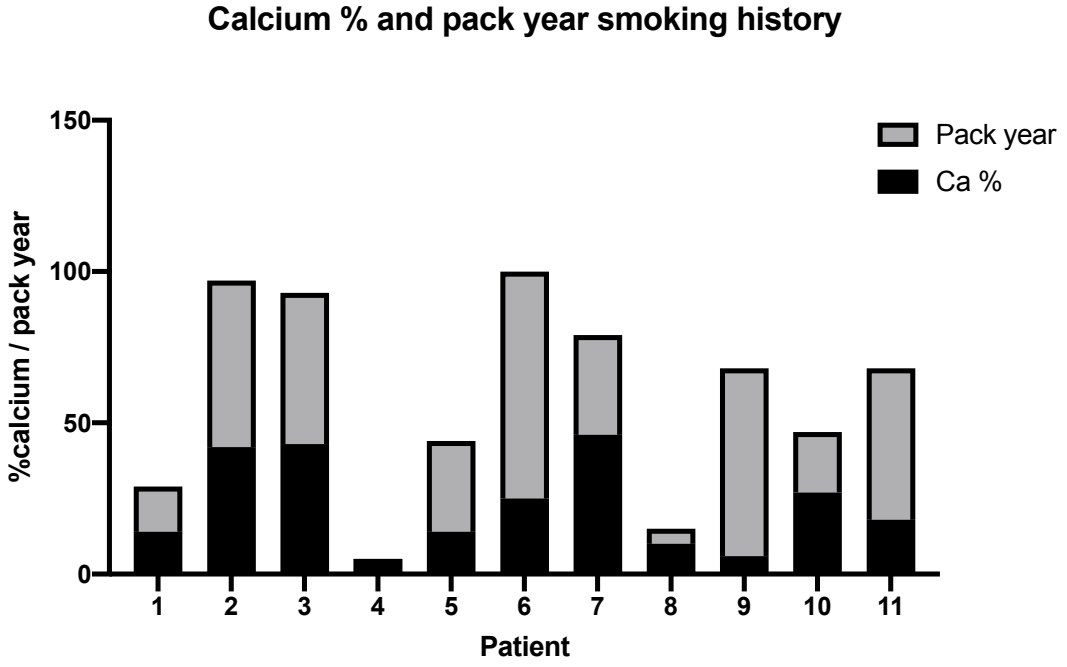
When analysing plaques for smokers and non-smokers, it was clear to see that the amount of calcium found within the plaque was significantly higher in the smoking groups when

compared to the non-smokers. This was evident in both carotid and femoral disease, as seen in graph 5A.

Unfortunately, there were not enough femoral samples to allow for adequate comparison between groups with varying demographics, but as seen in graph 5B; the percentage composition of calcium within the carotid plaque significantly increased in ex-smokers and smokers.

Interestingly, for both carotid and femoral disease, the percentage of calcium within the atherosclerotic plaque strongly correlated to the amount of pack years smoked, as shown in graph 6.

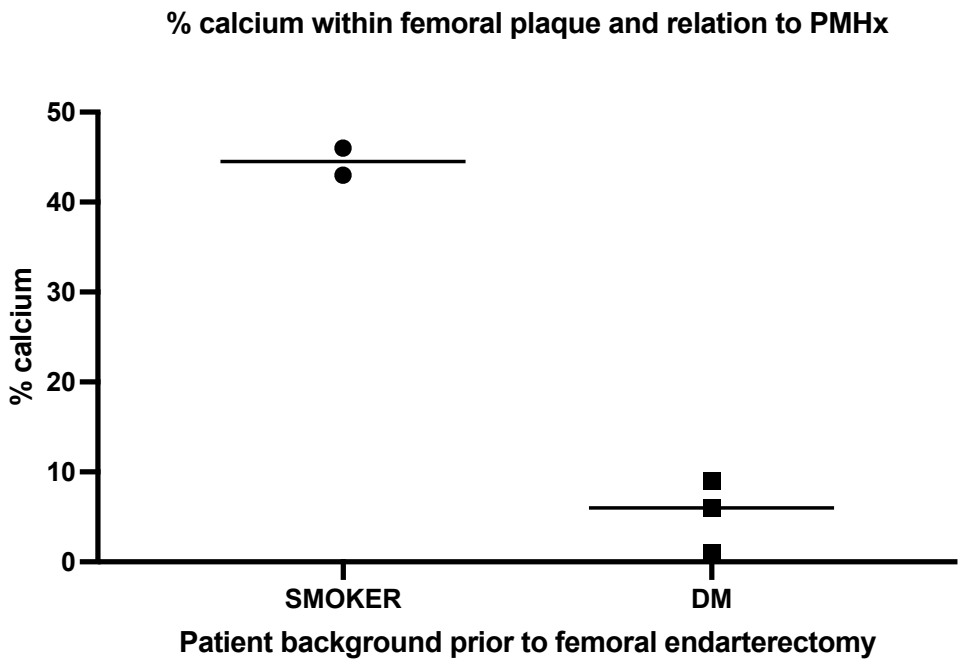
Graph 6: % of calcium and smoking history



Upon review of the femoral endarterectomy participants' past medical history, one clear and significant result was that those who were smokers (and this the main risk factor for their

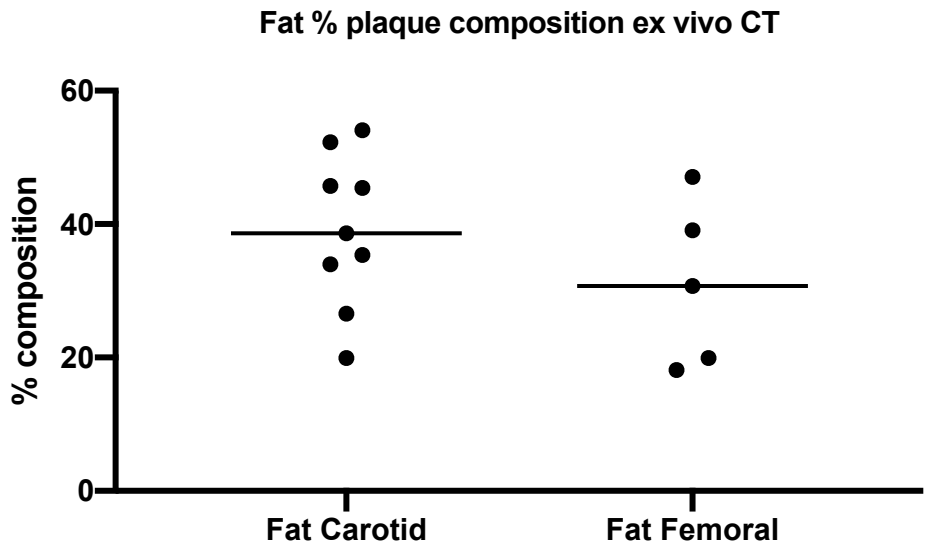
POAD) had a high percentage of calcium within their plaque, and those with diabetes mellitus (the risk factor for their PAOD) tended to have very low calcium composition (graph 7).

Graph 7: % of femoral plaque calcium and past medical history

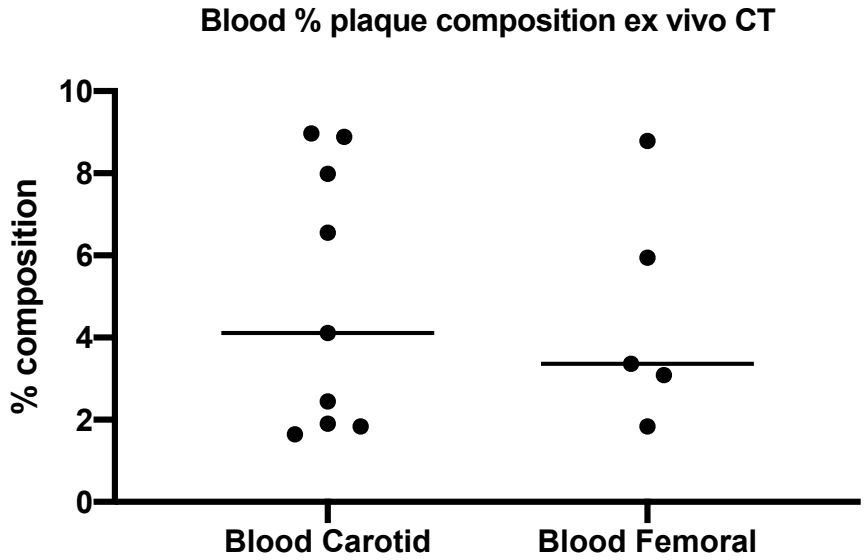


The rest of the plaque composition was analysed for both carotid and femoral, as a percentage of the total plaque volume. As can be seen in graphs 8, 9 and 10, the other plaque components were fairly similar in amount between the two types of specimen (this included fat, fibrous tissue, blood).

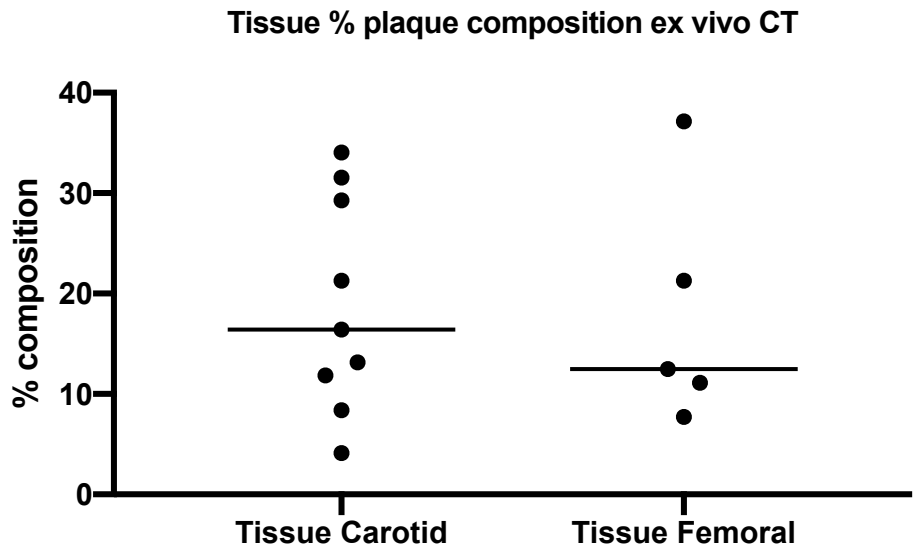
Graph 8: % of fat within carotid and femoral plaque



Graph 9: % of blood within carotid and femoral plaque



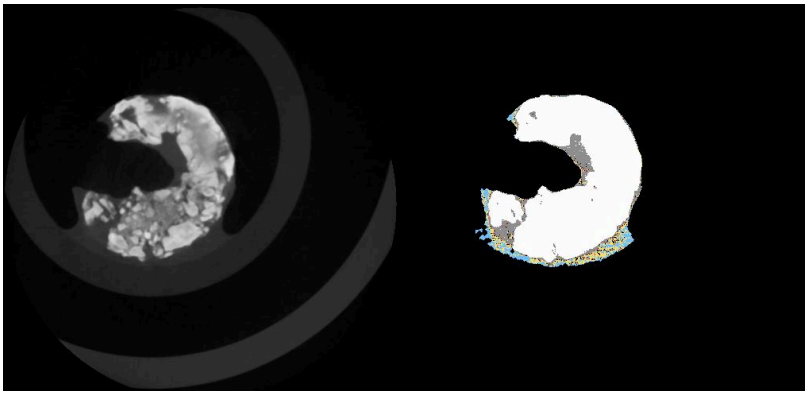
Graph 10: % of fibrous tissue within carotid and femoral plaque



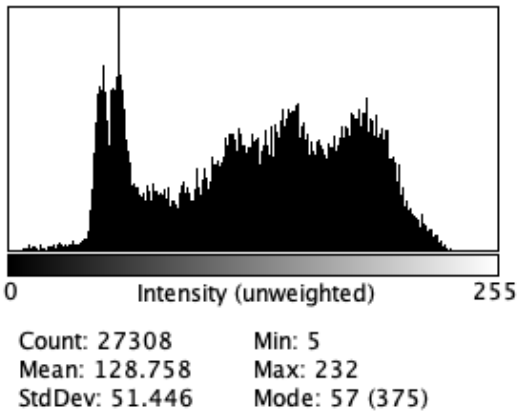
As calcium seemed to show the greatest variation between different plaques, it became subject for further characterisation using *Image J.*

Using the area of greatest plaque stenosis, it was possible to draw around a transverse section of plaque and analyse pixel intensity within the section. These results produced some interesting findings.

Picture 19: Carotid smoker; (A) micro CT transverse section, and computerised reconstruction using Hounsfield units, (B) pixel intensity graph of same transverse section

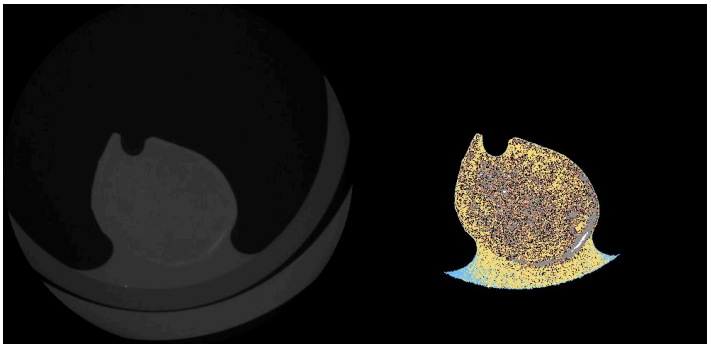


A

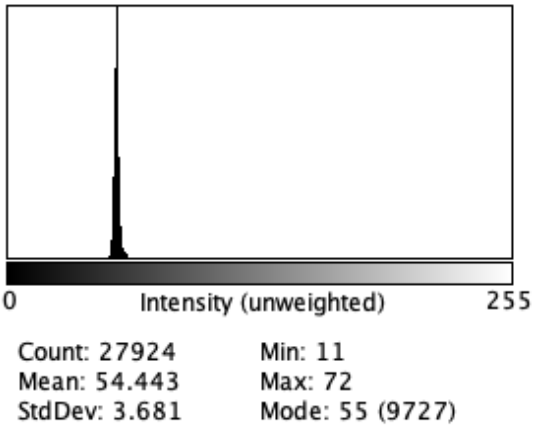


B

Picture 20: Carotid non-smoker; (A) micro CT transverse section, and computerised reconstruction using Hounsfield units, (B) pixel intensity graph of same transverse section

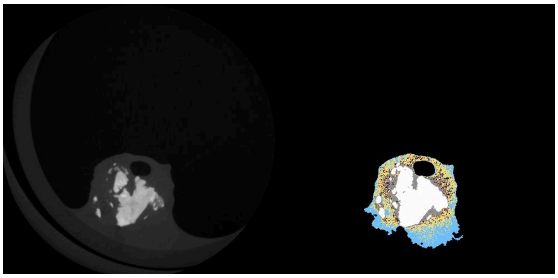


A

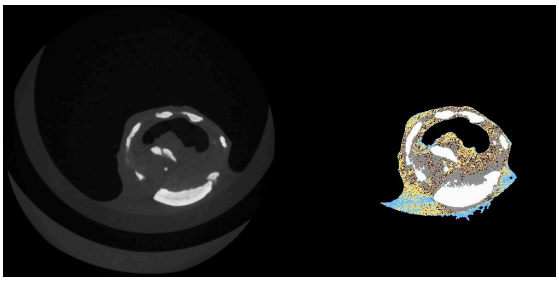


B

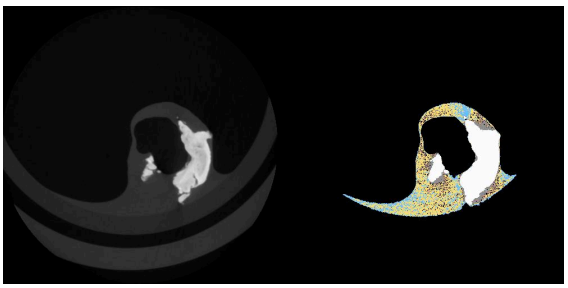
Picture 21: Carotid ex- smoker; (A) micro CT transverse sections, and computerised reconstructions using Hounsfield units, (B) pixel intensity graphs of same transverse sections



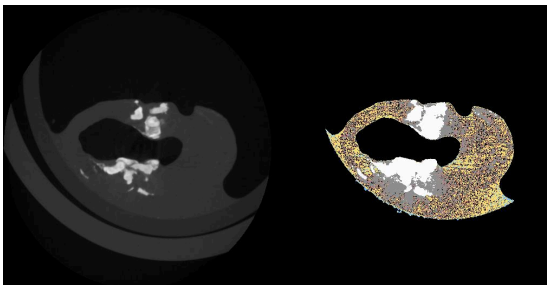
A1



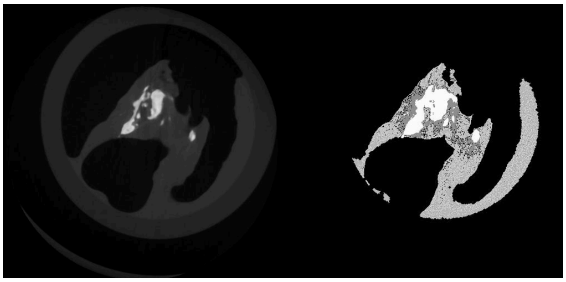
A2



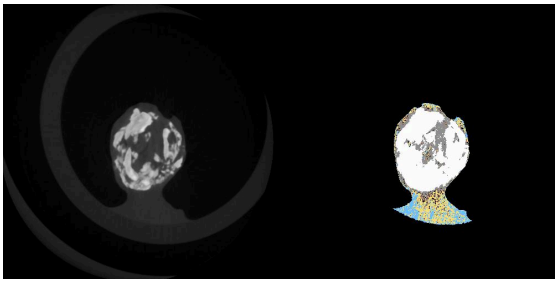
A3



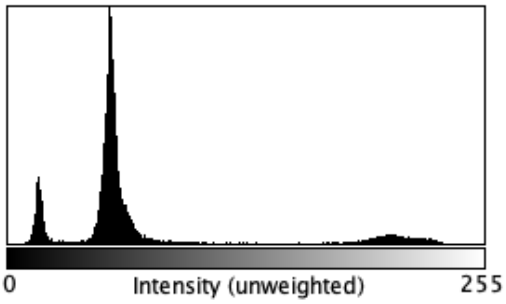
A4



A5

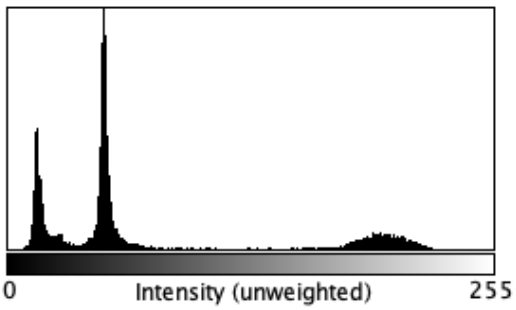


A6



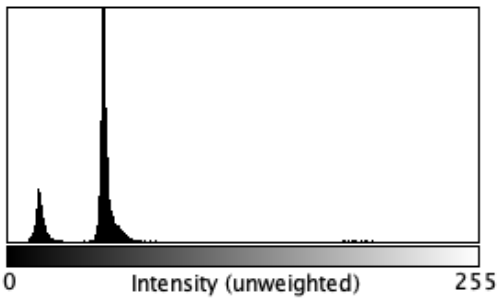
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Mean: 72.192 Max: 241
StdDev: 54.235 Mode: 54 (2559)

B1



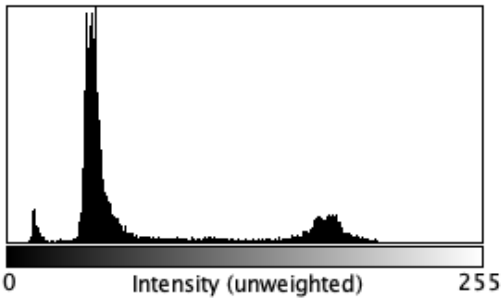
Count: 26510 Min: 3
Mean: 74.407 Max: 231
StdDev: 65.014 Mode: 50 (2258)

B2



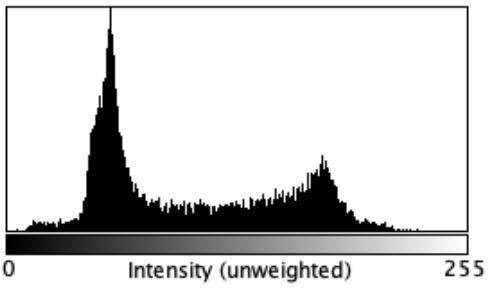
Count: 51143 Min: 6
Mean: 56.938 Max: 239
StdDev: 38.673 Mode: 52 (7436)

B3



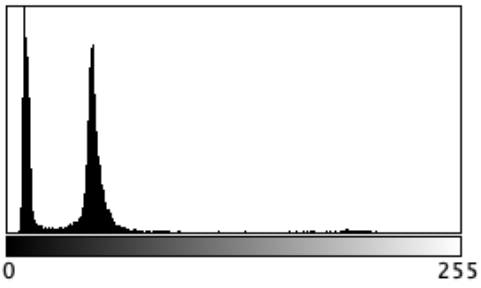
Count: 21003 Min: 5
Mean: 74.023 Max: 212
StdDev: 50.916 Mode: 47 (1367)

B4



Count: 17441 Min: 5
Mean: 101.690 Max: 230
StdDev: 52.329 Mode: 57 (501)

B5



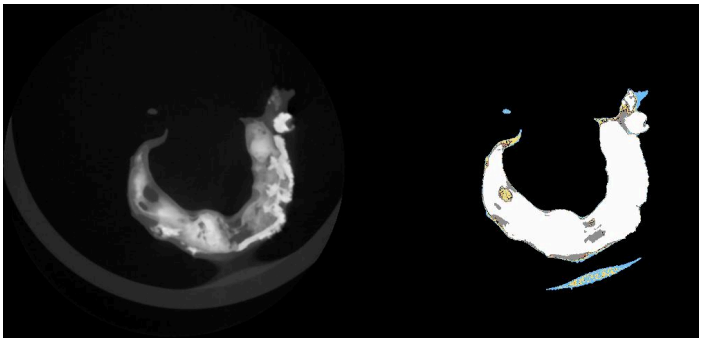
Count: 39568 Min: 4
Mean: 42.500 Max: 224
StdDev: 38.448 Mode: 9 (3103)

B6

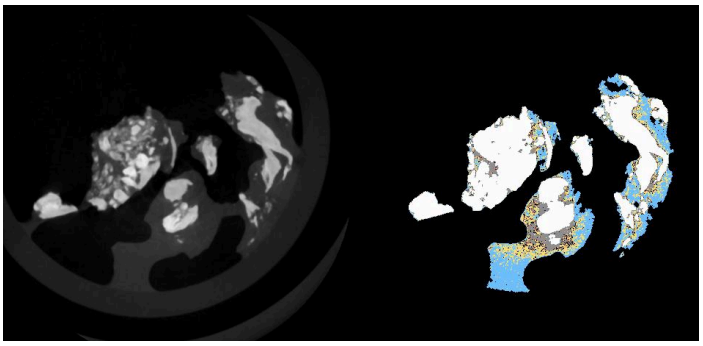
As you can see, the pixel intensity graphs depict the constituents of the plaque. Interestingly there are obvious differences between the carotid smoker (high calcium seen in the scan),

ex-smoker and non-smoker. As seen, there is one strong peak in the non-smoker scan. This same area is replicated in the ex-smoker, along with peaks both higher and lower. The carotid plaque of the smoker (significant calcium seen on scan) has much more heterogeneity and peaks much more towards the right (higher intensity).

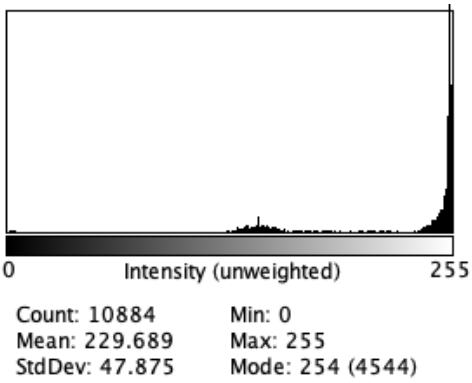
Picture 22: Femoral ex- smoker; (A) micro CT transverse section, and computerised reconstruction using Hounsfield units, (B) pixel intensity graph of same transverse sections



A1

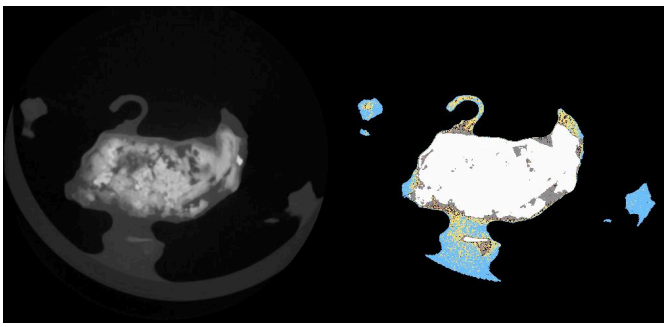


A2

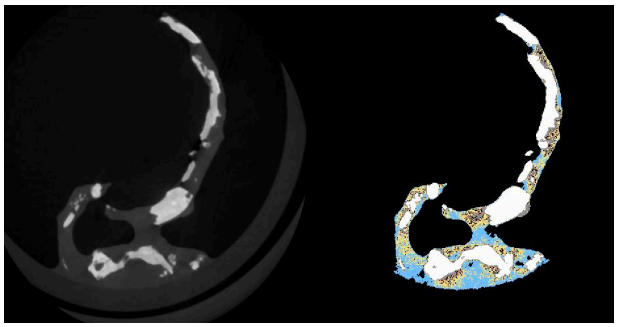


B1

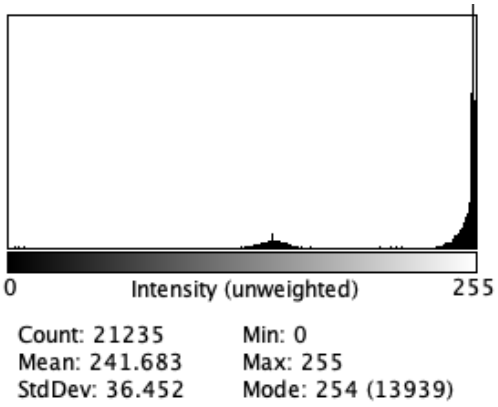
Picture 23: Femoral smoker; (A) micro CT transverse section, and computerised reconstruction using Hounsfield units, (B) pixel intensity graph of same transverse sections



A1

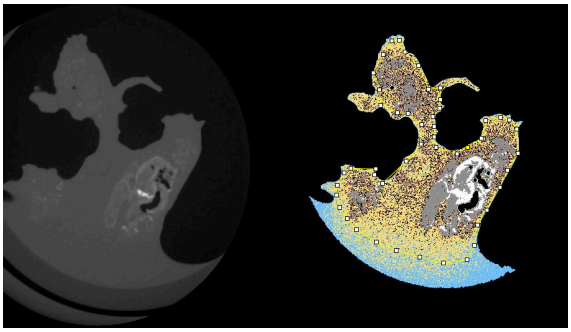


A2

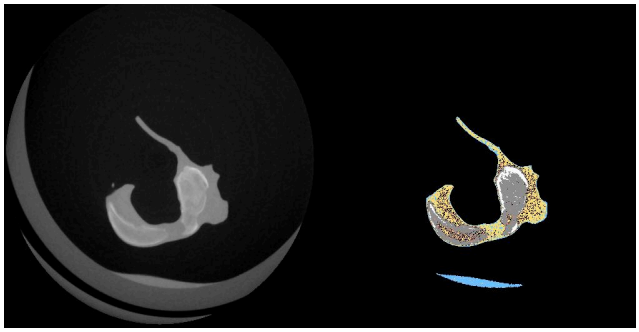


B1

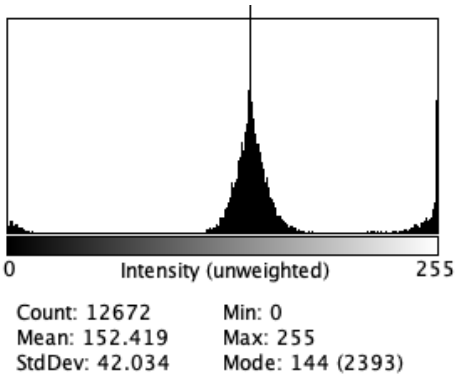
Picture 24: Femoral non- smoker; (A) micro CT transverse section, and computerised reconstruction using Hounsfield units, (B) pixel intensity graph of same transverse sections



A1



A2



B1

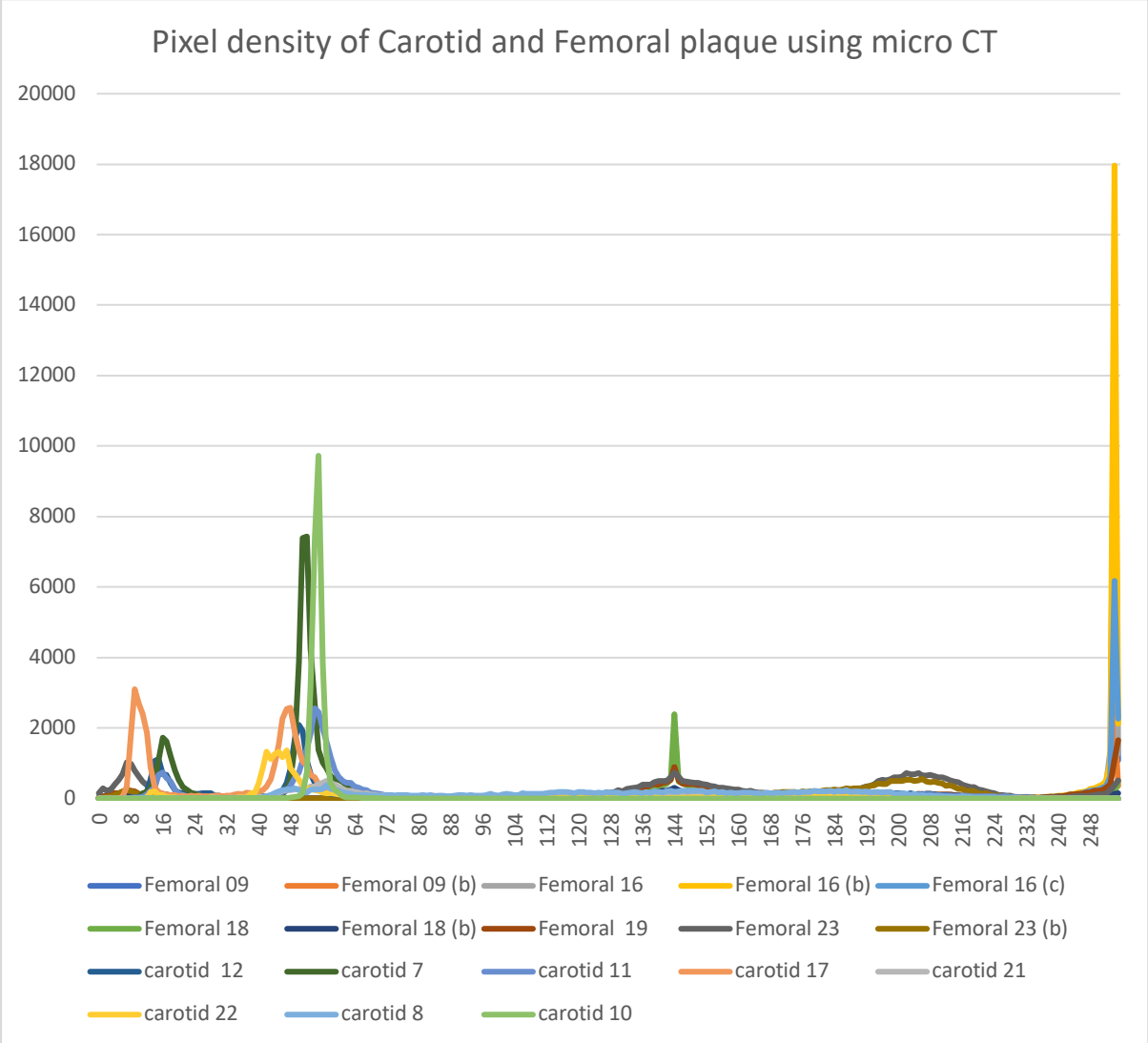
What is obvious from femoral plaques is that the pixel intensity graphs do not match those of the carotid, as expected.

As previously described, the raw data on plaque composition using Hounsfield units shows that composition elements between carotid and femoral plaque were not too dissimilar.

Patient demographics such as pack years of smoking directly affected composition. However, as can be seen here, the calcium **intensity** graphs are markedly different.

Femoral plaque appears to have a huge signal in the region of 255. Markedly higher than any of the carotid peaks of around 50 and 150.

Graph 11: Comparison of carotid and femoral pixel intensity through all plaque sections



Graph 11 shows the pixel density graphs of all the carotid and femoral plaques superimposed into each other. The results clearly depict differences between femoral and carotid calcium. It is not the fact that the calcium is in great quantities in the femoral plaque (disproved in earlier micro CT data), it is that the calcium present is much denser within the femoral tissue.

This is depicted in graph 11 where carotid plaques have density peaks towards the left hand side of the x axis (lower pixel density), and the femoral plaques along the far right of the x axis (higher pixel density).

These calcium differences support our macroscopic findings of much harder, crumbling tissue within femoral atherosclerosis, our issues with femoral sectioning, and the histological findings of possible bone like progenitor cells within the femoral plaque.

6.5 Ex vivo analysis of atherosclerotic plaque using (micro) magnetic resonance imaging

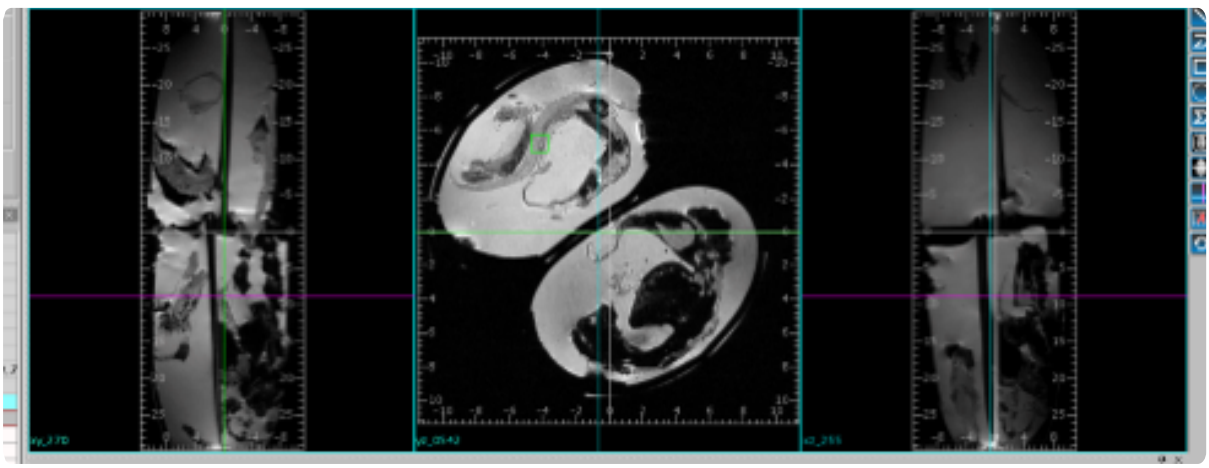
Micro MRI has been widely used in the biomedical field for both functional and anatomical imaging, although most often in small animals. It is particularly suitable for soft tissue, and therefore often preferred for scans of the brain, nerves and spinal cord.

Following a literature review to ensure feasibility for our desired outcomes; 6 plaques were embedded in gadolium-doped agar within a PTFE supporting plastic matrix, and subject to long

scan micro MRI. Only 6 plaques could be used for this analysis due to the National lockdown in response to covid-19. It was planned to scan all available plaques, but the chosen 6 (3 of each carotid and femoral) were pushed into overnight scanning during the final week of laboratory practice. Picture 25, 26.



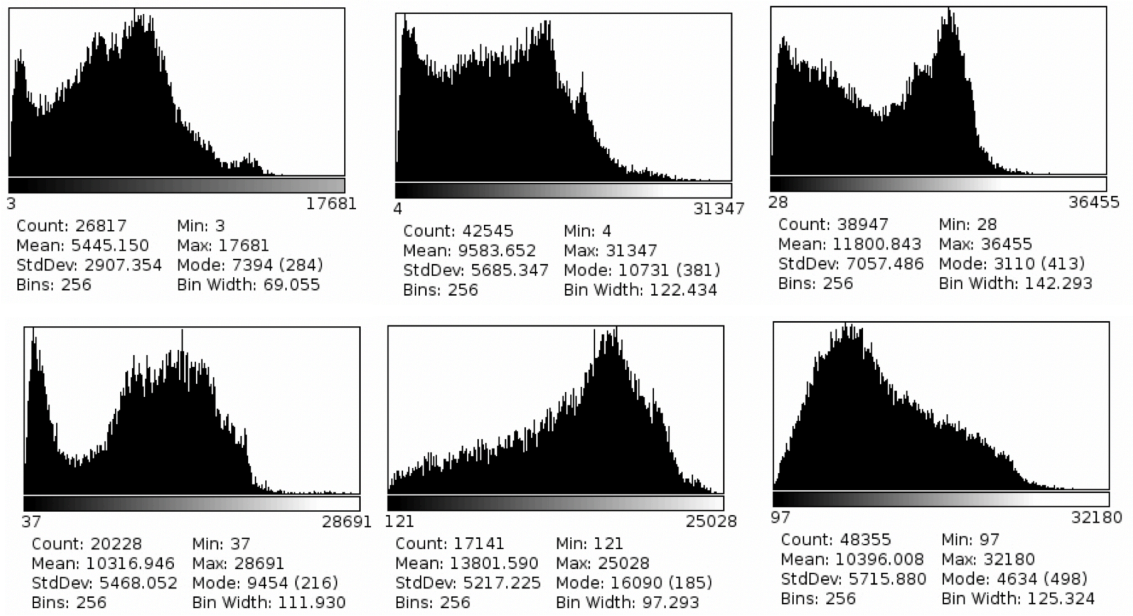
Picture 25: Micro MRI transverse section; 3 x carotid and 3 x femoral plaques



Picture 26: review of individual plaques using micro MRI

6.5.2 Carotid atherosclerosis

Picture 27: Pixel intensity graphs of micro MRI cross sectional imaging, for carotid atherosclerosis



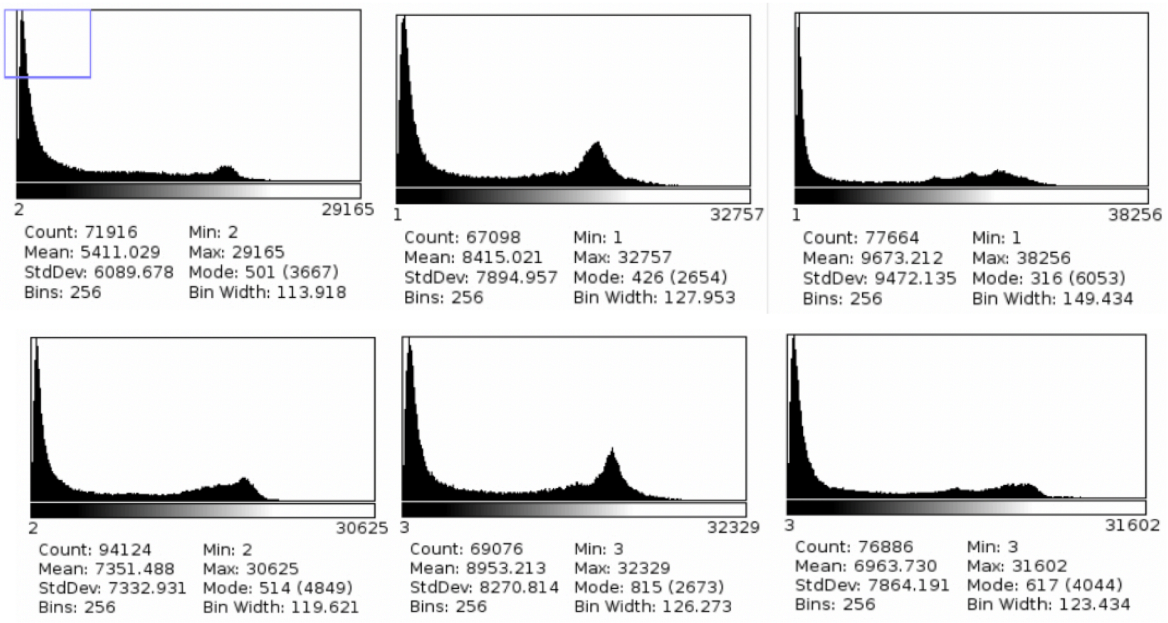
A B C

In the same format as described for micro CT, it was possible to mirror the calcium pixel intensity graphs for images gained with micro MRI. The area of maximal stenosis was highlighted and analysis performed in the same way.

As seen in the graphs above, pixel intensity for carotid disease is focused towards the middle of the x axis, which quite a spread in range over the graph.

6.5.3 Femoral atherosclerosis

Picture 28: Pixel intensity graphs of micro MRI cross sectional imaging, for femoral atherosclerosis



A B C

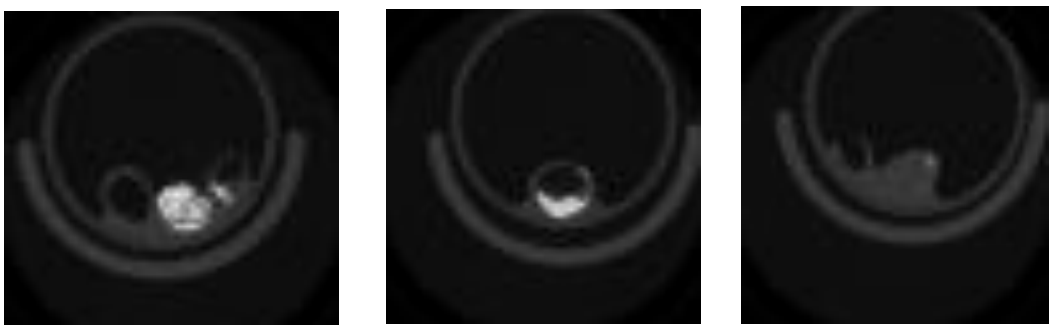
Looking at femoral atherosclerotic plaque with micro MRI; the significant peaks can be seen on the far left; a separate area, and waveform to the carotid peaks shown above.

This contrast from the micro CT data supports the visual opposition known with MRI and CT imaging; MRI tends to show calcium as dark and CT as white. Therefore the high peaks seen in the far right in micro CT are seen in the far left in micro MRI (Picture 28).

In addition to the comparison of symptomatic carotid plaque and femoral atherosclerotic legions, my confirmation viva assessors felt that comparable calcium results from *asymptomatic* carotid atherosclerosis would prevent 'not comparing like with like'.

Three asymptomatic carotid specimens were consequently recruited to the study. As stated previously, these plaques were enrolled without any patient identifiable information, to adhere to trust and ethics guidelines post COVID. Each plaque followed the same fixation processes as described in methods, prior to undergoing scanning with micro CT.

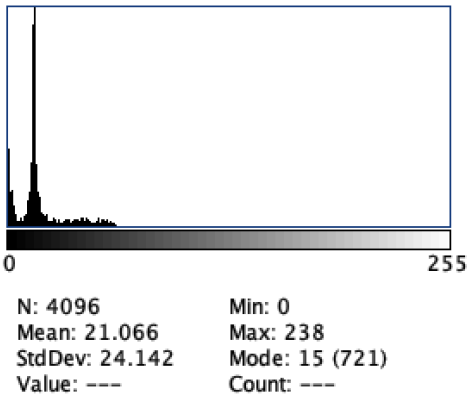
Picture 29: Micro CT of asymptomatic carotid atherosclerosis



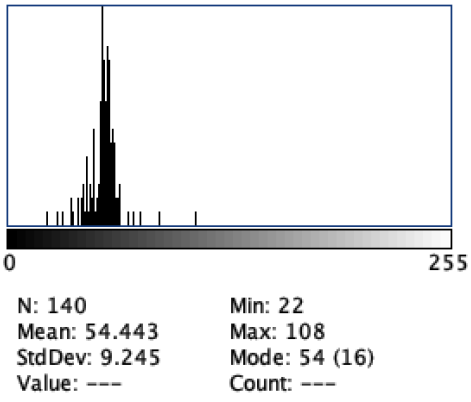
A B C

Asymptomatic carotid micro CT scans were analysed using hounsfield units, and calcium density graphs produced using image J (replicated method as for previous plaques).

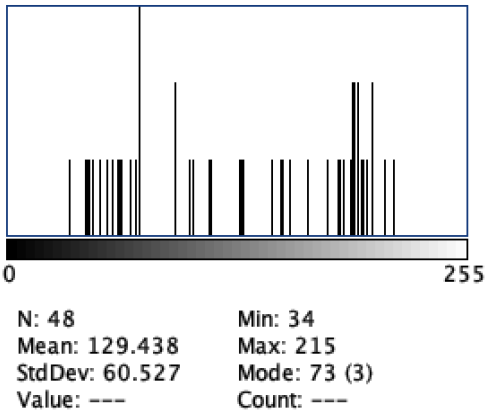
Picture 30: Pixel intensity graphs of micro CT cross sectional imaging, for asymptomatic carotid atherosclerosis



A



B



C

As shown in picture 29 and 30, the **density** of the calcium within the three asymptomatic carotid plaques remained in keeping with previous carotid samples.

6.6 Discussion

The results of this chapter have highlighted important findings in the composition of arterial atherosclerosis. We firstly stained both carotid and femoral atherosclerotic plaque sections; where we not only replicated equivalent sections seen in the literature, but were able to confirm the presence of calcium with von kossa staining, and highlight architectural differences between carotid and femoral plaque. This led onto the discovery of osteoblastic/osteoblastic type cells found only within femoral specimens.

Using ex vivo plaque imaging, it was possible to review the main components of atherosclerotic plaque and to what percentage they make up a legion.

- Smoking history has been shown to significantly correlate with the proportionate amount of calcium present within a plaque.
- Secondly, despite smoking history, the overall density of calcium within a plaque is significantly higher in femoral atherosclerosis.

We had hoped to take these results further, by using stains to differentiate the type of calcium present within our specimens. However, despite a thorough literature review, and discussions with both bone metabolism labs and our consultant histopathologist (E.F), we were not able to find a recognised stain that would show 'bone calcium' a separate entity from any other type of calcium.

‘all the commonly used calcium stains would stain both (we use von Kossa, and that definitely stains hydroxyapatite as well as non-bone mineralisation).’ – E.F (2024)

6.7 Conclusions

Carotid and femoral atherosclerosis may have similar components within their architecture, but the results shown in this chapter confirm that the calcium within femoral atherosclerosis is significantly more dense than found in carotid atherosclerosis.

We have also shown that femoral calcium may be or partially be produced from osteoblastic type progenitors in a more ‘bone like’ manor. This could explain the differences between carotid and femoral macroscopic structure, disease presentation and lack of improvement with current treatments.

7. Chapter 7: Further plaque analysis

7.1 PLEXIN D1

As part of the collaborative work with Prof Ellie Tzima's lab, it has been possible to look at the role of the novel mechanosensor PlexinD1 in the development of carotid and femoral atherosclerosis.

The staining and results analysis of carotid plaques were completed entirely by the Tzima lab, but this created an opportunity to contrast and compare femoral intima using the same techniques used on carotid tissue.

All femoral plaque recruitment, sectioning, staining and imaging was completed as part of this thesis, with result analysis and comparison supported by the Tzima group. Methods can be found in section 2.

7.1.2 Background

Endothelial cells (EC's) are extremely important to all vessels; arteries, veins and capillaries. EC's act as a barrier within blood vessels, but also an endocrine organ. They are able to sense flow through mechanoreceptors, and consequently dilate or constrict when required. They also play a role in the immune response, contributing to both atherogenic and athero-protective cascades (131).

The vascular endothelium prevents both lipoproteins and leucocytes from entering the vessel wall, therefore controlling parts of the pathway leading to atherosclerosis. Damage to the endothelium can lead to increased endothelial permeability, platelet aggregation, leucocyte adhesion and cytokine response.

Plexins are cell surface receptors that can regulate cellular activity by modulating adhesion structures and the cell cytoskeleton. Plexin D1, a member of the family, has been shown to not only participate in axonal guidance (132), but also mechanosensation (133). It is required as part of the endothelial response to sheer stress; and by detecting physical force within a vessel, PLXND1 can ultimately dictate atherosclerotic deposition. In atheroprone mice fed a high-fat diet, PLXND1 deficiency in endothelial cells decreased the plaque burden in the whole aorta (133).

The Tzima group have also shown, in currently unpublished data, that PLXND1 is upregulated in carotid plaque with higher levels of stenosis. There is currently no data on PLXND1 within femoral plaque.

PECAM-1 is a gylcoprotein which is expressed on endothelial cells, platelets and some leukocytes. Staining for PECAM-1 can therefore highlight the vessel endothelium. This is extremely important for vascular imaging, and can be seen in our images below.

DAPI is a fluorescent stain that binds strongly to adenine-thymine (AT) rich regions of DNA, consequently it can stain cells (both live and fixed).

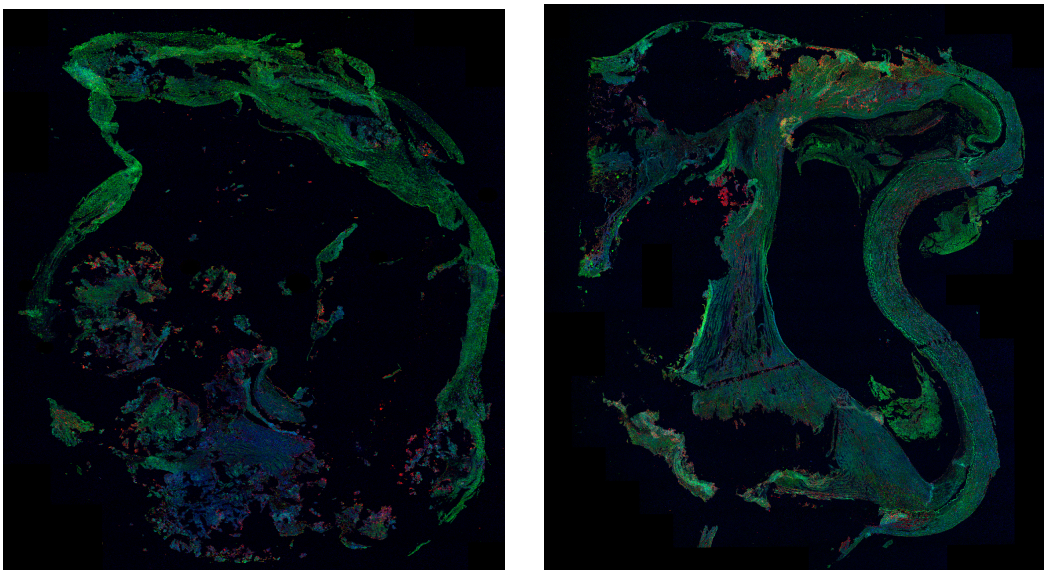
Our samples were stained with DAPI, and binding of PECAM-1 and PLEXIN D1, and showed the following results.

7.1.3 Results

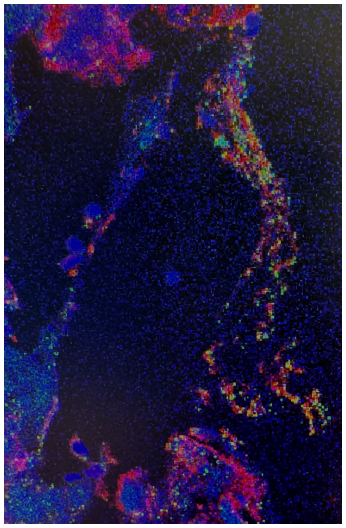
As can be seen in picture 31; although we were only able to stain a small number of femoral plaques, due to the same reasons mentioned previously, we were able to attain a few excellent sections.

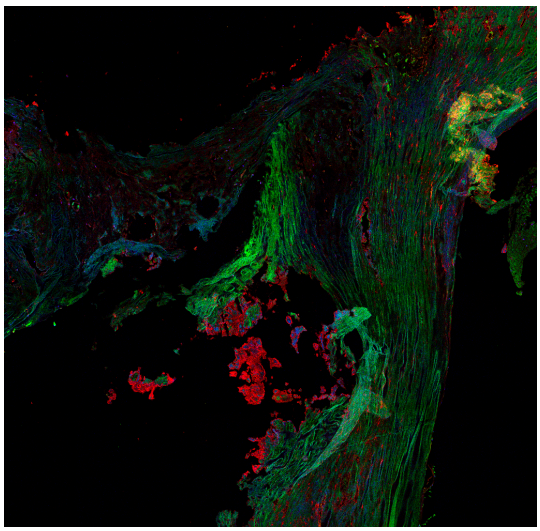
Some femoral specimens could not easily be sectioned, and others did not make it through the staining process.

Picture 31: Immunofluorescence staining of femoral atherosclerotic plaque, PECAM and PLEXIN D1



A B





C D

Picture 31 A&B show DAPI, PECAM and PLEXIN D1 within femoral atherosclerotic plaque. Of interest are the areas within the specimen where PECAM (green) and PLEXIN (red) co-localise, giving a 'yellow' colour (picture 31 C&D). Picture 29D also highlights expression of PLEXIN D1 within the plaque (seen in several different sections).

These results mimic the results for carotid sections, but due to low numbers of successful sections, cannot be used as scientific evidence.

7.1.4 Conclusions

Endothelial cells play an important role in the regulation of atherosclerotic disease. PLEXIN D1, an endothelial cell surface receptor, has previously been shown to play a part in atherosclerotic deposition. Here we see PLEXIN D1 co-localised with PECAM (i.e within the

vessel endothelium) of diseased plaque, as also seen in carotid atherosclerosis. This is an important finding, and will help to fuel future research in this area.

7.2 Socioeconomic status

7.2.1 Introduction

Socioeconomic status (SES) is a measure of a persons combined economic and social standing in the community, with particular attention to a persons' work, access to resources, education and income (134). SES has been linked to health and disability for centuries, with documentation extending to ancient Greece and Egypt. More recently it has been a growing interest in the research arena; combining basic science, public health and policy, and consequently having significant value for today's society.

As part of my confirmation viva, assessors felt that increasing the numbers enrolled in OxPVD should not be pursued. Instead, I was to accept current data following the COVID pandemic, and review something else such as socioeconomic status of the patients undergoing carotid or femoral endarterectomy.

This is an extremely interesting area; multiple studies have shown correlation with low SES and increasing cardiovascular morbidity and mortality, but there is little in the literature remarking specifically on carotid and femoral atherosclerosis. Lynch et al. in 1995 showed a strong association between low socioeconomic status and increased carotid atherosclerosis

(135). MacKenzie at al. in 2003 detailed an increase in symptomatic carotid disease in lower socioeconomic groups, but interestingly this did not correspond to increased numbers of carotid endarterectomies (136). The most recent work in this area by Diago at al. in 2023 again reiterated the above, but that education and SES can in fact interact with genetic predispositions and increase a person's cardiovascular risk (carotid artery thickness) (137).

With regards to femoral artery atherosclerosis, a few papers have highlighted aspects such as delayed groin wound healing in patients with lower SES (138), increased amputation rates (139), and greater reduction in mobility in lower social classes, but no papers have compared the SES of the population with carotid cf. femoral atherosclerosis.

To be able to carry out comparative analysis of SES between our two cohorts (carotid and femoral), it was also important to understand what data was required to do so. Many studies review a multitude of factors, such as education, income, size of house, to create a value on social status. This however requires significant epidemiological understanding or the help of population/public health researchers to ensure accuracy, as well as participant willingness to divulge personal information. There are also elements to SES questionnaires that can be subjective, rather than objective, and may create inaccuracy.

More crude methods to approximate SES have been shown to provide fairly accurate data. Danesh et al. looked at 26,000 postcodes in the UK and deemed a moderate and usable correlation between SES and postcode. Each postcode only serves around 15-20 houses on average in the UK, making it a more feasible option than in other countries (140).

7.2.2 Methods

Using the patients enrolled in OxPVD, I was able to review the hospital records and extract postcode data on each participant. As detailed above, studies in the UK have verified the use of postcode data to approximate SES, giving excellent results.

Each postcode was inputted into the **gov.org.uk** - Ministry of Housing, Communities & Local Government webpage: https://imd-by-postcode.opendatacommunities.org/imd/2019
This generated data on each postcode including deprivation rank, education, health and disability, income, employment, crime and housing from information in the last census. Results for this are tabulated in Table 10.

7.2.3 Results

Table 10: Study participant postcodes and socioeconomic status

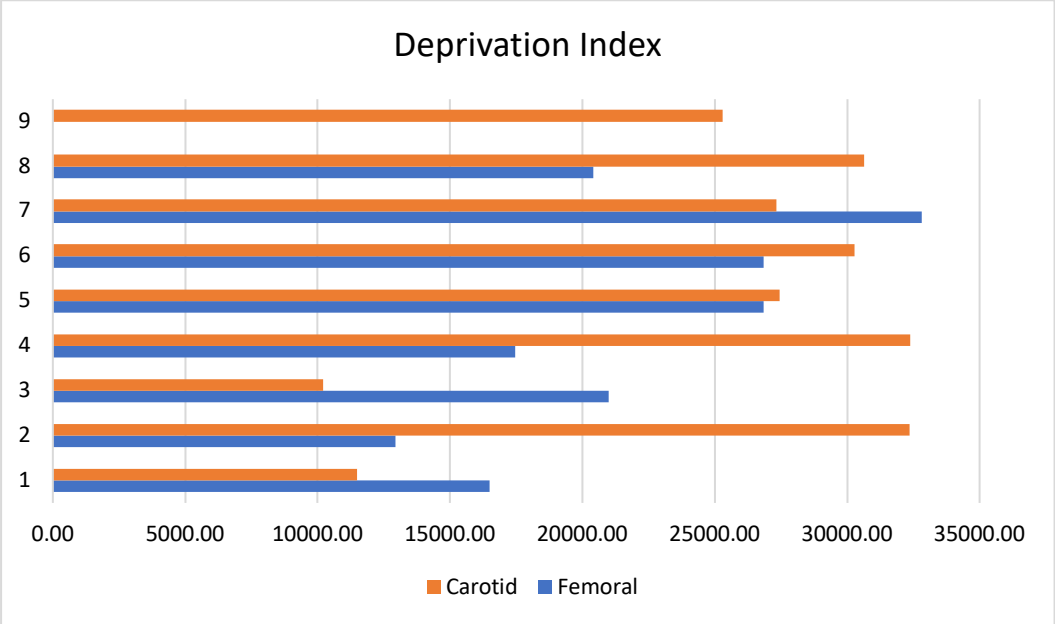
Postcode **Femoral**	**Deprivation Decile**	**Income Decile**	**Employment Decile**	**Education and Skills Decile**	**Health and Disability Decile**	**Crime Decile**	**Barriers to Housing and Services Decile**	**Living Environment Decile**
OX4 3LJ	6	6	7	3	6	3	6	5
HP20 2HF	4	4	4	3	5	4	5	10
OX14 3US	7	6	5	8	7	4	9	6
OX3 7XR	6	6	9	7	6	10	1	2
OX15 5PT	9	7	8	9	8	9	10	3

OX15 5PT	9	7	8	9	8	9	10	3
HP15 6TT	10	10	10	10	10	9	8	10
MK2 2LM								
OX3 7EH	7	6	7	7	7	5	5	4
Carotid								
SL3 8SR	4	4	6	4	4	2	3	3
RG4 8QJ	10	10	10	10	10	9	5	9
HP13 6XP	4	3	3	2	8	4	3	7
OX10 0PG	10	9	9	10	10	10	8	10
CF35 5BE								
OX2 6TG	9	9	10	10	10	8	4	2
OX7 3BT	10	8	9	7	10	8	9	9
SN7 8HU	9	8	8	9	10	10	6	3
HP13 6RA	10	9	9	10	10	7	5	8
OX11 7E7								
SL4 6AH	8	10	10	6	10	2	3	7

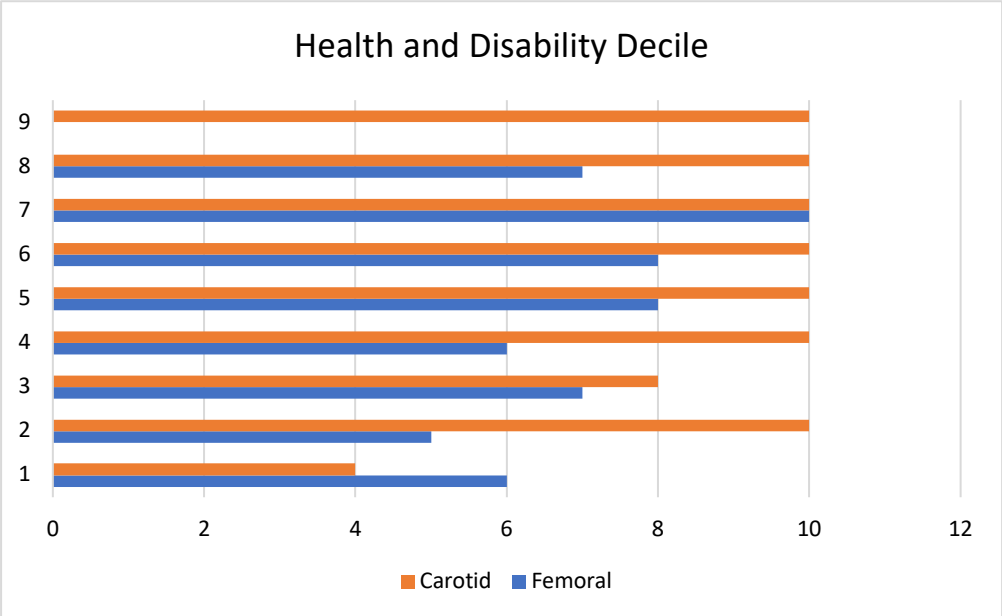
The results in table 10 highlight the key areas of deprivation, income, employment, education, health, crime, housing and living environment – linked to socioeconomic status, and the femoral and carotid OxPVD participants postcodes.

As can be seen in graph 12 below, the deprivation levels of the femoral patients were worse overall than for carotid patients. When looking at health and health and disability index, this was again poorer in the femoral cohort (graph 13). Interestingly, the education levels were marginally higher in the carotid group (graph 14), with overall higher employment index (graph 15).

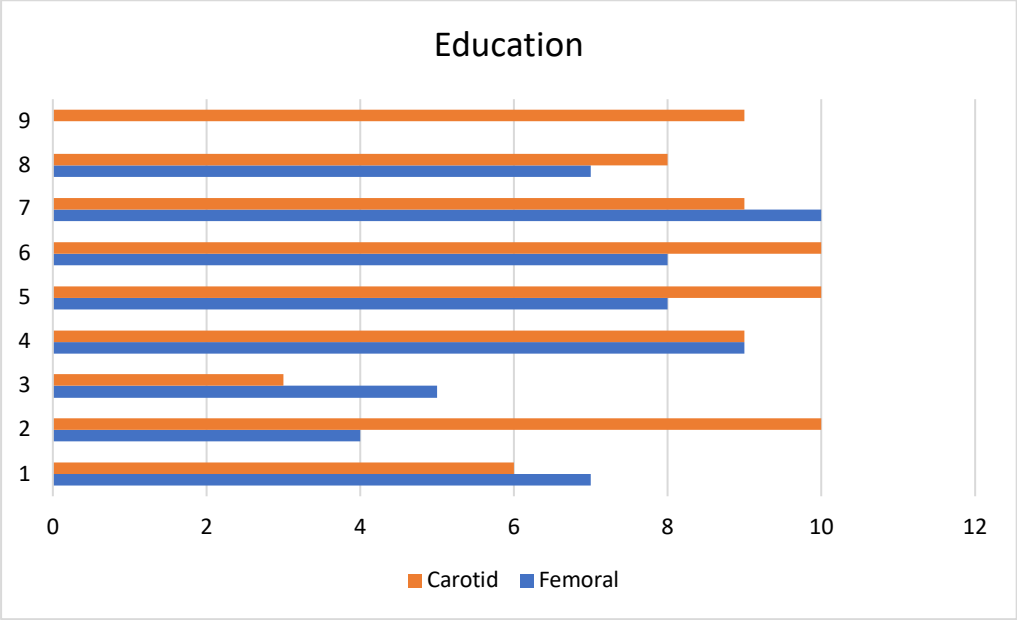
Graph 12: Deprivation index of carotid and femoral patients using postcode



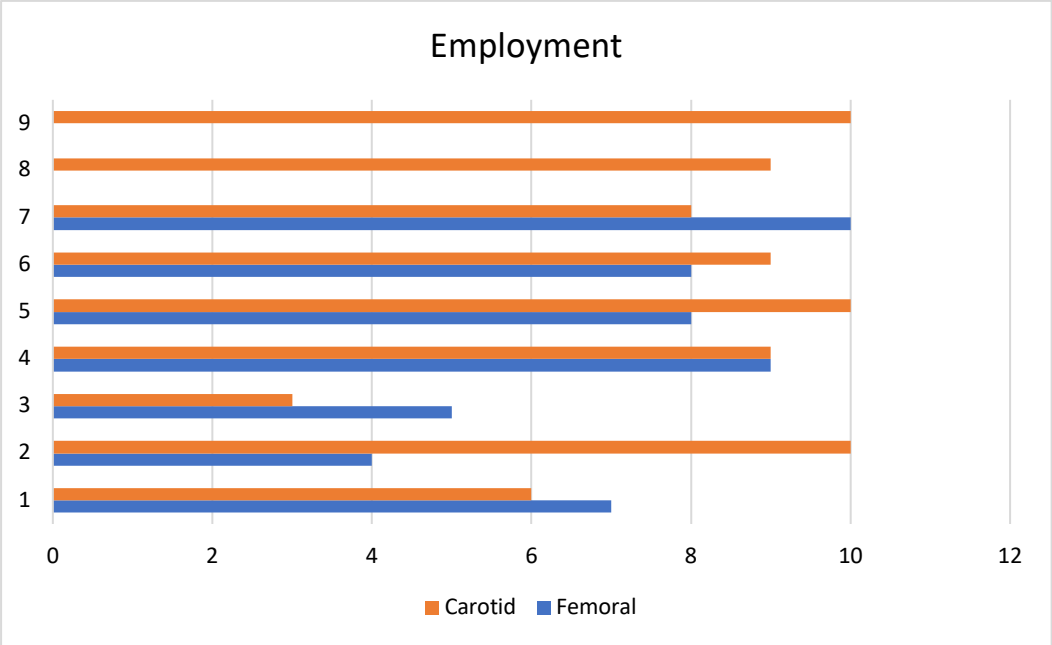
Graph 13: Health and disability of carotid and femoral patients using postcode



Graph 14: Education of carotid and femoral patients using postcode



Graph 15: Employment of carotid and femoral patients using postcode



7.2.4 Conclusions

Socioeconomic status is a growing area in research due to its significant benefits to public health and policy, and its consequent money saving implications. There are multiple ways in which SES can be determined; but they all try to approximate a persons' social standing in the community.

The method chosen for this chapter, was to review patients' postcodes as a crude measure of SES, to allow for carotid and femoral comparison. The data given for each participant included deprivation, health, education and employment. Result show that overall the patients undergoing carotid endarterectomy were marginally less deprived, with greater education and employment values, and improved health and disability scores.

There are however low numbers in each cohort, making it difficult to apply these results to larger population datasets. The results indicated in our study cohort highlight possible differences to SES between those patients with carotid and femoral atherosclerosis. Consequently, further research in the area may be warranted.

8. Conclusions

Atherosclerosis is a worldwide healthcare burden, causing significant morbidity, mortality and financial challenges to healthcare systems. Common risk factors of age, cholesterol and hypertension are all linked to atherosclerotic disease progression and current treatments for atherosclerosis is largely uniform.

Patients however present with atherosclerotic disease in different arterial beds, and overall patient outcomes do not neatly correlate with the extent of disease. Why is treatment more effective for certain types of atherosclerotic plaques than others? Why do certain patients experience a stroke but never an amputation?

25 years following the seminal paper by Dormandy et al. on 'the fate of the claudicant'; mortality rates remain at approximately 5%, the rate of amputation at 1-3% and around one quarter of patients continue to deteriorate from their disease. This is all despite the introduction of medical treatments and the evolution of endovascular measures.

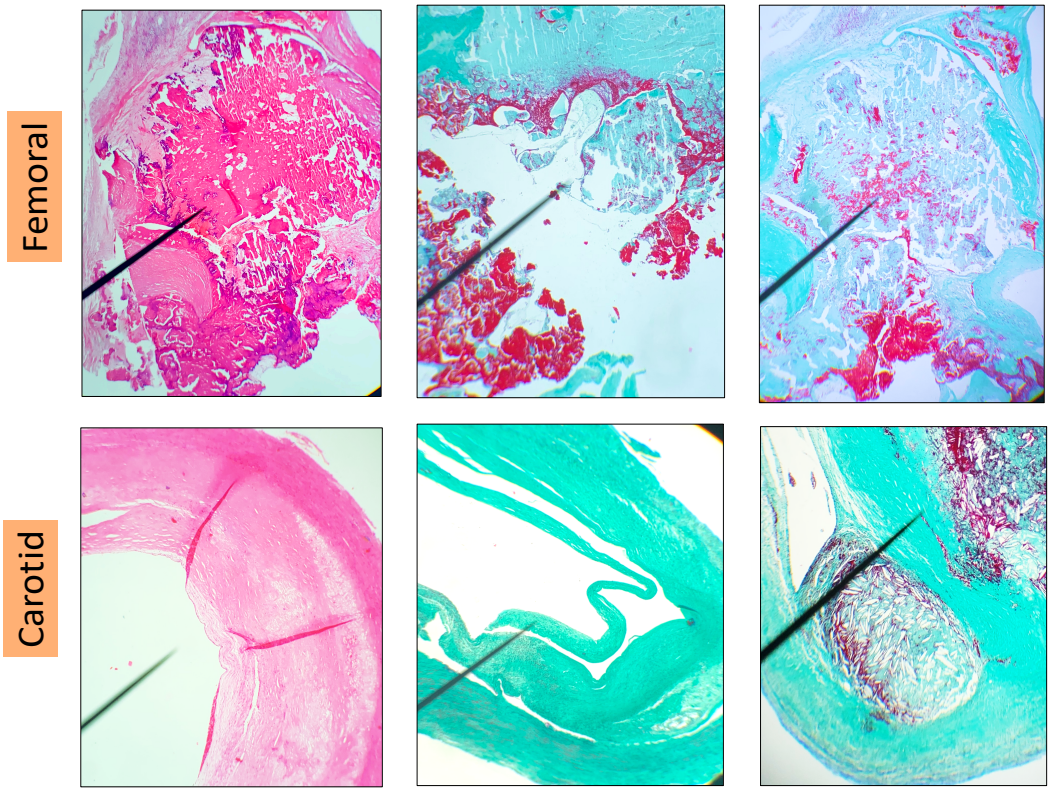
These questions raised significant doubts on the comparability of various types of atherosclerotic disease, particularly femoral and carotid; and lead to the recruitment of patients to OxPVD; patients undergoing either carotid or femoral endarterectomy.

In vivo plaque analysis showed some fairly significant findings; firstly that with the use of a coil array over the pelvis, it was possible to clearly image femoral atherosclerosis, but more importantly that femoral atherosclerosis was frequently seen as AHA 7 – calcified plaque. Comparatively, carotid disease was more commonly AHA 5/6 – fatty infiltration, fibro

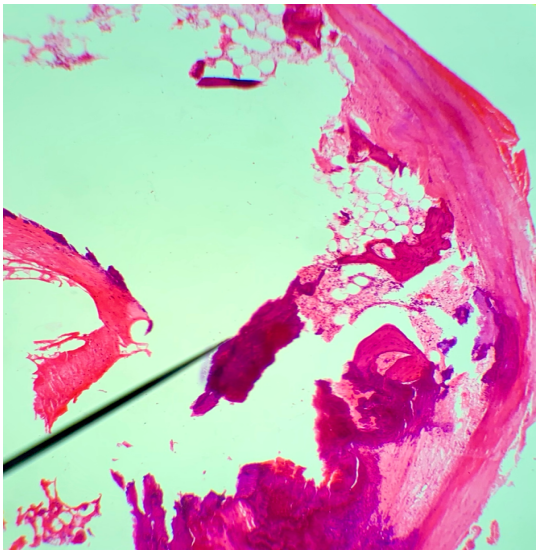
atheroma, with intraplaque haemorrhage. These results were also consistent with macroscopic subjective findings of the plaque ex vivo.

Each specimen was analysed using a variety of micro CT, micro MRI and histological stains to greater determine the plaque architecture.

The results from histological staining highlighted important findings in the composition of arterial atherosclerosis. Firstly both carotid and femoral atherosclerotic plaque sections where replicated equivalent sections seen in the literature. We were able to confirm the presence of calcium within the plaque with von kossa staining, and highlight architectural differences between carotid and femoral plaque as see below;



The most important and novel finding however was the presence osteoblastic/osteoblastic type cells found only within femoral specimens.



Using ex vivo plaque imaging (micro CT and micro MRI), it was possible to review the main components of atherosclerotic plaque and to what percentage they make up a legion. The key results from this work were that smoking history significantly correlated with the **amount** of calcium present within a plaque, and secondly; despite smoking history, the overall **density** of calcium within a plaque is significantly higher in femoral atherosclerosis.

The fact that femoral calcium may be or partially be produced from osteoblastic type progenitors in a more 'bone like' manor could explain the differences between carotid and femoral calcium density, its macroscopic structure, disease presentation and lack of improvement with current treatments.

The final finding from the OxPVD dataset was looking at the socioeconomic status of the study participants. Although overall numbers compared were low, comparison of the two groups showed that femoral patients were on average of a lower socio-economic class; more deprivation, poorer health and education, and with less employment.

The key differences explained throughout this thesis depict that carotid and femoral atherosclerosis are not entirely the same. It is important to recognise these differences for future research and the possibility of therapeutic targets that could improve patient outcomes.

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APPENDIX A

Table 1: Literature review of atherosclerotic plaque analysis (2016 onwards)

	Animal	Human tissue	Human serum
Carotid		Extracellular matrix proteomics identifies molecular signature of symptomatic carotid plaques. Langley SR, Willeit K, Didangelos A, Matic LP, Skroblin P, Barallobre-Barreiro J, Lengquist M, Rungger G, Kapustin A, Kedenko L, Molenaar C, Lu R, Barwari T, Suna G, Yin X, Iglseder B, Paulweber B, Willeit P, Shalhoub J, Pasterkamp G, Davies AH, Monaco C, Hedin U, Shanahan CM, Willeit J, Kiechl S, Mayr M The atheroma plaque secretome stimulates the mobilization of endothelial progenitor cells ex vivo. Vega FM, Gautier V, Fernandez-Ponce CM, Extremera MJ, Altelaar AFM, Millan J, Tellez JC, Hernandez-Campos JA, Conejero R, Bolivar J, Pardal R, Garcia-Cózar FJ, Aguado E, Heck AJR, Duran-Ruiz MC. J Mol Cell Cardiol. 2017 Apr;105:12-23. doi: 10.1016/j.yjmcc.2017.02.001. Epub 2017 Feb 20. Proteomic Profile of Unstable Atheroma Plaque: Increased Neutrophil Defensin 1, Clusterin, and	A study on the association of rs7950273 polymorphism in the PDGFD with ischaemic stroke in the Chinese Han population. Han J, Li H, Wu D, Zeng N, Wu Z, Cai L, Chen W, Zuo W, Zhang Y. Ann Hum Biol. 2016;43(1):78-80. doi: 10.3109/03014460.2015.1016552. (ISCHEMIRs: Finding a Way Through the Obstructed Cerebral Arteries. Nampoothiri SS, Menon HV, Das D, Krishnamurthy RG. Curr Drug Targets. 2016;17(7):800-10.)

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APPENDIX B

Human carotid plaque collection / processing / staining / and laser capture microdissection

(I) Plaque collection, division, and sample processing for FFPE/frozen sections

(II) Tissue processing and embedding for FFPE sections

(III) Sectioning on microtome and cryostat

(IV) IHC staining for CD68 and GPR109a for frozen sections

(V) Oil-red-O staining for lipid on frozen sections

(VI) Masson's trichrome staining for FFPE sections

(VII) H&E staining for FFPE sections

(I) Plaque sample collection and division

Material

1. 4% paraformaldehyde in PBS
2. OCT compound
3. 10% formic acid
4. Plastic histology mould and tissue cassette
5. Surgical scalpel or microtome blade + disposable forceps
6. PBS for washing
7. 1x 6-well plate with cover for washing and cutting
8. Dry-ice slurry (with 90% EtOH)

Procedure

Day 1

1. Make sure dry-ice is available and collect some in a box before sample collection
2. Make dry-ice slurry but pouring 90% EtOH into dry-ice *slowly*
3. Collect sample fresh from OT – note the proximal and distal end of the sample
4. Bring sample in PBS filled 50ml Falcon tube on ice back to lab *immediately*
5. Take pathology photo with paper ruler if needed (e.g. if special features noted)
6. Wash plaque in 6-well plate with PBS until cleared of blood

Day2

1. Check to see if plaque is well fixed
2. If not, take plaque out from fridge to RT and allow fixation for further 2-3 hours at RT
3. For calcified plaque, put the whole plaque in 10% formic acid for 24-48 hrs
4. After successful decalcification, rinse cassette in running tap water for >30mins to remove residual formic acid
5. Store sample cassettes in a beaker of 70% EtOH in 4^{o}C until tissue processing/embedding
6. Collect up to 20 cassettes before batch processing and embedding at WTCHG

(ii) Tissue processing and embedding for FFPE sections

Day 1 (at WTCHG, pm only)

1. Ensure tissue processer and embedder is booked in advance – email James Brown
2. Cut sample into 5mm segments and put in tissue cassette with the caudal(proximal) end facing down – label segments as CXXXW1, CXXXW2, CXXXW3 in a caud-to-cran order
3. Transfer cassette in 70% EtOH to WTCHG, preferably in the afternoon e.g. at 5pm
4. Switch on tissue processer, push "Up/Down" button to raise the rack. Rack 1 should be over the final bath (wax), if not push "Turn" button until Rack 1 in correct position
5. Set timer with arrow pointing to current time
6. Put samples in metal basket and clip to Rack 1
7. Push "Turn" button, and *while turning is in motion*, push "Automatic/Manual" button to activate the timer programme
8. Successful engagement of the automatic timer programme will result in the programme light switching on, and the rack making periodic floating movement
9. Tissue processer will then automatically switch on overnight and finish at around 8.30am
10. Ensure to arrive *before 8.30am* the following day to check and collect samples
11. Switch on the embedder to heat wax up before going home and leave notice to warn others not to switch it off overnight

Day 2 (at WTCHG, am only)

1. Sample should be in the last wax bath at 8.30am the next day, wait till the floating movement stop / programme light is off
2. Push the "Up/Down" button to raise the rack and basket, collect cassette into a beaker
3. Rinse the basket off any wax residue with hot tap water
4. Rush "Up/Down" button to lower the rack and switch tissue processer off.

5. Bring cassettes to the embedder, and put cassettes in the hot wax bath on the right side
6. Put empty plastic mould in the warming chamber on the left side
7. Pour small amount of wax in the plastic mould then embed sample, cool briefly to semi-solidify the sample position before pouring more wax on top; break the cassette and put labelled side on top, top up with more wax.
8. Leave in cold block for 30 minutes, before transferring to 4^{o}C fridge overnight. Alternatively, put paraffin blocks in -20^{o}C for 1 hour if planning to block later in the day

(iii) Sectioning on microtome and cryostat

For paraffin sections

1. Switch on water flotation bath to 45°C and slide dryer to 40°C
2. Switch on microtome
3. Collect paraffin blocks on ice
4. Trim block at 20um and cut section at 5-6um thickness
5. Remember to leave section on flotation bath (45°C) for long enough to flatten the section and to minimise tissue fold
6. Mount to sections per slide
7. Dry slide on dryer at 40°C for 1-2 hours, then swich dryer off and leave slides to further dry overnight at RT
8. Aim to cut 3 slides per block (1 for trichrome, 1 for IHC-p if needed, 1 spare)

**For frozen sections*

1. Ensure cryostat is booked in the neuroscience/ophthalmology lab at Level 5
2. Need to bring over: slides, OCT, scalpel, microtome blade, pencil, slide chamber
3. Switch on the cryostat and take out one mounting block to defrost (note – ensure the block is clean and the freezing base block is scraped, otherwise the block will get stuck frozen later!!)
4. Transfer OCT blocks on dry-ice, defrost a little and mount on block with OCT
5. Trim block at 20-40um amd cut section at 10um thickness
6. Air dry slides for 20 mins at RT before fixation and staining
7. Aim to cut 3 slides per block (1 for Oil-red-O, 1 for IHC-f, 1 for trichrome)

(vi) Masson's trichrome staining for FFPE sections

Material

1. Merck Masson's trichrome (1.00485.0001) – Azophloxine (red, solution 1), Tungstophosphoric acid orange G (orange, solution 2), Light green SF (green, solution 3)
2. 1% acetic acid (will need about 1L per round of staining)
3. Histoclear and EtOH in 100%, 96%, 70% dilution

Procedure

1.	Histoclear	5 mins x2
2.	100% EtOH	30 secs x2
3.	96% EtOH	30 secs x2
4.	70% EtOH	30 secs x2
5.	Rinse in 1% acetic acid briefly	few seconds
6.	**Solution 1** (Azophloxine, red)	**9 mins**
7.	Rinse in 1% acetic acid	30 secs
8.	**Solution 2** (Tungstophosphoric acid orange G)	**2 mins**
9.	Rinse in 1% acetic acid	30 secs
10.	**Solution 3** (Light green SF)	**2 mins** (1 mins if thick sections)
11.	Rinse in 1% acetic acid	30 secs
12.	70% EtOH	1 mins
13.	96% EtOH	30 secs x2
14.	100% EtOH	30 secs x2
15.	Histoclear	5 mins x2
16.	Mount with NeoMount	

For trichrome staining for frozen sections, slides should be 4% PFA fixed for 1 hour and rinse in water before 1% acetic acid in step 5 above

(vii) Haematoxylin and eosin staining for FFPE sections

Material

1. Gill 2 Haematoxylin (Sigma) – this is a progressive haematoxylin stain
2. Eosin Y alcoholic (Sigma) – this gives good staining but is alcoholic so evaporate quickly
3. Histoclear and EtOH in 100%, 96%, 70% dilution
4. NeoMount

Procedure

1.	Thoroughly dry FFPE sections (staining time is empirical and the timing below is optimised for 4-5 um sections)	
2.	Run down to water	
3.	**Gill 2 haematoxylin**	**2.5 mins**
4.	Rinse in **tap water** (more alkaline than ddH2O)	few changes until water is clear
5.	Dip in 70% EtOH	15 secs
6.	Dip in 95% EtOH	15 secs
7.	**Eosin Y Alcoholic**	**1 mins**
8.	Rinse in water	30 secs
9.	Dehydrate, clear, and mount in NeoMount	

APPENDIX C - ETHICS