on

Carotid Plaque

Modification.

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

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March 2002
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Acknowledgements.

My sincere thanks go to Professor PRF Bell for giving me the opportunity to perform the following work during my stay at Leicester University. I would like to thank him for his constant support, excellent supervision and invaluable advice. I would also like to thank the Royal College of Surgeons of England for the financial support that has enabled me to finish this work, and all the other staff at the Medical Sciences department at Leicester University in particular Dr Louise Jones without whose help sections of this work would not have been possible.

This work has taught me a little bit of surgery and small amount of science, but more importantly it has been a life experience that I will never forget.
Publications and Presentations resulting from this Thesis.

Grant Awards

June 1999.
Awarded the Kate Weeks fellowship by the Royal College of Surgeons of England. I was awarded this fellowship following submission of a proposal for a research project in open competition on a national basis. This was used to fund my M.D research project on the role of doxycycline in influencing carotid plaque stability.

Publications.

The Influence of Carotid Plaque Morphology on the Development of Cerebral Symptoms.
B. Axisa, AR Naylor, PRF Bell, MM Thompson.
Published, Vascular Surgery, USA.

A simple and reliable method of doxycycline determination in human plasma and biological tissues.
B. Axisa, AR Naylor, PRF Bell, MM Thompson.
Published, Journal of Chromatography B.

Doxycycline lowers MMP-1 production in carotid plaques.
B. Axisa, A.R. Naylor, N. London, PRF. Bell, MM Thompson
Published, Stroke. 2002 Dec;33(12):2858-64.

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Association of Surgeons of Great Britain and Ireland.
Doxycycline penetrates the carotid plaque and inhibits the MMP-9 secretion by macrophages in vitro, May 2000.

American Heart Association, USA
November 2000, New Orleans USA.
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Abstracts

British Journal of Surgery
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June 2000

Circulation
A Prospective Randomised Trial of Doxycycline in Patients undergoing Carotid Endarterectomy
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Chapter 1

Atherosclerosis of the Carotid Artery and its Clinical Manifestations
Vascular diseases of the nervous system are amongst the most frequent causes of admission to hospital. The annual incidence in the U.K. varies regionally between 66-160/100,000, with a prevalence of around 500/100 000, of which one third are severely disabled [1, 2]. Stroke patients utilise about 10% of NHS bed days, and use up to 5% of the annual expenditure [3]. One year after onset, up to 25% of stroke patients will have died and about 35% of the survivors will be dependant on some form of daily assistance [3].

1.0 Stroke

Stroke is a generic term, lacking pathological meaning. It can be defined as a disease secondary to disorders of the blood or blood supply to the brain, with symptoms lasting longer than 24 hours [4]. There are two broad categories of stroke: ischaemic or haemorrhagic. Haemorrhagic stroke occurs in about 15% of cases and results from bleeding into the brain parenchyma from a variety of causes e.g.: aneurysms, arteriovenous malformation, neoplasms, anticoagulant therapy etc. Ischaemic stroke is much more common than haemorrhagic stroke, being responsible for about 85% of cases.

Causes of ischaemic stroke

The causes of ischaemic stroke are summarised in table 1 on the next page. By far the commonest cause is atheromatous disease of the extracranial vessels. The earliest report associating stroke and extracranial artery disease was made by C Miller Fisher, who in 1951 related atherosclerotic disease at the carotid bifurcation to ischemic symptoms in the ipsilateral eye and brain [5]. This thesis concentrates on the role of the carotid plaque in the production of cerebral symptoms, and only this aspect of stroke aetiology will therefore be discussed further.
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Table 1: Causes of ischaemic stroke
1.1 Risk factors for Stroke

The traditional risk factors for stroke are the risk factors for the development of atherosclerotic disease, i.e. age, sex, race, hypertension, smoking, hyperlipidaemia and diabetes. In addition several other associations have been identified in recent years which are specific to stroke per se.

Traditional risk factors.

Age

Of all the factors that have been shown to have an effect on the development of atherosclerosis, age has the strongest and most consistent association. Lesions appear in the aorta in the first decade of life, in the coronary arteries in the second, and in the cerebral vessels in the third [6, 7].

Race

Several studies have shown a large geographical variation in the incidence of stroke, however this may be a reflection of different diagnostic criteria, medical facilities, and differences in age sex distributions between populations [8]. In a study on a multiethnic population in south London, 21% of whom were black, Stewart et al found that the incidence of strokes in a black population were nearly twice as high as that in the white population (2.21/1000 compared to 1.25/1000) [9].

Hypertension

A large number of prospective randomised trials have shown that hypertension is the single most important risk factor for stroke in both men and women [10-15]. A review of 14 prospective randomised trials has shown that a
reduction in diastolic blood pressure of 5-6mmHg is associated with a reduction in stroke mortality of 35-40% [16].

The relative influence of diastolic and systolic blood pressure on the risk of stroke varies between different studies. In a 12-year follow up of men in the Oslo study, diastolic blood pressure was a stronger predictor of stroke mortality than systolic blood pressure [17]. In a 20 year study in Lithuanian men, both systolic and diastolic blood pressure were strongly associated with an increased risk of stroke mortality [18]. Similarly, in the Renfrew/Paisley study in Scotland, both systolic and diastolic blood pressure were positively associated with increased risk, and the relative risk for a 1-SD increase in systolic and diastolic blood pressure were similar, suggesting that either is a good indicator of future mortality risk [19].

In a number of countries, a decline in mean blood pressure following starting antihypertensive therapy was associated with a significant decline in stroke rate [14] [20]

**Smoking**

Smoking has been shown to be a very strong risk factor for stroke in many epidemiological studies [21-26]. In a meta analysis of thirty-two separate studies the overall relative risk of stroke associated with cigarette smoking was 1.5, the figure rising to 1.9 for cerebral infarction, with the risk associated with smoking being higher in the lower age groups [27]. A dose response relationship between the number of cigarettes smoked and relative risk has also been observed, and there is a small increased risk in women compared to men [27, 28]. Other studies have shown that cessation lowers the risk of stroke to that of non-smokers [21, 28].
The relative risk of stroke among hypertensive smokers is five times that among normotensive smokers but 20 times that of normotensive non-smokers [29].

**Serum cholesterol**

The association between elevated cholesterol levels and stroke is not clear cut, with some studies showing an increased risk and others no association [30]. In a meta-analysis by Quizibalbash, elevated cholesterol levels above 5.7mmol/L were associated with an increased pooled risk of 1.3, but the Prospective Studies Collaboration failed to show an association between cholesterol and incidence of stroke in an analysis of 13000 strokes in 450,000 people [31, 32]. However in the latter study most of the cohorts studied did not include non fatal strokes, and different stroke subtypes were not clearly delineated. Thus a positive association with ischaemic stroke could be counterbalanced with a negative association with haemorrhagic stroke [32, 33]. The level of risk of ischaemic stroke varies with the relative proportions of HDL and LDL cholesterol, with the former having a protective and the latter a deleterious effect [34, 35].

**Diabetes Mellitus**

A number of studies have correlated an increased risk of stroke and mortality in diabetics [36, 37] [38]. In the Framingham Study, the risk of stroke was 2.6-fold higher in men with NIDDM and 3.8-fold higher in women with NIDDM than in nondiabetic subjects of the corresponding sex [39]. Furthermore, diabetic patients have more severe strokes and a higher mortality rate after stroke [40]. In the study by Hart et al, men and women who were diabetic had a 3 fold greater stroke
mortality compared with non diabetics [19]. Diabetes was the strongest risk factor for stroke mortality in Finland [41].

As in the case of cholesterol, the data on the association of blood glucose levels and risk of stroke in non-diabetics varies. In a study by Hart et al, higher rates of stroke were seen in the top 5% of glucose levels for men and women, although this only reached significance in women [19]. In the Whitehall study, middle aged men with glucose tolerance values in the top 5% of the glucose distribution were found to have double the risk of stroke mortality [42]. These results suggest a threshold effect of glucose with stroke incidence and mortality among non diabetics. Similarly, non fasting glucose was positively associated with ischaemic stroke mortality in the British Regional Heart Study and in an Israeli study, but it was not found to be an indicator of stroke risk in a 12 year follow up of men without diabetes in a study in Oslo [43, 44].

Emerging risk factors for stroke

Chlamydia Pneumoniae and other chronic infections

A large number of studies have identified Chlamydia Pneumoniae in atheromatous plaque and its presence has also been associated with carotid plaque instability and an increased risk of stroke [45-48] [49]. Although a causative effect of the organism in atheromatous plaques has not been proved, the presence of the organisms might be an important factor in plaque progression [50, 51].

The potential role of Chlamydia Pneumoniae in carotid plaque destabilisation will be further discussed in chapter 4. Studies have shown that eradication of Chlamydia Pneumoniae is possible using macrolide antibiotics. One study using roxithromicin has shown a reduction in the number of plaques positive
for Chlamydia Pneumoniae mRNA in 12 out of sixteen plaques from patients on placebo to only five out of sixteen patients on the active antibiotic [52]. Another study using azithromycin has shown a reduction in symptoms in patients with unstable angina who were administered the antibiotic, suggesting that this organism may be involved in acute atheromatous plaque instability to be discussed later [53].

Other infectious agents including cytomegalovirus, herpes simplex virus type one and helicobacter pylori have been identified in carotid plaques and it has been suggested that their presence may predispose to plaque instability and stroke [54, 55]. Further studies are needed to elucidate the exact role of these organisms in the production of cerebral symptoms.

**Homocysteine levels**

Homocysteine is a sulphur containing aminoacid produced by the demethylation of methionine. Marked elevations of homocysteine in the blood due to rare inherited defects of enzymes involved in its metabolism are associated with premature cardiovascular disease [56]. Moderately elevated levels of the aminoacid, reflecting the normal genetic polymorphisms, are common in the general population [57].

In a prospective case control study on a cohort of middle aged men in the UK, the British Regional Heart Study cohort, a strong independent association between homocysteine concentration and stroke was observed [58]. Similarly, in the Rotterdam study and Framingham cohorts of patients, the risk of stroke and myocardial infarction increased directly with total homocysteine levels [59, 60]. However, in another cohort of middle aged men and women in Finland, no association was observed [61]. Additional prospective studies are required, with
sufficient power to characterise the form of association between homocysteine and risk, linear or threshold, and to study the interactions between homocysteine and other risk factors; as modest effects of homocysteine levels on stroke rates will have profound implications on public health [62].

Fibrinogen levels

Elevated fibrinogen levels have been associated with an increased risk of stroke in a number of studies [63-65]. In a meta analysis of six prospective epidemiologic studies involving a total of 92147 patient years, Ernst et al found that elevated fibrinogen levels were associated with myocardial infarction and stroke [66]. However, fibrinogen is an acute phase reactant, and its levels are affected by a variety of confounding factors which have themselves been found to increase the risk of stroke e.g. age, smoking, diabetes and LDL[67-69] . More data from prospective studies is needed in order to assess the effect of fibrinogen levels as a separate risk factor in stroke.

1.2 Pathogenesis of atherosclerosis.

Although several theories about the pathogenesis of atherosclerosis have been put forward, the most widely accepted is the 'modified response to injury' hypothesis first put forward by Russell Ross in 1976 [70, 71]. This theory has subsequently been modified several times in view of emerging data [72-76]. It regards atherosclerosis as an inflammatory- proliferative response in the artery wall in response to various forms of insult to the endothelium and smooth muscle cells, under the influence of various cytokines and other inflammatory mediators released by the inflammatory cell infiltrate [73].
Lesion prone sites.

Although atherosclerosis is regarded as a systemic disorder, a number of sites have a predilection for development of atherosclerosis. These lesion prone sites include branch points and areas of curvature within arteries and emphasise the importance of focal haemodynamic factors in the development of atherosclerosis [77].

These lesion prone areas differ structurally and functionally from non-lesion prone sites. They are characterised by increased permeability of the endothelium to plasma proteins such as albumin, fibrinogen, and LDL cholesterol [77, 78] [80]. The glycocalyx coat of the endothelial surface is also reduced in thickness, and endothelial cell turnover is greater in these areas [81]. They are also the sites that show a marked increase in intimal cholesterol accumulation in animals challenged with a high cholesterol diet [81].

Accumulation and modification of lipoproteins.

Endothelial injury from a variety of causes (e.g. smoking, shear stresses), increases the permeability of the cells and allows low density lipoprotein and other large molecules to penetrate to the subendothelium [82-86]. This process is directly proportional to the concentration of circulating lipoproteins [87]. Studies have shown that for any given concentration of lipoprotein in the plasma, the fraction of lipoprotein retained in the artery wall was a greater determinant of fatty streak development [88]. Once beneath the endothelium, low density lipoprotein, which has a high affinity for glycosaminoglycan, becomes trapped, and is subject to a variety of changes which result in the formation of modified lipoprotein. One modification which is believed to be of particular importance is oxidation, either by
free oxygen radicals or by lipoxygenases of endothelial and macrophage origin [89-91]. This process yields a variety of short chain aldehydes, ketones, and other substances which can become covalently crosslinked to the apoprotein B moiety of LDL and can thus bind to the scavenger receptor on macrophages allowing internalisation [92]. Another important modification is glycation, which can be a very important mechanism underlying the rapid progression of atherosclerotic disease associated with diabetes [86, 93, 94].

Modified LDL has a number of biological functions. It is a chemoattractant for peripheral blood monocytes, and it augments the expression and synthesis of monocyte chemoattractant protein-1 (MCP-1), and the synthesis of interleukin-1(IL-1) and macrophage colony-stimulating factor (M-CSF) [95]. It inhibits monocyte migration and causes inactivation of endothelium derived relaxing factor. It is also cytotoxic, a factor which is believed to be very important in the formation of the necrotic core associated with lesion progression to be discussed later. Finally it is an antigenic molecule, and this may be important in inducing an associated autoimmune element which has been described in the pathogenesis of atherosclerosis [79, 86].

LDL uptake is mediated by low density lipoprotein receptors which are under negative feedback control, thereby limiting the amount that can be taken into a particular cell. In contrast, modified low density lipoprotein uptake is mediated by scavenger receptors, which are not subject to any feedback control [96]. This allows the macrophages and smooth muscle cells to take up large amounts of lipoprotein resulting in the formation of foam cells, so called because the droplets of lipid in the cytoplasm give them a foamy appearance.
Other lipoproteins, including Lp(a) and small dense lipoproteins, are also subject to modification and may be involved in the formation of the atherosclerotic plaque [97].

The importance of oxLDL in atherosclerosis was first established through the use of the antioxidant probucol in the Watanabe Hyperlipaemic rabbit (WHHL). Treatment of these animals with probucol results in a lower percentage of diseased vessels, and it also appears to be capable of reducing foam cell formation and may affect monocyte recruitment in the early phases of hypercholesterolaemia [98, 99]. Adherence of monocytes at lesion prone sites was reduced in cholesterol fed rabbits treated with probucol [81]. A large trial carried out in Sweden to test the effects of probucol on hypercholesterolaemic individuals (n=303), the Probucol Quantitative Regression Swedish Trial (PQRST), has not shown any significant effect on reduction in atheroma in the femoral artery of the treated group as compared to controls [100]. Failure to achieve a clinical benefit was partly attributed to a 35% reduction in HDL cholesterol levels in patients on probucol as compared to control, HDL being the principal lipoprotein responsible for removing cholesterol from the tissues [101].

**Monocyte recruitment and activation: The Fatty Streak**

The earliest visible atherosclerotic lesion is the fatty streak. Fatty streaks are the result of a marked focal increase in monocytes at the sites of atherogenesis. The monocytes attach to the underlying endothelium by means of cytoplasmic processes, which appear to facilitate their migration through the endothelium into the intima.
Adhesion of monocytes is not a random phenomenon but involves the surface expression of endothelial leucocyte adhesion molecules (ELAM's), vascular cell adhesion molecules (VCAM's), and the secretion of a variety of cytokines, such as interleukin-1β and monocyte colony stimulating factor [74].

Following adherence to the endothelial cells, the monocytes migrate to the subendothelial space under the influence of a number of chemoattractant molecules [79]. The most important is monocyte chemoattractant protein-1 (MCP-1), derived from smooth muscle cells, for which the cells express specific receptors. Other chemoattractants include oxidatively modified LDL, and transforming growth factor-β (TGF-β) [74, 86].

Once in the subendothelial space, the monocytes undergo activation differentiation to become macrophages, and secrete a variety of cytokines that augment the local inflammatory process resulting in the atherosclerotic lesion [102]. The cells are influenced by migration inhibition factors, such as oxidised LDL to remain in the intima of the vessel wall. They accumulate large amounts of modified lipoprotein to form foam cells and the resulting focal accumulation of lipid laden macrophages and smooth muscle cells is called the fatty streak [96].

Macrophages are the principal source of inflammatory mediators in the atheromatous microenvironment [73, 86]. They can internalise oxLDL through the scavenger receptor and through a putative oxLDL receptor, and can oxidise LDL through several pathways including lipoxygenase enzymes. They secrete numerous growth factors, in particular PDGF, IL-1, and TNF-α, which can in turn stimulate PDGF gene expression by smooth muscle cells and endothelium. One particular form of atherosclerosis where macrophages and T lymphocytes occur in large numbers are the atherosclerotic lesions associated with transplant rejection. It is
postulated that a localised immune response may exacerbate the macrophage /T cell interactions and macrophage smooth muscle interactions leading to a pronounced proliferation of these cells. A similar immunologic process may be operative in atherosclerosis [86].

The critical role the macrophages play in the inflammatory response leading to lesion progression suggests that finding means of controlling macrophage participation at all levels could be critical in modifying lesion progression [86].

**Smooth muscle proliferation: The Fibrous Plaque.**

The fatty streak can regress, but very often undergoes progression to form a fibrous plaque [97]. This consists of a fibrotic cap consisting of smooth muscle cells recruited from the media. Smooth muscle cell migration is under the influence of PDGF and insulin-like growth factor-1 (IGF-1), released by platelets adhering to the dysfunctional endothelium over the plaque and from endothelial cells, macrophages and smooth muscle cells in the lesion [103-105]. PDGF and other mitogens including insulin-like growth factor (IGF-1), basic fibroblast growth factor (b-FGF), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) stimulate these cells to undergo mitosis [106]. Beneath the fibrous cap, the fibrotic lesion consists of foam cells and debris derived from cell necrosis as well as free cholesterol crystals and deposits of calcium. Necrosis in the deeper layers is believed to result from the cytotoxic effect of modified lipoproteins, the release of free radicals, and possibly ischaemia due to the increased diffusion distance between the tissues and blood. Calcium deposits in the deeper layers of the plaque are attributable either to the crystallisation of
calcium on pre-existing cholesterol crystals or to the osteogenic activity of cells within the lesion [107, 108].

A very important factor leading to plaque progression is the adherence of platelets to areas of focal loss of endothelial cells. This focal loss results from shear stresses placed on dysfunctional endothelial cells and the toxic effect of free radicals and oxidised lipoproteins released from the underlying foam cells [84]. Adherent platelets release a number of factors which promote lesion development. One of these is heparinase, which degrades heparan sulphate, a polysaccharide in the extracellular matrix that normally inhibits smooth muscle cell migration. Loss of endothelial cells also results in reduced secretion of prostacyclin (PGI2) and endothelial derived relaxing factor-nitric oxide. Prostacyclin normally inhibits platelet aggregation and nitric oxide is a potent vasodilator and inhibitor of platelet adhesion and aggregation [109, 110]. The reduction in heparan sulphate and reduced secretion of PGI2 and nitric oxide may permit smooth muscle cells in the arterial media to change from a contractile to synthetic state, which enables them to release enzymes facilitating migration from the media into the intima, and to synthesise extracellular matrix [111] [112]). While in the synthetic state, smooth muscle cells can also respond to PDGF that they secrete as well as to other growth stimulators. Their synthetic activity will determine the matrix content of a lesion, and thus the smooth muscle cell plays the principal role in the fibroproliferative component of atherosclerosis [86].

Changes in the relative proportions of macrophages and smooth muscle cells in the atheromatous plaque have been associated with the propensity of a plaque to become unstable, with a relative increase in the content of plaque macrophages and a decrease in plaque smooth muscle cells being associated with plaque instability
Advanced lesion formation - The Complicated Plaque.

The fibrotic plaque may be stable for a number of years, but under certain circumstances may develop fissures or ulceration, intraplaque haemorrhage, and overlying thrombus formation, resulting in a complicated plaque. The relationship of these gross morphological changes in relation to development of symptoms in the carotid atheromatous plaque will be discussed in chapter 3.

Factors responsible for plaque rupture include turbulence or mechanical shear stress, intraplaque haemorrhage, or the release of matrix degrading enzymes by macrophages present in the lesion. The latter group of enzymes and their relation to atheromatous plaque stability will be discussed in chapter 4, with particular reference to their role in carotid plaque stability.

1.3 Clinical manifestations of Carotid Artery Disease

Disease at the origin of the internal carotid artery is not necessarily associated with the production of cerebral symptoms. Data from the ECST and NASCET trials as well as other similar trials has shown that the incidence of symptoms increases with the degree of stenosis, but even in patients with up to eighty percent stenosis of the internal carotid artery, only 32% are symptomatic, with the rest being asymptomatic. The recent increase in the use of duplex ultrasound in assessing carotid artery disease has further emphasised the fact that a
large number of patients with disease of the internal carotid artery are asymptomatic.

The most common clinical manifestations of carotid artery disease are transient ischaemic attacks, amaurosis fugax, and a variety of stroke syndromes which vary according to the main artery affected.

Transient ischaemic attacks

Transient ischaemic attacks are episodes of focal neurological deficit which resolve in 24 hours or less and leave no residual deficit. They are important harbingers of cerebral infarction. A reduction in cerebral blood flow below 20-30mL /100g /min produces neurological symptoms. The development of infarction is a consequence of the degree of reduced flow and the duration of such a reduction. If flow is restored within a critical period, ischaemic symptoms may partially or completely resolve. TIA's are largely due to emboli which subsequently break down or are dislodged to smaller vessels distally.

Transient ischaemic attacks produce symptoms depending on the area of the brain affected, and symptoms include transient hemiparesis, hemisensory disturbance, dysphasia, dysarthria, or any other symptom complex to be described below.

Following a TIA, 5-10% of patients will develop infarction in each year of follow up. However, not all patients who have cerebral infarction have had a warning TIA.
Amaurosis fugax

Amaurosis fugax is an episode of transient monocular blindness. It is a special form of TIA caused by retinal embolism from the carotid circulation. As in the case of TIA's, it is a warning of a potential episode of cerebral infarction.

Large vessel occlusion syndromes

Internal carotid artery

The full clinical picture consists of contralateral hemiplegia, contralateral hemisensory disturbance, homonymous hemianopia of the contralateral side, and gaze palsy to the opposite side (i.e. eyes deviated to side of lesion). If the dominant hemisphere is involved, global aphasia will result. A partial Horner's syndrome may develop on the side of the occlusion due to involvement of sympathetic fibres on the internal carotid wall. The clinical syndrome of carotid artery occlusion results in a mortality rate as high as 25%.

The outcome of carotid occlusion depends on collateral blood flow from the circle of Willis, but in addition the external carotid artery may provide flow to the anterior and middle cerebral arteries via the meningeal branches and retrogradely through the ophthalmic artery to the internal carotid artery.

Anterior cerebral artery

The clinical picture depends on the site of the occlusion. Occlusion proximal to the anterior communicating artery is usually well tolerated because of cross flow. Distal occlusion results in weakness and cortical sensory loss in the contralateral lower limb with associated incontinence. Proximal occlusion when both anterior cerebral arteries arise from the same side results in cerebral paraplegia with lower
limb weakness, sensory loss, incontinence, and presence of grasp, snout and
palmomental reflexes.

Middle cerebral artery

Occlusion of the middle cerebral artery results in contralateral hemiplegia
(with relative sparing of the leg), contralateral hemianaesthesia and hemianopia,
and aphasia if the dominant hemisphere is involved. If cortical branches are
affected separately, the clinical picture is less severe, for example involvement of
the parietal branches alone may produce Wernicke's dysphasia with no limb
weakness or sensory loss.

Posterior cerebral artery

The posterior cerebral artery supplies midbrain structures, the choroid
plexus, and posterior thalamus. Cortical branches supply the undersurface of the
temporal lobe, occipital and visual cortex.

Occlusion of the posterior cerebral artery will result either a midbrain or a
thalamic syndrome, or a mixture of both. Occlusion of branches supplying the
midbrain result in a third nerve palsy with contralateral hemiplegia (Weber's
Syndrome)

Occlusion of thalamic branches will result in cerebral symptoms such as
chorea or hemiballismus with hemisensory disturbance.

Effect of collateral circulation.

The result of occlusion and stenosis of the vessels supplying the brain
depends both on the rate of development of the lesion as well as on the
effectiveness of the collateral circulation. If the blood supply to a region of the
brain can be restored quickly enough, a reversible neurological deficit ensues (Tia
or Amaurosis Fugax). If flow is not restored, a permanent deficit will occur whose extent depends on the size of the ischaemic area. In clinical practice the clinical syndromes described above are not clear cut, and variable manifestations of two or more syndromes occur depending on the area supplied by the compromised vessels.
Chapter 2

The Clinical Management of Carotid Artery Disease
Carotid artery disease is managed either by medical treatment or surgical intervention. Management depends on the degree of stenosis produced by the atheromatous plaque, on the presence or absence of symptoms, and on the state of the contralateral artery. Patients who do not qualify for operation are treated by best medical management.

2.0 Best Medical Management.

Best medical management involves optimisation of risk factors. This includes cessation of smoking, treatment of hypertension, hypercholesterolaemia and diabetes, antiplatelet therapy, and control of cardiac arrhythmias including anticoagulation for atrial fibrillation if not contraindicated.

Smoking.

In the British regional heart study, which included 7735 men aged 40-59 years, current smokers had a 4 fold relative risk of stroke compared with non smokers, while ex-smokers showed a lower risk than current smokers but a greater risk than non smokers in a 12.75 year follow up [116]. The benefit of giving up smoking was seen within 5 years of giving up smoking. Light smokers reverted to the risk of those who had never smoked while heavy smokers retained an increased risk over never smokers [116]. The greatest absolute benefit of stopping smoking was seen in hypertensive subjects [117, 118]. A number of other studies have also shown a beneficial effect of cessation of smoking similar to the above [25, 26, 119, 120].
Hypertension

Hypertension is the most important risk factor in the development of stroke. It is also the risk factor most amenable to treatment [20, 121]. The results from 18 controlled trials show a reduction in relative risk of stroke of 25-47% among treated hypertensive patients[122]. This reduction applies both to the elderly and to younger patients, but the absolute reductions are greater among the elderly and the number of patients with hypertension that need to be treated to prevent a stroke is lower in the elderly because they have a higher risk of stroke [121]. The reductions in relative but not absolute risk appear to be similar for both isolated systolic hypertension and combined systolic and diastolic hypertension in the elderly [123]. In addition, in a review of nine trials including a total of 6752 patients, drug treatment reduced the incidence of stroke recurrence compared to controls (RR 0.72) [124].

Hypercholesterolaemia

Although as discussed earlier a concrete association between levels of cholesterol and stroke is not clear cut, a large number of studies using the cholesterol lowering agents HmgCoA reductase inhibitors have shown a significant reduction in stroke rates in patients treated with these drugs [125, 126]. In a meta-analysis of 462 strokes in 20438 patients, Blau et al found a 31% reduction in the risk of stroke in patients on HmgCoA reductase inhibitors compared to controls [127]. The reduction in stroke rates may not be solely dependant on cholesterol lowering mechanisms but may involve effects on endothelial cells, platelets, macrophages, and smooth muscle cells [126, 128-130]. Other possible mechanisms include an effect on matrixmetalloproteinase activity in carotid atheroma with consequent plaque stabilisation, which will be discussed fully in chapter 4.
Diabetes

Diabetes increases the risk and severity of stroke, and the complications of diabetes including nephropathy, retinopathy, and micro and macroangiopathy are all associated with an increased risk of stroke [131, 132]. In addition to its effect on atherosclerosis, uncontrolled diabetes results in proliferative lesions in the brain microvasculature resulting in occlusion and stroke [133]. Tight diabetic control reduces the rate of progression of these complications and reduces the overall stroke risk.

Atrial fibrillation

Atrial fibrillation is associated with an increased risk of ischaemic stroke. On average, patients with AF have a stroke risk of 4.5% per year. Anticoagulation reduces this to around 1.5% per year, a 70% relative risk reduction [134]. Data pooled from five randomised trials comparing the effect of warfarin or aspirin with controls in patients having fibrillation, has shown that in women, warfarin reduced the risk of stroke by 84%, compared with 60% in men. Aspirin at a dose of 75mgs daily reduced the risk of stroke by 18%, while at a dose of 325mgs there was a 44% risk reduction [135, 136]. There was no significant difference in the annual rate of bleeding in the control (1%), aspirin (1%), and warfarin group (1.3%) [135]. These results have been replicated in other studies [137, 138]. The value of warfarin therapy in patients less than 75 years old is less clear because of a high risk of hemorrhagic complications [137]. Current practice guidelines for stroke prophylaxis recommend warfarin (target INR 2.5: range 2.0 to 3.0) for AF patients at high risk for stroke including those over 75 years of age or younger patients with additional risk factors [139, 140]. Aspirin should be reserved for low risk patients or those unable to take warfarin [141].
2.1 Surgical Management of carotid artery disease

The present accepted policies for operative intervention in carotid artery disease include:

1. Symptomatic severe stenosis of the carotid artery on the side ipsilateral to the symptoms (severe stenosis is defined as stenosis of more than 70% producing a velocity increase of more than 200cms/s on Doppler ultrasound [142])

2. Asymptomatic severe stenosis of the carotid artery if the contralateral side is occluded

3. Asymptomatic patients who are included in the European Asymptomatic Carotid Surgery Trial (ACST).

Similar guidelines for operation have been published by the American Heart Association committee in the United States [143].

The first carotid endarterectomy was carried out in 1953 by Dr Michael DeBakey, although he did not publish his technique till 1975 [143a]. Eastcott et al published the first report of an operation to treat disease of the extracranial carotid arteries in 1954, where a diseased segment of artery at the bifurcation of the common carotid was excised and the artery reanastomosed the ends [143b]. In the following decade the operation was performed in an ever-increasing number of patients, although no trials had been carried out to ascertain its efficacy. It then became apparent that the operation itself had a significant degree of mortality and morbidity and that a proportion of operations resulted in strokes, which the operation originally set out to prevent.

In view of this two large international clinical trials as well as several smaller trials set out to establish its efficacy in preventing stroke and defining more clearly the subgroups of patients most likely to benefit from the operation. The major trials dealt
with the role of the operation in symptomatic and asymptomatic subgroups, and these will now be discussed in turn.

**Symptomatic carotid stenosis.**

Three large multicentre trials were carried on patients with symptomatic carotid stenosis; the European Carotid Surgery Trial (ECST), and the North American Symptomatic Carotid Surgery Trial [144], and the Veterans Affairs Cooperative Studies trial, and all published their results in 1991 [145-147]. The ECST enrolled 3024 patients until its termination in 1996, and followed patients for a period of five years [145]. Patients were divided into three subgroups depending on the severity of stenosis: 0-29%, 30-69%, and 70-99%. The NASCET study included 2946 patients with a stenosis between 30 and 99% [146]. The Veterans Affairs Trial included 189 men with a stenosis of more than 50% [148]. The results and conclusions from all three studies were similar.

All three studies used angiography to assess the degree of stenosis in the diseased artery. In the NASCET and Veterans Affairs trials the percentage stenosis was measured by the ratio of the diameter of the diseased artery at the point of greatest stenosis with the diameter of the normal artery beyond the carotid bulb. The ECST trial measured the ratio of the narrowest segment of the internal carotid artery or the distal common carotid if more diseased to the estimated normal arterial wall at the level of the stenosis. The ECST trial therefore graded stenoses as higher than if they had been scored with the NASCET method. However the corresponding values for the two trials have been calculated and the data for the different patient subgroups is still comparable [149, 150]. During the period that the major trials were going on duplex scanning has taken over angiography as the most commonly used method of assessment of carotid artery disease, and in many centres this is the only method of
imaging used to assess disease prior to operation [151-153]. Angiography itself has a small but important risk of stroke, which can rise as high as 3.7% if patients with symptomatic disease are considered [154]. In practice this has the effect of effectively improving the overall outcome of surgically treated patients and reducing the morbidity of screening when compared to the major trials.

Severe stenosis

The results for patients with severe (70-99%) stenosis are shown on table 1. The operation carried a perioperative risk of any stroke or death of 5.8 - 7.5%, and a 2.1 to 3.7% risk of major stroke or death. However, the initial operative risk was soon offset by a reduction of ipsilateral stroke from 26% in the medical patients to 9% in the surgical patients at 24 months in the NASCET trial, and from 16.8% to 2.8% in the ECST [155]. This represents an absolute risk reduction of 14-17% over 2 to 3 years. There was a similar positive effect of surgery for all strokes (including contralateral, vertebrobasilar, and haemorrhagic), but the greatest effect was noted in the ipsilateral stroke risk. The greatest benefit from the operation was derived until 3 years after the operation. Beyond this point, the curves for the medical and surgical groups converged and ran parallel to each other. The medical group had the greatest risk of stroke in the first 2 years after randomisation, following which the risk fell drastically to the same levels as the surgical group, probably due to stabilisation of the plaque or possibly development of collaterals [155, 156].

<table>
<thead>
<tr>
<th>OUTCOME</th>
<th>ECST</th>
<th>NASCET</th>
<th>V.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute reduction in ipsilateral strokes</td>
<td>14%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td>Absolute reduction in all major strokes and death</td>
<td>9.6%</td>
<td>16.5%</td>
<td>17.7%</td>
</tr>
<tr>
<td>All perioperative stroke and death.</td>
<td>7.5%</td>
<td>5.8%</td>
<td></td>
</tr>
<tr>
<td>Perioperative major stroke and death</td>
<td>3.7%</td>
<td>2.1%</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Results of ECST, NASCET and Veterans Affairs trials in patients with severe symptomatic stenoses (70-99%).
Figure 1. Kaplan-Meier survival curves to show survival free of major stroke (with non-stroke deaths occurring more than 30 days after surgery censored) in surgery and control patients with 80-99% stenosis of symptomatic carotid artery. The slope of the curve is proportional to the risk of major stroke. The slopes of both groups are parallel after 2 years, showing that the risk in both groups are the same.

Adapted from the European Carotid Surgery Trialists' Collaborative Group (Lancet 1998; 351: 1379-87).

The data shows that assuming an operative risk of 7.5%, 17 strokes are prevented for every 100 endarterectomies carried out. This means that six operations are necessary to prevent one stroke over the next 30 months. If the operative risk is reduced to 5% (absolute risk reduction of 19.5%), one stroke is prevented for every five operations carried out. In its final report in 1998, the ECST trial collaborators reviewed the results and added the sex and age of the patient into the analysis. Addition of these variables revealed that in general men derive more benefit than women, and the stroke free life expectancy is dependent on age (as expected). They also revised the cut off point at which they recommended surgery to 80% as opposed to the previous cut off point of 70%. However, it was recognised that any clinical
decision needs to take into account the local surgical expertise and complications record, and any decision to operate must consider all these variables [157].

**Moderate stenosis**

In the ECST trial, 1599 patients with moderately stenosed internal carotid arteries (30-69%) were randomised to receive medical or surgical treatment, (60 and 40%) respectively [158]. The trial showed that no benefit could be obtained over a period of 4-5 years in patients with 50-69% stenosis or over a period of 6-7 years in patients with 30-49% stenosis[158].

The NASCET trial, which randomised 1118 patients to medical and 1108 patients to surgical treatment showed a marginally significant reduction in the 5 year rate of ipsilateral stroke from 22.2% in the medically treated group to 15.7% in the surgically treated group (6.5% absolute risk reduction) [159]. This means that 15 operations would have to be performed to prevent one stroke over five years assuming the same level of expertise and low complication rate in the trial[159]. In the group with 30-39% stenosis, the failure rate was not significantly lower in the endarterectomy group compared to the medically treated group (14.9% and 18.7% respectively, P=0.16)[159]. The consensus of opinion at present is that carotid endarterectomy is not beneficial in patients with symptomatic moderate stenosis[156, 159]. Future studies including further information about plaques which are most likely to be symptomatic from noninvasive investigations like analysis of duplex ultrasound images may allow identification of a subgroup of patients with moderate stenosis who are likely to benefit from carotid endarterectomy.
Mild stenosis

Patients with mild stenosis (0-29%), were only investigated in the ECST trial [160]. This showed no benefit from surgery in this subgroup, and over the time of follow up and in the number of patients treated, surgery was actually shown to be detrimental. The study showed that in the next three years following a symptomatic episode in patients with this degree of stenosis, there were almost no strokes to be prevented (only one ipsilateral ischaemic stroke occurred over three years of follow up in the 374 patients randomised), and any benefits of surgery were far outweighed by its early risks [160].

Asymptomatic disease

There were 5 major trials investigating the efficacy of carotid endarterectomy in carotid artery disease:

<table>
<thead>
<tr>
<th>STUDY</th>
<th>Patients</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid Artery Stenos with Asymptomatic Narrowing: Operation Versus Aspirin Casanova study Group.</td>
<td>410</td>
<td>1991</td>
</tr>
<tr>
<td>Mayo Asymptomatic carotid Endarterectomy Group Mayo Asymptomatic Carotid Endarterectomy Study Group</td>
<td>71</td>
<td>1992</td>
</tr>
<tr>
<td>Asymptomatic Carotid Stenosis Trial Veterans Affairs Cooperative study group</td>
<td>444</td>
<td>1993</td>
</tr>
<tr>
<td>Asymptomatic Carotid Atherosclerosis Study Thirty-nine clinical sites across the United States and Canada</td>
<td>1662</td>
<td>1995</td>
</tr>
<tr>
<td>European Asymptomatic Carotid Surgery Trial St Mary's Hospital London.</td>
<td>1850</td>
<td>Ongoing</td>
</tr>
</tbody>
</table>

Table 2: Trials investigating the role of carotid endarterectomy in asymptomatic stenosis.
The Asymptomatic Carotid Atherosclerosis Study recruited 1662 patients with asymptomatic carotid artery stenosis of 60% or greater and patients were randomised to best medical treatment or endarterectomy [161]. After a median follow up of 2.7 years, the aggregate risk over five years for ipsilateral stroke and any peroperative stroke or death was 5.1% for surgical patients, and 11% for patients treated medically. (Aggregate risk reduction of 53%). It concluded that patients with asymptomatic carotid artery stenosis of 60% or greater and whose general health makes them good candidates for elective surgery will have a reduced 5-year risk of ipsilateral stroke if carotid endarterectomy performed with less than 3% perioperative morbidity and mortality is added to aggressive management of modifiable risk factors [161].

The Veterans Affairs Trial included 444 men with a stenosis of more than 50%, and showed a reduction in ipsilateral neurologic events (Tia and stroke) from 8.0% in the surgical group compared with 20.6% in the medical group [162]. However, there were no significant differences in two groups when analysis of all strokes and all deaths at 30 days post surgery was performed. The study estimated that a cohort of at least 3000 patients would be necessary to prove a significant benefit at if these figures were maintained [162].

The CASANOVA (Carotid Artery Stenosis with Asymptomatic Narrowing: Operation Versus Aspirin) study has been criticised for its methodology and found no significant difference in the number of neurological deficits in surgical versus medical groups [163]. The Mayo group recruited 71 patients but the trial was terminated early because of a significantly higher number of myocardial infarctions and transient cerebral ischemic events in the surgical group than in the medical group [164]. No data has been published yet from the European Asymptomatic Carotid Surgery Trial (ACST), and recruitment continues [165].
In a recent meta analysis published by the Cochrane collaboration all these trials were reviewed and the authors concluded that although there is some evidence favouring CEA for asymptomatic carotid stenosis, the effect is at best barely significant statistically, and extremely small in terms of absolute risk reduction [165]. Therefore, extreme caution should be exercised in translating the results into clinical practice. In particular, only those centres with a demonstrated low perioperative complication rate should contemplate performing CEA in patients with asymptomatic carotid stenosis [165].

2.3 Carotid Endarterectomy

The operation consists of opening up the internal carotid artery and "shelling out" the atheromatous intima. It can be performed under general or local anaesthesia. The patient lies supine with the head supported on a neck ring and turned slightly towards the opposite side. The pinna is bent forward and fixed with tape to allow access to the sternomastoid to be raised from the mastoid process should high exposure of the artery become necessary.

Depending on whether a vein or patch graft is going to be used a suitable segment of cephalic vein should also be identified and prepped if a vein patch is to be used.

An oblique skin incision is made along the anterior border of the sternomastoid muscle extending from the mastoid process to the sternoclavicular joint. The subcutaneous tissue and platysma are divided along the same line. The incision passes through cutaneous nerves and will therefore result in an area of permanent numbness beneath the line of the jaw. However the greater auricular nerve can usually be preserved.
The anterior facial vein is then identified and divided between ligatures. This allows the jugular vein to be displaced posteriorly. The hypoglossal nerve which may loop very low in the neck at this level should be identified and protected. The lower pole of the parotid gland lies superiorly and this may have to be mobilised to improve access taking care to avoid damage to the facial nerve.

The carotid sheath is then opened initially from the lower end to expose the common carotid artery. The vagus nerve lies posterior and deep to the artery in this position. A sling is then passed along the common carotid artery and the exposure is the continued in a cephalad direction in order to expose the bifurcation and then the internal carotid artery, which lies posterior to the external carotid at this level. The artery is then carefully dissected to allow the passage of a sling around it. The vessel should be manipulated as little as possible during the dissection in order to avoid dislodging any atheromatous debris or thrombus from the artery, with resultant dire consequences. If necessary the hypoglossal nerve can be slung upwards to protect it.

The origin of the external carotid artery is also dissected and a rubber sling passed around it.

**Brain protection during surgery.**

When the internal carotid artery is clamped intraoperatively, the blood supply to the hemisphere on the operative side is dependent on the adequacy of the collateral circulation from the Circle of Willis. If this is inadequate a shunt may be necessary to maintain flow to the brain during the procedure. Two methods are used to assess the adequacy of the collateral circulation during operation. Internal carotid artery stump pressures are measured by cannulating the artery after clamping and then connecting the probe to a transducer. The pressure measured in this way is a reflection of the adequacy of the circulation through the Circle of Willis. A mean pressure of about
60mmHg and a pulsatile waveform is often said to indicate adequate perfusion of the relevant hemisphere. If these criteria are not met then the artery is shunted. The second method involves monitoring cerebral function using electroencephalography (EEG), which produces abnormal patterns should the hemisphere become ischaemic during clamping.

Some surgeons always use a shunt, while others prefer to use a shunt selectively. There is a small amount of risk associated with shunt use due to damage to the intima and air embolism. However these risks become negligible with familiarity of use.

**Removal of the atheroma.**

A dose of heparin (5000 units) is given intravenously and sufficient time allowed for it to circulate. Clamps are applied to the internal carotid, common carotid and external carotid in that order. A longitudinal arteriotomy is then made in the common carotid artery which is extended into the internal carotid artery.

The Javid shunt is then clamped in the centre with a haemostat and inserted into the common carotid artery and retained with a ring clamp. It is then flushed by releasing the haemostat at the centre to avoid introducing any air emboli, and made into a loop. The distal end is carefully inserted into the internal carotid artery avoiding damage to the intima, and the central clamp released.

The assistant then holds open the loop of the shunt and the endarterectomy is commenced. Once the plane between the intima and media is identified and entered, dissection can usually be carried round the whole circumference using a Watson Cheyne or MacDonalds blunt dissector. Once round the circumference, the atheroma can be cut across, and the dissection continued distally into the internal carotid artery, were the intima either feathers off of is cut neatly. A number of interrupted sutures
(Kunlin sutures) are placed to secure the upper edge of the intima to prevent dissection. Some surgeons tack the lower end of the endarterectomy as well.

Once the endarterectomy has been carried out, the endarterectomised surface is flushed with heparinised saline making sure all debris is removed. The arteriotomy is then closed either primarily or using a vein, PTFE, or Dacron patch [166]. The rationale behind using a patch is that it reduces the risks of early postoperative thrombosis or late restenosis. Early carotid thrombosis occurs in about 3% of CEA's, and late restenosis affects about 13% of patients but the latter rarely have any clinical sequelae. Patch closure increases clamping time and synthetic material is susceptible to infection. In a review of six trials on 794 patients to address this question, the Cochrane Collaboration have performed a meta-analysis which seems to suggest a beneficial effect of patching on perioperative stroke and restenosis when compared to primary closure [167]. However, this finding has not been reproduced in other trials and larger trials are necessary to define the best method of closure [168].

After closure and removal of the shunt the clamps are removed starting with the internal carotid. This allows retrograde flow of blood and any air to escape through the suture line. The external carotid is removed next and finally the common carotid.

**Closure**

Layered closure is performed using absorbable sutures, and one or two drains are left at and around the site of the arteriotomy to drain any bleeding.

**Complications**

1. **Bradycardia and hypotension.** These are believed to be due to stimulation of the nerve to the carotid body. These are managed by careful fluid replacement and administration of atropine as necessary.
2. **Stroke.** If this occurs in the first few hours after surgery the patient is taken back to theatre and the endarterectomy re-explored, as this may be due to thrombotic occlusion of the artery.

3. **Neck haematoma.** A small haematoma may be allowed to resolve spontaneously but a large haematoma will need operative drainage.

4. **Cranial nerve lesions.** The operation may result in partial or complete loss of function of four cranial nerves. The cervical branch of the facial nerve may be traumatised usually by excessive traction, but this usually recovers completely.

   The hypoglossal nerve may be cut but is usually partially damaged resulting in deviation of the tongue to the affected side. This is also usually temporary if due to excessive traction.

   The glossopharyngeal nerve is rarely damaged due to its location well superior to the carotid bifurcation, but if damaged the deficit is quite troublesome as it produces difficulty in swallowing.

   Injury to the vagus nerve is also rare due to its deep location, though it may be damaged when slinging the artery. Injury results in hoarseness which may be temporary or permanent.

**Follow-up.**

Follow up varies with local protocols but in general patients are seen six weeks after surgery, and six months later when they are discharged. No routine follow up scans are performed except in patients recruited in clinical trials.
Chapter 3

The Influence of Carotid Plaque Morphology on the Development of Cerebral Symptoms.
3.0 Introduction

In recent years the results of randomised prospective trials have clearly defined the beneficial effect of carotid endarterectomy in the management of carotid artery disease. The ECST and the NASCET trials have both shown conclusively that symptomatic patients with severe stenosis of the internal carotid artery have a significant reduction in the risk of stroke following carotid endarterectomy. [144, 155]. Figure 1 illustrates a summary of the data from the medical arm of the two studies, which effectively reflects the natural history of the disease, showing an approximate doubling of the risk of stroke in patients with severe (70-99%) stenosis as opposed to patients with mild degrees of stenosis (0-29%). As a corollary, the graph shows that a large proportion of patients with severe carotid artery disease may actually remain asymptomatic, emphasising the role of factors other than stenosis in the production of symptoms.

![Figure 1. Graph showing percentage number of symptomatic patients with increasing degrees of stenosis. (Data compiled from ECST and NASCET trials).](image-url)

Clearly the way forward is to identify potentially unstable plaques that are most likely to undergo plaque disruption and give rise to the catastrophic nature of atherothrombosis. The experimental work has therefore shown that factors other than stenosis are important in determining the clinical significance of carotid stenosis and that the role of carotid endarterectomy may itself in order to improve clinical outcome from carotid artery disease.

*Carotid endarterectomy: a product for life?*
Similarly, data from the asymptomatic carotid atherosclerosis study [169], has shown that unoperated patients with a greater than 60% diameter reducing carotid artery stenosis had only an 11% risk for ipsilateral hemispheric stroke at 5 years and a 19.2% 5-year risk for ipsilateral transient ischaemic attack or stroke.[169] Holdsworth et al found that from a cohort of 2590 patients, 151 asymptomatic patients had 80-99% stenosis and another 120 asymptomatic patients had occluded arteries [170]. On the other hand, a number of patients with mild stenotic lesions less than 50% proceed to develop stroke and this adverse clinical outcome might be averted by carotid endarterectomy. The introduction of duplex scanning of the carotid vessels has further emphasised the presence of severe disease in some asymptomatic patients, and only mild disease in some of the symptomatic ones. It is therefore apparent that factors in addition to the degree of stenosis are responsible for determining clinical outcome from carotid artery disease.

Clearly the way forward, is to identify potentially unstable plaques that are more likely to undergo acute disruption and give rise to symptoms, which would give the surgeon the opportunity to operate selectively on patients who are at greater risk. Carotid plaques can produce ischaemia either by stenosis at the origin of the internal carotid artery limiting blood flow or by undergoing disruption and giving rise to emboli to the brain. The Framingham study showed a high incidence of embolisation in stroke patients that was not of cardiac origin [2]. Attention was therefore turned in the last two decades to the morphology of the carotid plaque itself in order to try to identify factors which are indicative of instability and to identify the underlying biochemical factors resulting in acute disruption and cerebral symptoms. A large number of studies have now been published attempting
to identify histological features of unstable plaques and to correlate specific plaque morphology with the development of symptoms [6-13]. In addition, in recent years a lot of research has been undertaken in order to try to assess plaque morphology using non invasive means such as B mode ultrasound, MRI and even helical CT scanning [171-175]. Many authors have described an association between heterogenous plaques identified using B mode ultrasound and development of symptoms, even when stenotic progression was taken into account. Although the technology is still in its infancy, future developments such as 3- dimensional U/S will certainly give much more information about plaque morphology.

The reports in the literature regarding plaque morphology have broadly classified plaques into simple and complicated. Simple plaques consist of a central core of lipid surrounded by smooth muscle cells, collagen and elastic fibres. A fibrous cap of varying thickness separates the lipid core from the lumen. Complicated plaques are either ulcerated, have varying degrees of intraplaque haemorrhage, or have overlying thrombus. The aim of this study is to review the literature examining the relationship between plaque morphology and the production of symptoms, and to discuss the factors resulting in some of the inconsistencies.

3.1 INTRAPLAQUE HAEMORRHAGE

The pathogenesis of carotid artery intraplaque haemorrhage is unclear. A typical carotid plaque with large area of intraplaque haemorrhage is shown in figure 2. The reported incidence of plaque haemorrhage in the literature has varied from 7.4% to 87% [176] [177]. Three potential sources have been described: rupture of vessels derived from the vasa vasorum at the base of the lesion, rupture of superficial
vessels (possibly derived from the lumen), and dissection of blood from the arterial lumen through an ulcer or fissure [178-180]. The pathogenetic mechanisms of how intraplaque haemorrhage may give rise to cerebral ischaemia are also unclear. Two possible mechanisms have been proposed. First, bleeding into a plaque can lead to an acute increase in plaque volume, thus causing luminal narrowing or obstruction. Secondly, extensive intraplaque haemorrhage can cause an increase in intraplaque pressure causing the plaque to rupture and expel blood clot or atheromatous debris into the arterial lumen. The first mechanism will result in haemodynamic ischaemia, while the second mechanism will cause arterio-arterial embolism [181-183].

Figure 2. Carotid plaque with a large area of macroscopic intraplaque haemorrhage.

There is disagreement in the literature about the significance of the correlation between haemorrhage and cerebral symptoms. Some authors have found a positive association, whereas others have failed to demonstrate any correlation [183-187].
This is partly the result of different definitions of haemorrhage, some authors only considering haemorrhage to be significant if it constituted more than 50% of the plaque surface area [188]. Some authors included microscopic plaque haemorrhage in their definition, [189] while others have attempted to subclassify haemorrhage into groups including degree of extension into the plaque [190] [191]. A further area of disagreement is in the description of symptomatic plaques; some groups have used clinical criteria to describe symptoms while others have measured microembolisation rates in order to assess the degree of plaque instability [187]. In order to try and evaluate the significance of intraplaque haemorrhage, the data from the studies shown in the table 1 was reviewed, and is depicted graphically in figure 3.

<table>
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<td>Avril</td>
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<tr>
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<td>2</td>
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<td>21</td>
</tr>
<tr>
<td>Park</td>
<td>191</td>
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</tr>
</tbody>
</table>

Table 1. Incidence of symptoms in association with intraplaque haemorrhage.
In their study on 376 carotid endarterectomy patients, Imparato and his colleagues [188] have examined the gross morphological characteristics of plaques and found intramural haemorrhage in 30.6% of plaques. Haemorrhage was significantly correlated with symptomatic plaque, and was also the only factor significantly more correlated with focal symptomatic plaques as opposed to asymptomatic and non focal symptomatic plaques. In addition, intraplaque haemorrhage demonstrated a significant positive correlation with increasing stenosis (P<0.001), and when the degree of stenosis is also taken into account, the difference only appears to be significant in stenoses of > than 70%, with 43% of the symptomatic plaques having intraplaque haemorrhage compared with only 22% of the plaques from asymptomatic patients. These findings have been supported by the studies of Lusby et al [183], Avril et al [176], and Park et al[186]. Ammar et al found a significant correlation between symptomatic and asymptomatic plaques only when repeat
haemorrhages were taken into account, the symptomatic plaques having significantly more multiple haemorrhages than the asymptomatic ones. However little information about the degree of stenosis is given in this study and possibly a higher degree of percentage stenosis in the asymptomatic group may affect the result in the light of the findings of the previous researchers cited earlier [177]. However, these above results have not been reflected in all the studies. Hatsukami et al measured the volume of plaque haemorrhage identified histologically in 43 plaques from arteries with severe stenosis and found no correlation either between haemorrhage and symptoms or between the volume of haemorrhage and symptoms [190]. Von Maravic also had similar results with respect to symptom association with both haemorrhage and volume of haemorrhage [191]. Bornstein et al [192] and Van Damme et al [193] also examined plaques histologically with respect to age of haemorrhage and timing of symptoms but found no association between recent haemorrhages and symptoms as opposed to the results of Lusby et al [183] who reported an increase in the symptomatology associated with plaques containing recent haemorrhages. They also divided haemorrhage into superficial and deep and found that more haemorrhages occurred deeply in the plaque. This may reflect the site of fragile neovascularisation and lends support to the theory of intraplaque haemorrhage originating from these vessels. Interestingly, Sitzer et al who measured microembolisation rates in 39 plaques from patients with significant stenoses did not find an increased microembolisation rate in patients with intraplaque haemorrhage as well as no association with symptoms [187]. This observation lends support to the theory that intraplaque haemorrhage produces symptoms by plaque disruption rather by direct narrowing of the arterial wall resulting in haemodynamic compromise.
With this conflicting evidence it is difficult to draw conclusions on the role of intraplaque haemorrhage in producing cerebral ischaemia. Standardisation of data collection and analysis of plaques (macroscopic and microscopic), as well as accurate definitions is the only way forward to answer this question. In addition the degree of stenosis is an important consideration and the symptomatic and asymptomatic groups must be matched for degree of stenosis in order to eliminate confounding factors.

3.2 Ulceration

Carotid plaque ulceration is defined as a disruption of the arterial intima with exposure of the underlying thrombogenic layers of the plaque (Figure 4). The shearing stresses on the intima that occur across a stenosed region of the carotid artery due to increasing velocity have been implicated in the pathogenesis of carotid plaque ulceration. The incidence of carotid plaque ulceration have variously reported to occur in between 43%[194] and 71% [186] of plaques.

Figure 4: Ulcerated carotid plaque with complete intimal disruption.
Ulceration has also been the subject of considerable disagreement as a prognostic indicator for cerebral embolic disease. Several studies have attempted to quantify the significance of plaque ulceration in relation to development of stroke, however these have yielded conflicting data. The data from six studies is listed in table 2 and depicted graphically in figure 5.

The largest study was that carried out by Park and colleagues [186]. He reported an overall incidence of 71% ulceration on macroscopic examination of carotid endarterectomy specimens from 1008 patients with degrees of stenosis varying between < 75% in 14.2% and > 75% in the remainder, and he found a significant association between plaque ulceration and development of symptoms. This correlation was also reported by a number of other researchers [176, 187, 195-197]. In the study by Eliasziw et al diagnosis of ulceration was made by examination of carotid angiograms. However, studies by Streifler et al found that the sensitivity and specificity of detecting ulcerated plaques were only 45.9% and 74.1% respectively [198]. Both Eliasziw and Averil noted an increased incidence of ulceration with increasing degrees of stenosis, as was also shown by Bassiouny et al [199], although the latter group did not show an association of ulceration with symptom development in significantly stenotic patients. Sitzer et al also measured microembolisation rates in patients with ulceration and found that 16 out of 17 patients had microemboli detectable by transcranial Doppler monitoring, and 50% of these had more than 5 microemboli per hour. No concomitant neurological symptoms were observed during the recording [187]. This raises the possibility that all plaque ulcerations are emitting microemboli if monitoring is carried out for long enough, and possibly all ulcers are subclinically 'symptomatic' in this context.
Interestingly, in the same study an association between luminal thrombus and plaque ulceration was also observed, further emphasising the role of ulceration as a source of cerebral emboli.

In contrast to the above findings Imparato et al found no significant difference between the number of symptomatic plaques with ulcerations compared to asymptomatic plaques. Similarly Bassiouny et al conclude that ulceration and surface thrombi that may lead to cerebral emboli are prominent features in markedly stenotic plaques even when symptoms are absent. Carr et al [189] found a significant association between gross plaque ulceration and symptomatic plaques, but there was no association between microscopic ulceration and symptoms.

As with intraplaque haemorrhage future studies should ideally be accompanied by more standardised definitions and ways of analysing the macro and microscopic features of ulceration. In particular the degrees of stenosis of the arteries should be described using comparable criteria in order to enable comparison of similar groups of patients.

<table>
<thead>
<tr>
<th>Author</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>Carr</td>
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<td>Averil</td>
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<td>Park</td>
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<td>231</td>
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<tr>
<td>Sitzer</td>
<td>15</td>
<td>2</td>
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</table>

Table 2. Data from six reports showing relation of symptoms to ulceration.
Figure 5. Graph depicting data in table 2. The result may be partly skewed because of the large number of patients in the study by Park et al.

3.3 Intraluminal thrombus

Rather surprisingly, intraluminal thrombosis is another area where there has been considerable controversy regarding its role in the production of cerebral symptoms. One would expect a carotid plaque with overlying thrombus to be virtually pathognomonic of the unstable plaque. However, analysis of the data from the four studies shown in table 3 shows that statistically there is no significant association between intraluminal thrombus overlying a carotid plaque and the development of symptoms. The data is depicted graphically in figure 6.
Table 3: Data from 5 reports showing incidence of symptoms with presence of intraluminal thrombosis.

<table>
<thead>
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<th></th>
<th>Thrombus</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>Imparato et al.</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>Avril et al.</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>Bassiouny et al.</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Sitzer et al</td>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>

A possible explanation for this rather incongruous observation may lie in the fact that during their formation thrombi adhere to the plaque surface, and subsequent dislodgement results in the production of cerebral ischaemia and possibly stroke.
depending on several factors including the state of the collateral circulation. Plaques which become dislodged early after they form presumably give rise to symptoms, but will not be found at operation. On the other hand, a thrombus which is sufficiently well adhered to the arterial wall to withstand the trauma of operative manipulation of the artery may not give rise to symptoms and eventually become incorporated into the plaque.

A similar concept has been put forward by Fisher et al to explain why apparently uncomplicated plaques may have given rise to symptoms. The detection of plaque disruption appears to be time related, in that specimens obtained shortly after clinical symptoms occur are more likely to show abnormality [200]. Specimens obtained weeks after symptoms occur are less likely to show intimal disruption because intimal repair probably has occurred. Fisher et al therefore propose that acute plaque disruption could lead to overlying thrombosis. This may lead to cerebral ischaemia either as a consequence of acute plaque expansion due to intraplaque haemorrhage from the arterial lumen, or due to distal embolisation of thrombus. With intimal repair, the luminal thrombus could be incorporated into the carotid plaque. This results in plaque enlargement and may or may not have been symptomatic [201]. A similar sequence of events can be construed to occur following acute haemorrhage into a plaque which disrupts the plaque surface and results in superimposed thrombosis. This model may account for normal looking plaques from symptomatic patients and possible complicated plaques from asymptomatic patients. Interestingly, Bassiouny et al found no correlation between luminal thrombosis and symptoms in patients with severe stenosis of their carotid artery but symptomatic stenotic plaques were associated with luminal thrombosis.
(55%) compared to asymptomatic nonstenotic plaques (0%) in a statistically significant manner (P<0.01). [199]

3.4 Non invasive carotid plaque characterisation.
Several studies have attempted to identify a relationship between carotid plaque morphology using B mode ultrasonography and the development of carotid symptoms. Reilly et al first showed in 1983 that the echo patterns in the B mode image of a carotid plaque could be related to the composition of the plaque. They classified the appearance of carotid plaques into homogeneous and heterogeneous types. The homogeneous types consisted of uniformly high to medium level echoes, whereas the heterogeneous plaques consisted of mixed high, medium and low level echoes. They found that the homogeneous pattern correlated with a fibrous lesion on pathological examination, and that the heterogeneous lesion correlated with the presence of intraplaque haemorrhage, ulceration, loose stroma containing lipids and cholesterol and proteinaceous deposits [202].

These findings were subsequently followed up by Gray Weale and colleagues who classified plaques into four major subtypes: echolucent (type 1), echolucent with small echogenic areas (type 2), echogenic with small echolucent areas (type 3), and echogenic (type 4). Symptomatic patients were found to have predominantly type 1 and 2 lesions, whereas the asymptomatic patients had predominantly type 3 and 4 lesions. In addition, a significant relationship was found between ultrasound types 1 and 2 and the presence of either intraplaque haemorrhage or ulceration in the endarterectomy specimens [175]. These findings were subsequently reproduced by other groups [203].
Several other authors in recent years have attempted to further define these subgroups by introducing objective criteria for plaque assessment using computerised image analysis of plaque echotexture. El Barghouty et al have analysed carotid plaques using computerised image analysis software and have attempted to quantify the degree of plaque heterogeneity by quantifying the Gray scale median (GSM) of the plaque. This value is a measure of the plaque echogenicity and ranges from zero, which is totally black (soft areas) to 255 which corresponds to a white (dense, fibrotic or calcified) area on the plaque. They have described a correlation between unstable plaques and the gray scale median of the plaque [204]. In a later study the same group have also defined points of reference which allow standardisation of plaque analysis in such a way that the density of the plaque is corrected for and compared with the density of blood and adventitia within the same lesion [205]. The same group as well as others group have also correlated the gray scale median with the incidence of cerebral infarction on CT scan, plaques with a gray scale median of more than 50 being associated with a 9% incidence of infarction as opposed to those with a GSM of less than 50 which had a 40% incidence of cerebral infarction in both studies [206] [207].

Other authors have attempted to characterise plaque morphology and appearance using helical computed tomography scanning and MRI. Estes et al have shown that helical computed tomography scanning can differentiate between plaques containing calcium, fibrous tissue, and lipid in the stroma, as verified by histological examination [171]. Gortler et al demonstrated a correlation between carotid plaque histology and appearance on MRI in vitro, and Toussaint has subsequently shown that a the technique could also be used to characterise plaques in vivo [208].
Plaque analysis using B mode ultrasonography is still in its early stages of development, however ultrasound offers a tremendous advancement in plaque assessment over the traditional angiographic criteria as it allows assessment of the actual composition of the plaque. However with the rapid advancements in technology, such as the development of three dimensional ultrasound which allows a more comprehensive assessment of the carotid plaque, the future holds promise that non invasive plaque assessment will evolve and will eventually play a very important role in delineating a subgroup of patients who have a predisposition to develop symptoms and could benefit from carotid endarterectomy but do not fit into the present criteria for surgical intervention.

**Conclusion**

Examination of the literature suggests that the propensity of a carotid plaque to produce symptoms depends on more than just the degree of obstruction it produces. Though the Ecst and Nascet trials have been extremely useful in defining the role of carotid endarterectomy in patients with severe stenosis, a subgroup of patients with less severe stenosis with potentially unstable plaques who may benefit from operative intervention still remains to be defined. Though the results of different studies are in conflict with regards to the relative importance of plaque complications and their role in producing cerebral ischaemia, the bulk of evidence suggests that carotid plaque morphology does play a significant role in determining clinical outcome. More standardised ways of analysing and reporting aspects of carotid plaque morphology are necessary in order to achieve more comparable results. In addition technological advancements in non invasive assessment of plaque morphology will certainly be extremely useful improving patient selection.
Chapter 4

The Matrix Metalloproteinases and Carotid Artery Disease

Potential for Pharmacologic Carotid Plaque Stabilisation
The carotid plaque is a dynamic structure and its composition has a direct effect on its propensity to become unstable and rupture. The extracellular matrix of the plaque is continuously being remodelled. The MMP class of enzymes play an important role in the extracellular matrix turnover, and dysregulated metalloproteinase activity potentially contributes to acute plaque rupture. The salient characteristics and regulation of this class of enzymes and their relation to carotid plaque instability will now be reviewed followed by a discussion of potential pharmacological inhibitors which can be used to modulate their activity.

4.0 THE MATRIX METALLOPROTEINASES

The matrix metalloproteinases (MMP's), are a class of zinc and calcium dependant endopeptidases of which up to twenty three members have been characterised to date (Table 1). Between them they can degrade all the components of the extracellular matrix [209-211]. [212]

These enzymes are involved in a large number of physiological processes including wound healing, angiogenesis, parturition, and in the normal turnover of the matrix of blood vessel walls. In addition they have been implicated in pathological processes such as arthritis, periodontitis, glomerulonephritis, degenerative central nervous system disorders such as multiple sclerosis, and in the cancer spread and metastasis [213-217]. In recent years they have been implicated in the development of atherosclerosis and aneurysmal disease [218-220].
<table>
<thead>
<tr>
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<tr>
<td><strong>Collagenases</strong></td>
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<tr>
<td>Interstitial</td>
<td>Collagen types I, II, III, VI, X, gelatins, PG.</td>
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Table I. Major types of MMP's and substrate specificities. PG: proteoglycan
Classification.

Several classifications of MMP's have been proposed based on the sequence of identification, molecular size, and substrate specificity. They may be subdivided into two major classes: the secreted MMP's, which include the collagenases, the gelatinases, and the stromelysins; and the recently discovered membrane type MMP's (MT-MMP's), which are an integral part of the plasma membrane rather than secreted proteins. The major members as well as the substrate specificities of the main MMP's are shown in table 1 on the previous page.

The secreted MMP's

Structure.

The soluble MMP's share common features which allow them to be classified as a group [221]. They are secreted in a latent proform requiring activation, contain zinc at their active site, and they function best at a neutral pH. They are inhibited by specific tissue inhibitors of metalloproteinases (TIMPS).[222]

The structure of the soluble MMP's is highly conserved. There is up to 70% similarity in the amino acid sequences between members of the same group and about 50% similarity between members from different classes [223]. The soluble MMP's share a basic structure consisting of five domains:

1. A signal peptide
2. A propeptide containing a cysteine residue which interacts with the zinc ion at the active site and is important in maintaining the latency of the enzyme
3. A catalytic site which contains the zinc binding site
4. A hinge region which bridges the catalytic site to the carboxyterminal end of the molecule

5. A carboxyterminal domain which resembles haemopexin or vitronectin and is responsible for conferring substrate specificity.[224]

Matrilysin is the smallest MMP and does not contain the carboxyterminal domain and the hinge region [210, 221]. The two gelatinases have three fibronectin like sequences in their catalytic domain which are thought to facilitate binding of the enzymes to their substrate [225].

Regulation of MMP activity

Regulation of MMP activity occurs at three different levels: transcription, activation of the latent proenzymes, and inhibition of the active enzymes. Multiple regulatory pathways exist at each level, and compensation can occur at each level by activation of other systems should one pathway become inactivated. This principle may be important when assessing the effect of pharmacological agents on MMP activity which will be discussed later.

Transcriptional regulation.

Many cytokines and growth factors have been shown to affect the expression of MMP's [226, 227]. Interleukin -1, platelet derived growth factor, TNFα, and interferon γ have all been shown to stimulate MMP expression [226, 228, 229] [230, 231]. Other cytokines such as TGF β, heparin, and corticosteroids have an inhibitory effect. [226]
The promoter regions of the MMP genes have been studied in detail and share some common features. The promoter regions of interstitial collagenase, stromelysins 1 and 2, metalloelastase, and matrilysin all contain binding sites for activator protein-1 (AP-1) and the polyomavirus enhancer A-binding protein-3 site (BP-3), are important in regulating expression of these genes [209, 225, 226]. In contrast, the promoter region of gelatinase A (MMP-2), lacks a TATA box and the binding sites for AP-1 and BP-3, however it contains a binding site for transcription factor SP1 [226, 232]. (A TATA box comprises a thymine-adenine sequence in the promoter region of a gene which is recognised by transcription factors responsible for initiating transcription by RNA polymerases). This difference in the promoter profile might explain the fact that gelatinase A is constitutively expressed, whereas the other MMP's are mainly inducible.

Recent studies have shown that increased levels of the oxygen radical nitric oxide (NO), which is produced by nitric oxide synthase can decrease MMP-2 and MMP-9 expression by vascular smooth muscle cells [233]. Similar inhibition of MMP expression was observed in rat mesangial cells, were NO was found to inhibit the increase in MMP-9 mRNA levels induced by interleukin-1 [234]. In addition, increased nitric oxide activity in aortic smooth muscle cells was associated with increased TIMP-1 expression [233].

Proenzyme activation

In the proenzyme state, proteolytic activity is prevented by an association of the cysteine residue in the pro domain with the zinc residue in the active site [235]. Cleavage of the pro domain results in a conformational change that activates the enzyme, a process that has been referred to as the 'cysteine switch' [236]. Plasmin is
one such enzyme that can bring about this proteolytic cleavage. Plasminogen activation by urokinase can activate stromelysin which then activates other MMP's, resulting in a fivefold increase in their proteolytic activity [237]. In addition plasmin activation of collagenase makes this enzyme more susceptible to proteolytic cleavage by stromelysin. Cell surface receptors for urokinase plasminogen activator on macrophages and other sources of MMP's such as smooth muscle cells and tumour cells is thought to localise proteolysis to the surface of the cell [238]. The plasminogen/plasmin activator system is susceptible to inhibition by plasminogen activator inhibitors (PAI's), and the balance between PAI's and tissue plasminogen activator may play an important role in balancing the level of degradative activity in the extracellular matrix. [226] Studies using targeted mutagenesis in Apo E deficient mice, which develop atherosclerotic lesions if fed on a high cholesterol diet, have shown that deficiency in the urokinase type plasminogen activator gene protects them from macrophage mediated destruction of the medial elastic lamina and microaneurysm formation. The ability of macrophages to penetrate the elastic lamina depends on their ability to produce MMP 12 which is then activated by plasmin. These findings suggest that plasmin may have a physiological role activating the MMP proenzymes [239]. Indeed, in the absence of u-PA, macrophages were not able to convert pro MMP's into their active form in a reconstituted system [240].

In addition to the above system, several other mechanisms of MMP activation have been described. Membrane type MMP's 1 and 2 can activate MMP-2, and it is postulated that other membrane bound enzymes can activate other MMP's in a similar manner [241, 242] [243]. Other enzymes, such as bacterial proteases and
leucocyte derived proteases like neutrophil elastase and mast cell proteases have been shown to activate MMP's in vitro and may have a similar role in vivo [244] [245]. In addition, reactive oxygen species produced by activated macrophages have also been shown to activate MMP's in vitro and may possibly have an important role in vivo in bringing about the imbalance in MMP degradative activity seen in certain inflammatory/infective states [246, 247].

**Tissue inhibitors of metalloproteinases (TIMPS).**

The third level of control of MMP activity is at the level of enzyme inhibition by a group of natural inhibitors called tissue inhibitors of metalloproteinases (TIMPS). Four TIMPS have been characterised to date [248]. They are synthesised by the same cell types producing MMP's, and counteract excessive proteolytic activity by these enzymes [249]. Structurally, the TIMPS are similar and have 12 identically conserved cysteine residues forming six disulphide bonds, which results in folding of the molecules that appears to be essential for their activity [250]. The TIMPS interact in a 1:1 molar ratio with the active MMP's and form high affinity irreversible complexes inhibiting their activity. In addition interaction with the proenzyme forms inhibiting activation also occurs [251]. TIMP 2 interacts specifically with MMP 2, and like the enzyme is constitutively expressed. Expression of the other TIMP's is inducible, and subject to many physiological and pharmacological agents [242]. The inducibility of a certain inhibitor is both cell and type specific. For example TGF-β stimulates TIMP -1 expression, as do PDGF and IL-1. Dexamethasone inhibits TIMP-1 expression but stimulates TIMP-3 expression [226].
In addition to MMP inhibition, recent evidence has shown that TIMP-2 binds to MT-MMP and the resulting complex can actually activate MMP-2 [252, 253]. Higher concentrations of TIMP-2 would result in inhibition of the enzyme. [252]

In addition to MMP inhibition, TIMPS have been shown to have other effects such as growth promoting properties and stimulation of erythroid precursors, however the exact physiological role of these functions has not been established [248, 254, 255].

Membrane type matrix metalloproteinases.

The membrane type matrix metalloproteinases were first described by Sato et al in 1995, and 5 members have been characterised to date.[256] [257]. In addition to the 5 structural domains present in the secreted MMP's, the membrane type MMP's also have a transmembrane domain at the C terminus which binds the enzyme to the cell membrane, and an intracellular domain which may be involved in intracellular regulation of activity.[257-259] [260].

The exact physiological role of the MT-MMP's has not yet been elucidated. Their patterns of expression suggest a complex picture with overlapping expression in both normal and tumour tissues. They have been identified in the cell surface of brain, heart, lung, placenta, vascular and embryonic tissues among others[261-264]. In addition, MT1-MMP has been investigated extensively and found to be expressed in malignant tumours from human brain, colon, pancreas, liver, ovary and cervix [265-267]. Increasing evidence is accumulating that MT-MMP1, 2, 3, and 5 act as cell surface activators of MMP-2, localising the proteolytic activity at the surface of the cell [257]. Pro collagenase 3 (MMP-13), has also recently been
shown to be activated by MT1-MMP [258]. Purified MT1-MMP, MT2-MMP and MT3-MMP can degrade fibronectin, laminin, type I and III collagens, nidogen, tenascin, aggrecan, and perlacan [268-270]. MT3-MMP appears to be able to degrade denatured type I collagen, type III collagen, and fibronectin [271].

The MT-MMP's themselves are synthesised in latent forms and activation is required for them to exert any proteolytic function. The mechanism responsible for MT-MMP activation appears to be mediated by members of the proprotein convertase family which can cleave off the prodomain at the carboxyl side of the enzyme [270]. Thus a proprotein convertase/MT-MMP/MMP cascade could be potentially responsible for extracellular matrix turnover at the level of zymogen activation [257]. Human saphenous vein smooth muscle cells were found to constitutively express MT1-MMP, and in atheromatous arteries, MT1-MMP expression was found to colocalise with the smooth muscle and macrophages. Interleukin-1 α, TNF-α, and oxidised LDL increases expression of MT1-MMP by smooth muscle cells, and TNF-α also increases expression of MT1-MMP by human monocyte derived macrophages [272]. The shoulders and regions of foam cell accumulation displayed locally increased expression of 92-kD gelatinase, stromelysin, and interstitial collagenase. These findings suggest a potential role of these inflammatory mediators in augmenting MT-MMP activity and proteolysis in atheromatous plaque. The tissue inhibitors of matrix metalloproteinases have also been shown to inhibit MT-MMP'S in vitro suggesting that they may be involved in the regulation of these enzymes in a similar way to their soluble counterparts.[268]

4.1 The Carotid Plaque.
The advanced carotid atherosclerotic plaque consists of a lipid core which is separated from the vessel lumen by a fibrous cap of varying thickness. The lipid core consists of necrotic cells, mainly macrophages, free cholesterol crystals and varying degrees of calcification. The fibrous cap surrounding the plaque separates the thrombogenic central core from the flowing blood. This cap may fracture and rupture, resulting in acute disruption of the plaque and consequent clinical symptoms. The histological and biochemical changes underlying acute plaque disruption have been the subject of intensive research in recent years, and a number of characteristics have been found to be associated with unstable plaques.

**Histological features.**

Carr et al examined 44 carotid endarterectomy specimens with similar mean percentage stenosis and risk factors. Patients with symptomatic carotid artery disease were found to have more frequent plaque rupture, fibrous cap thinning, cap foam cell infiltration and intraplaque fibrin than asymptomatic plaques. In addition, symptomatic plaques have been shown to contain more cholesterol, calcium and less collagen and proteoglycans than asymptomatic plaques. Lipid-laden plaques with low levels of collagen are associated with plaque ulceration, subintimal hemorrhage, and ischemic neurologic symptoms [273]. The lipid cores have been shown to consist of two types, avascular or mildly vascularised and highly vascularised. In about 60% of cases these vessels reacted with CD 34+, a marker of neovessels, and they were often surrounded by mononuclear infiltrates [114, 274]. These neovessels are very fragile and are supported by a thin basement membrane. Increased proteolytic activity in vicinity of these primitive neovessels could destabilise them resulting in intraplaque haemorrhage [114, 275].
Immunohistology

Immunohistochemical analysis of carotid plaques shows an extensive inflammatory infiltrate of CD 68+ macrophages, T lymphocytes, scarce B lymphocytes and mast cells at sites of plaque rupture [113-115, 276]. These infiltrates are prominent in the caps, shoulders and bases of the plaques in up to 85% of complicated cases, and they are only present in 46% of uncomplicated plaques [274]. Milei et al have measured the macrophage and T cell content of plaques from patients with stable and unstable carotid plaques. The macrophage rich areas were larger in symptomatic plaques than in asymptomatic plaques, and the presence of the cellular infiltrate was associated with microembolization [113]. Similar infiltrates have been identified in coronary atherosclerotic plaques, and the same mechanisms underlying coronary plaque disruption are probably operative in carotid plaques [277]. There is a complex interaction between the different cell types involved in the development and subsequent instability of the atherosclerotic plaque. Both macrophages and T cells produce numerous cytokines which exert an autocrine and paracrine effect on surrounding macrophages, T cells, smooth muscle cells, and endothelial cells.

Dysregulated extracellular matrix metabolism is believed to underlie complicated plaques. Macrophages, smooth muscle cells, endothelial cells and T lymphocytes have all been shown to produce MMP's under the influence of cytokines in both in vivo and in vitro. Shah et al demonstrated that human monocyte derived macrophages induce collagen breakdown when incubated with fibrous caps of human atherosclerotic plaques in vitro. This was associated with cellular expression
and zymographic evidence of activated MMP 1 and 2 in the supernatant of the cultured cells, and was abolished in the presence of an MMP inhibitor [278]. Galis et al investigated the expression of MMP's in atherosclerotic plaques from coronary specimens, and in uninvolved arteries. Normal arteries stained uniformly for MMP 2 and TIMPS 1 and 2. Atheromata were found to contain MMP1, 3 and 9 in addition to MMP 2. These enzymes were localised to macrophages, smooth muscle cells, lymphocytes, and endothelium. Increased MMP expression was localised to the fibrous cap, the shoulders regions, and the base of the lesion's lipid core. Additionally, this study revealed that control arterial tissue contained the zymogen form of MMP 2, whereas atherosclerotic tissue contained both pro MMP 9 and the activated forms of both gelatinases [279].

Mertens et al have determined the metalloproteinase activity in carotid endarterectomy specimens and found that in areas of significant plaque stenosis, MMP 9 activity was 260% higher than in an equivalent non stenotic area in the same vessel [280]. Brown et al identified active synthesis of MMP 9 by macrophages, smooth muscle cells and lymphocytes in coronary plaques from patients with unstable angina, while only 3 out of ten patients with stable angina were actively synthesising MMP 9. This was the first study to correlate the expression of a metalloproteinase enzyme and clinical evidence of plaque instability [281].

Similar patterns of MMP activity have been identified in carotid atherosclerotic plaques and the mechanisms of acute plaque disruption may be similar. Galis et al have shown by in situ zymography that in freshly excised atherosclerotic specimens, gelatinolytic activity localised in the same areas of increased MMP expression in unstable regions of the plaques. Plaques' shoulders and regions of
foam cell accumulation displayed locally increased expression of 92-kD gelatinase, stromelysin, and MMP-1. [279]. Nikkari et al also found intense MMP 1 expression in unstable regions of atherosclerotic plaques, and there was a strong correlation between the percentage of the lipid core perimeter occupied by haemorrhage and the percentage of the lipid core perimeter positive for MMP 1. MMP 1 was not detected in non atherosclerotic arteries [275]. Similarly Sukova et al have demonstrated increases levels of MMP-1 and MMP-13 in atheromatous carotid plaques compared to fibrous plaques. In addition collagenase cleaved type 1 collagen colocalised with MMP-1 and MMP-13 positive macrophages [282]. Loftus et al have studied carotid plaques from recently symptomatic, asymptomatic, and patients who had symptoms up to six months before surgery. They found significantly increased MMP-9 expression in the recently symptomatic group when compared to the other groups. Furthermore the MMP-9 level was significantly higher in plaques undergoing spontaneous embolisation as well as in those with histological evidence of plaque instability [283].

Increased expression of matrix degrading proteases per se does not imply increased proteolytic activity, as the enzymes have to be activated, and are subject to local inhibitory control by other molecules such as TIMPS [249]. The balance of these two opposing forces will determine the degree of proteolytic activity and ultimately plaque stability. In pathological conditions associated with local release of cytokines in the vessel wall, enhanced regional expression of MMP's may contribute to regional migration of smooth muscle cells and weakening of matrix that favour plaque rupture [228].
It is unclear what causes a plaque to suddenly become unstable. Any agent which modulates the delicate balance in the chronic inflammatory milieu in the atheromatous plaque can possibly trigger acute plaque disruption. A possible infective aetiology has been implicated in recent years. Infection with Chlamydia Pneumoniae has been found to be associated with both coronary and carotid atherosclerotic plaques, where it usually localises in smooth muscle cells and macrophages [46, 284-287]. The organism has been shown to induce expression of MMP's by macrophages [288]. Interestingly, in one study patients with unstable angina who were treated with the anti Chlamydial agent azithromycin had an improvement in their symptoms [289]. Infection with this organism, or possibly reactivation of latent infection could provide a plausible mechanism of inducing plaque instability. However, a number of other studies have failed to demonstrate this association between unstable plaques and infection with this organism and suggest that it may just be associated with diseased lesions rather than be a cause. [290, 291]. Further studies are needed to confirm or refute a causal relationship.[292]

Reactive oxygen species produced by activated macrophages have also been shown to activate MMP's [247], which could provide a mechanism of plaque destabilisation in acute infection (possibly systemic) or any mechanism which transiently increases the level of activity of cells present in a chronic inflammatory response[247]. Other studies have shown that oxidised lipoproteins which have long been associated with atherosclerotic disease may augment cytokine and ultimately MMP expression by atheroma cells, mainly the macrophages. Lipid laden macrophages have been shown to elaborate MMP's capable of degrading the
major constituents of the extracellular matrix even without further stimulation [247, 293]. Alteration of the lipoprotein milieu of the plaque may therefore provide yet another avenue for potential control of acute plaque instability [294].

The complex processes underlying plaque instability provide multiple points of possible therapeutic intervention. The association of increased MMP activity with unstable carotid plaques and development of symptoms provides a potential avenue for therapeutic carotid plaque stabilisation, and several classes of compounds are being investigated at present which will now be discussed.

4.2 POTENTIAL THERAPEUTIC INTERVENTIONS.

The major classes of drugs that have either been used or have the potential of modulating MMP activity can be divided into five types:

1. Tetracyclines such as doxycycline and minocycline,
2. HMG-Co A inhibitor class of drugs (statins), such as fluvastatin and simvastatin,
3. Macrolide antibiotics such as roxithromycin and azithromycin,
4. Antioxidant drugs such as N-acetyl cysteine, Probucol and alpha-tocopherol (vitamin E).
5. Non steroidal anti inflammatory drugs, such as aspirin

(See Table 2 overleaf)

Tetracyclines

The tetracyclines, particularly doxycycline and minocycline have been shown to inhibit MMP activity in a number of in vivo and in vitro studies. In addition, a
group of similar compounds which lack antibacterial activity, the chemically modified Tetracyclines have also been shown to have similar inhibitory effects.

<table>
<thead>
<tr>
<th>Class of drugs</th>
<th>Generic names</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracyclines</td>
<td>Doxycycline</td>
<td>Direct enzymatic inhibition</td>
</tr>
<tr>
<td></td>
<td>minocycline</td>
<td>Inhibition of transcription</td>
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<td></td>
<td></td>
<td>Reduced iNOS expression</td>
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<tr>
<td></td>
<td></td>
<td>Eradication of C. Pneumoniae</td>
</tr>
<tr>
<td>HMG CoA reductase inhibitors</td>
<td>Simvastatin</td>
<td>Inhibition of secretion</td>
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<td></td>
<td>Fluvastatin</td>
<td>Indirect</td>
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<td></td>
<td>Pravastatin</td>
<td>♦ Reduced cholesterol in macrophages</td>
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<td></td>
<td></td>
<td>♦ Hydroxyl radical scavenging activity</td>
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<td></td>
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<td>♦ Upregulation of nitric oxide synthetase</td>
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<tr>
<td>Antioxidant therapy</td>
<td>N-acetyl</td>
<td>Free radical scavenging</td>
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<td></td>
<td>cysteine</td>
<td>Reduced free radical production</td>
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<td></td>
<td>Probucol</td>
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<td></td>
<td>Vitamin C</td>
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<tr>
<td>Macrolide antibiotics</td>
<td>Erythromycin</td>
<td>Eradication of Chlamydia Pneumoniae</td>
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<tr>
<td></td>
<td>Roxithromicin</td>
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<tr>
<td>Non steroidal anti-inflammatory</td>
<td>Aspirin</td>
<td>Direct enzymatic inhibition</td>
</tr>
<tr>
<td>Drugs</td>
<td>Piroxicam</td>
<td>Possible synergistic effect with tetracyclines.</td>
</tr>
</tbody>
</table>

Table 2. Major classes of compounds involved in MMP inhibition.

**Mechanism of action of tetracyclines.**

The exact mechanism of action is not yet clear, and several levels of inhibition may be operative.
Transcriptional level

Doxycycline and the chemically modified Tetracylines have been shown to inhibit expression of MMP's at the level of mRNA expression in several studies [295-298]. Hanemaaijer et al have shown that doxycycline and chemically modified tetracyclines inhibit the synthesis of MMP-9 by human endothelial cells. No effect was observed on the expression of MMP-2 and the MMP inhibitors TIMP-1 and TIMP-2 [295, 299]. However Cakir et al demonstrated inhibition of MMP-2 synthesis by doxycycline and CMT's from human skin keratinocytes, suggesting that different cell types may have different levels of susceptibility to inhibition [298].

Inhibition of enzymatic activity

Tetracyclines are potent chelators of divalent metal ions and it has been proposed that they achieve their effect by binding to enzyme bound zinc and reducing the calcium concentration [300]. Smith et al have shown that activation of recombinant human neutrophil procollagenase in the presence of doxycycline results in fragmentation of the enzyme with loss of enzymatic activity. Incubation of the active enzyme with doxycycline also results in loss of enzymatic activity. Fragmentation of the enzyme was also found to occur in the presence of calcium chelating agents, and was inhibited by addition of calcium. This suggests that doxycycline alters that configuration of the procollagenase by binding associated calcium, which results in a conformational change that renders the enzyme more susceptible to proteolysis with irreversible loss of enzyme activity. [301]. Studies using truncated enzymes suggest that doxycycline disrupts the haemopexin-like
domain of MMP-13 and the catalytic domain of MMP-8, inactivating the enzymes. MMP-1 was similarly inhibited but required higher concentrations of doxycycline to achieve this effect [302] [303].

Inhibition of inducible nitric oxide (iNOS) expression

Doxycycline and CMT-3 augmented iNOS mRNA degradation and decreased nitric oxide production in macrophages [304-306]. Incubation of mesangial cells and macrophages with CMT-3 and CMT-8 resulted in time- and dose-dependent inhibition of NO production [305, 307]. Although the above effect might be beneficial as it reduces nitric oxide induced pro-MMP activation, a reduction in nitric oxide levels has been associated with increased cytokine induced MMP expression in mesangial cells [234]. Conversely, increased nitric oxide activity in aortic smooth muscle cells has been associated with reduced MMP-2 and MMP-9 production [233]. The overall effect of the Tetracyclines on nitric oxide and its effect on MMP activity in vascular tissue still needs to be investigated.

Eradication of Chlamydia Pneumoniae

Tetracyclines are also the antibiotics of choice against Chlamydia Pneumoniae organisms, and therefore may affect atherosclerotic plaque stability by elimination of this organism.

In vivo trials.

Several clinical studies have been carried out on both human and animal subjects which have correlated the above findings in vivo. Doxycycline has been used in the treatment of periodontal disease in a number of clinical trials, and has been shown
to inhibit MMP mediated periodontal tissue destruction even when used in sub­
antimicrobial doses.[308] [303, 309-312]. This has now also been approved by the
FDA as an adjunct to scaling and root planing in the management of periodontal
disease [313]. Similarly doxycycline has been used in the treatment of osteoarthritis
and rheumatoid arthritis with reported reduction in MMP activity and clinical
benefit in one study [314-316].

Studies in animal models in which aortic aneurysms were induced using an infusion
of pancreatic elastase have shown a significant reduction in aortic diameter in a
group of animals given doxycycline, associated with a reduction in MMP-9 activity
and degradation of medial elastin seen in a control group [317]. Thompson et al
have shown a significant reduction in MMP-2 and MMP-9 expression in the aortic
aneurysm tissue following short term treatment with doxycycline. This raises the
possibility of a clinically significant effect on inhibiting aneurysm expansion in
vivo [318].

The serum concentration of doxycycline varies between 1.7-5.7μg/mL (6-10μM).
after a single oral administration of 100-200mg [319]. In vitro studies have shown
that higher levels of doxycycline are necessary to significantly inhibit MMP
activity. However the level of inhibition is both dose dependant and also varies
according to the type of enzyme, so a clinical benefit is still possible at
conventional therapeutic doses [295, 302, 320, 321]. Franklin et al have reported
mean tissue levels 2.9μg/mL of tetracycline in aortic aneurysm wall after intravenous
administration of 500mgs of tetracycline. To date no data on doxycycline levels in
vascular tissue has been published, but one similar levels of doxycycline may be
present after oral administration [322].
The above studies all point to a promising therapeutic potential of doxycycline in inhibiting MMP activity in the carotid plaque resulting in plaque stabilisation. Clinical trials to assess the potential role of doxycycline in carotid artery disease are currently underway.

HMG-CoA reductase inhibitors (The statins)

The HMG-CoA reductase inhibitors, which include fluvastatin, simvastatin and pravastatin among others, are another group of drugs that are being intensively investigated as potential atheromatous plaque stabilising agents. First used for their antihypercholesterolaemic properties, substantial evidence now points to other mechanisms through which they may retard the development of atherosclerosis and stabilise established atheromatous plaques [323-327]. As with the tetracyclines, direct and indirect effects on MMP secretion or activation of action may be operative.

Direct effect: Inhibition of MMP secretion

Fluvastatin has been shown to inhibit the secretion of MMP-9 from both human and mouse derived macrophages in a dose dependant manner, at a concentration as low as 5 micromol/L [323]. The inhibitory effect was overcome by simultaneous addition of mevalonate, a precursor of isoprenoids. Similar data were obtained with simvastatin [323].

Indirect effects

1. Reduction the in accumulation of cholesterol by macrophages
HMG-CoA reductase inhibitors block cholesterol esterification and endocytosis of modified lipoproteins by macrophages [328]. Interestingly, vastatin activity was more pronounced in cholesterol loaded macrophages (ie foam cells) rather than in normal cells [329].

2. Hydroxyl radical scavenging activity
Fluvastatin but not the other statins was shown to have hydroxyl radical scavenging activity similar to that of alpha tocopherol [330].

3. Upregulation of nitric oxide synthetase
Simvastatin, lovastatin and mevastatin have been shown to upregulate nitric oxide synthase expression by human saphenous vein endothelial cells predominantly by post transcriptional mechanisms [331] [332], which has been associated with reduced MMP-2 and MMP-9 and increased TIMP-2 production [233].

Clinical trials.
Several clinical trials assessing the efficacy of the HMG CoA reductase inhibitors in preventing stroke have been carried out, and several meta-analyses of these studies have been published [125, 127, 333-336]. These trials were conducted to assess the risk of stroke from the perspective of cholesterol lowering but the conclusions may possibly be related to other mechanisms of action as discussed above. The relative risk reduction varied between 27% and 31% in treatment as opposed to placebo groups [127, 336]. Interestingly, one study concluded that use of cholesterol modifying diets as well as Clofibrate to reduce serum cholesterol was not associated with any decrease in stroke rates. [337].
Antioxidant Therapy

As discussed previously, reactive oxygen species have been shown to both induce expression of MMP's by macrophage derived foam cells as well as activate MMP's. Studies using the antioxidant agent N-acetyl-L-cysteine (NAC), an ROS scavenger, decreased not only gelatinolytic activity but also gelatinase expression by macrophage derived foam cells in vitro [338]. Antioxidant therapy also appears to affect the cellular composition of the atheromatous lesion, reducing primarily the monocyte macrophage content [339]. Recent studies using healthy volunteers on dietary supplementation with vitamin E have shown a reduced production of reactive oxygen species by macrophages, reduced interleukin-1 production and reduced adherence after stimulation with lipopolysaccharide [340]. Other antioxidant agents such as Probucol and vitamin C, which have shown potential clinical benefits in other MMP mediated processes such as angioplasty restenosis, may also prove useful in future in vivo experiments to test the possibility of using antioxidant therapy to stabilise atherosclerotic plaques [341, 342].

Macrolide antibiotics such as roxithromycin and azithromycin

The macrolide antibiotics, including roxithromycin and azithromycin have not been shown to have any direct effect on MMP activity, but may in turn out to be very useful drugs to treat Chlamydia Pneumoniae once the role of an infective aetiology in plaque destabilisation has been clarified further.

Non steroidal anti inflammatory drugs such as aspirin.
et al have also shown that several nonsteroidal inflammatory agents including the commonly used drugs piroxicam, meloxicam and sulindac can inhibit collagenase activity in vitro. The effect was reversed after dialysis of the drug enzyme complex. [344]. This has potential important implications as the nonsteroidal agent aspirin is currently accepted standard treatment for patients with carotid and coronary artery disease due to its antiplatelet aggregant properties, and a further potential mode of action by MMP inhibition remains to be evaluated in clinical trials [344]

Conclusion

The mechanisms described above suggest that a large number of new therapeutic approaches could help to prevent or modulate the course of atherosclerotic disease. The future holds promise that improved understanding of the underlying pathophysiological processes will help to direct therapy to preventing the potentially devastating clinical consequences of carotid atherosclerotic disease.
Chapter 5

A Trial of Doxycycline Therapy in Patients undergoing Carotid Endarterectomy
On the basis of the background discussed in the previous chapters, it is apparent that the carotid plaque is a very dynamic structure whose connective tissue matrix is continuously undergoing remodelling. The balance of the proteolytic and synthetic processes within the plaque determine its stability. MMP's seem to play a critical role in this process, and their susceptibility to inhibition by doxycycline makes them potential targets for pharmacological plaque stabilisation.

In view of this it was hypothesised that administration of doxycycline to patients with carotid artery disease could potentially reduce the levels of MMP's within carotid plaques and result in plaque stabilisation.

5.0 HYPOTHESIS

Doxycycline penetrates the carotid plaque and reduces matrix metalloproteinase expression. This may potentially result in a change in plaque histology.

Objectives of this thesis

1. To determine if doxycycline penetrates the carotid plaque at therapeutic doses and to quantify the level achieved in atheromatous plaque.

2. To determine the effect of doxycycline on MMP expression by monocyte derived macrophages

3. To determine the effect of doxycycline on MMP-1,2,3, and 9, and TIMP-1,2, levels in carotid plaques

4. To quantify any effect of doxycycline administration on carotid plaque histology.
5.1 Study design

Author's note
I was not involved in the initial preparations and joined after the initial stage was formulated. Other members of the study team were responsible for ethics committee approval, basic study design and initial funding. I subsequently recruited all the patients, performed all the experimental procedures, performed all the analyses, and presented the data and wrote the associated papers.

A prospective randomised double blind trial was conducted at the Leicester Royal Infirmary, UK. Patients undergoing endarterectomy for carotid artery disease were recruited between September 1998 and December 1999. Patients were randomised to doxycycline 100mg b.d or placebo from the time of booking until the operation. Ethics committee approval was obtained to carry out the study, and every patient recruited was asked to sign a consent form after being informed about the background of the trial. The indications for operation were symptomatic severe stenosis (more than 70% on duplex ultrasound scanning) ipsilateral to the side of symptoms, severe stenosis with occlusion of the contralateral side, and asymptomatic patients who were included in the Asymptomatic Carotid Atherosclerosis Trial [169].
Exclusion criteria included known hypersensitivity to tetracyclines, anticoagulant treatment, history of hypersensitivity, and patients with systemic lupus erythematosus (exacerbations of systemic lupus may occur).

Estimation of sample size and power calculations
The null hypothesis was defined as there being no difference in the levels of MMP-1,2,3,9, and TIMP-1,2 between the placebo and doxycycline treated groups. No data was available from previous clinical studies in human patients regarding the potential level of MMP inhibition by doxycycline in atherosclerotic plaques. An estimate of effect size was therefore based on in vitro studies and studies investigating the effect of doxycycline on MMP levels in other fields.
Studies by Smith et al using recombinant human MMP-8 have shown an inhibition of 61% of its activity upon activation by APMA in the presence of 10\(\mu\)M doxycycline. Even higher levels of inhibition were observed with higher concentrations of doxycycline.[301] In studies by Cakir et al using canine osteosarcoma cells, doxycycline significantly reduced MMP-1 activity at doses of 10 and 20 \(\mu\)g/ml by 35% and 50%, respectively. These cells were not endogenously producing MMP-1 and therefore an additional effect on MMP expression (at mRNA level) could potentially result in much higher inhibition [296].

In an interesting study by Smith et al on patients undergoing total hip replacement who were given varying combinations of doxycycline, it was found that a dose of doxycycline of 100mgs b.d. for five days reduced collagenase levels by about 50%. (exact level not specified in paper) [347]. Golub et al used low dose doxycycline (20mgs/day or b.d.) in patients with chronic periodontitis and found a reduction in collagenase activity of up to 80% [303a]. This has been reproduced in subsequent studies by the same group.

On the basis data from the above studies an \textit{a priori} analysis was carried out to estimate the sample size required. The power level of the study was set at 80% in accordance with usual practise. This gives a possible false negative rate (type II error) of 20% (\(\beta\) level). The \(\alpha\) level was set at 0.05, giving a possible false positive rate (type I error) of 5%. Effect size was estimated to be 0.7 between the treated and untreated groups, taking into account that doxycycline seems to have an effect on multiple levels of MMP regulation (mRNA expression, enzyme degradation, direct MMP inhibition). Using the G Power statistical package, a total sample size of 68 patients would be necessary to prove or disprove the null hypothesis.

Primary outcome measures were levels of doxycycline in carotid plaques, and levels of MMP-1,2,3, and 9 and TIMP-1 and 2 in the treatment compared to control groups.
Secondary outcome measures were the incidence of stroke or occlusion of the artery under study (the side where the operation was to be carried out). If a patient developed a stroke or occluded he was stopped from the trial.

The distribution of the tablets was done exclusively by the trial pharmacist, thereby eliminating any possible allocation bias. The patients were deemed to be in the trial until the endarterectomy was carried out, following which no further follow up was carried out.

**Study Cohort**

A total of 100 patients were enrolled with an age range from 43-83 years (median 66 years). A full history including symptom classification, risk factors, drug history, and a full physical examination were carried out on enrolment.

From the 100 patients recruited, a total of 81 patients eventually proceeded to carotid endarterectomy, and 17 patients were not operated due to the various reasons shown on the trial flow diagram shown in the next page.

Mean times to operation were 65.9 days (13-157 days). On the day of admission the patients had a blood sample collected for quantification of serum doxycycline. The remaining capsules were collected and a capsule count carried out as a measure of compliance. Embolisation rates were determined by transcranial doppler monitoring of the middle cerebral artery for half an hour, and the data collected for future analysis.

The carotid endarterectomy specimen was collected at the time of operation and divided longitudinally in two equal samples. One half was stored in liquid nitrogen for enzymatic quantification and the other half fixed in formalin and later paraffin embedded for histological analysis.
5.2 Trial Flow Diagram

Table 1 Clinical Presentation

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVA</td>
<td>41%</td>
</tr>
<tr>
<td>TIA</td>
<td>14%</td>
</tr>
<tr>
<td>Stroke</td>
<td>14%</td>
</tr>
<tr>
<td>Ischaemia</td>
<td>14%</td>
</tr>
</tbody>
</table>

Notes:

1. One patient was operated bilaterally using same drug sample; 3 pots were reallocated to patients who ran out of tablets at the beginning of the trial, and one patient was operated bilaterally using the same pot. Therefore a total of 98 carotids entered the trial.
2. 2 specimens from operated group were not collected.
5.3 Patient Characteristics

The characteristics of the patients included in the trial are summarised in tables 1 and 2. A stroke was defined as a focal neurological deficit lasting more than 24 hours. A transient ischaemic attack was defined as a focal neurological deficit which resolved completely in 24 hours. Amaurosis Fugax was defined as a transient episode of monocular blindness lasting less than 24 hours. Central retinal artery occlusion was deemed to have occurred in patients with monocular blindness persisting beyond 24 hours. The relative frequency of the different presentations are shown in table 1.

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVA</td>
<td>34%</td>
</tr>
<tr>
<td>TIA</td>
<td>27%</td>
</tr>
<tr>
<td>Amaurosis Fugax</td>
<td>26%</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>7%</td>
</tr>
<tr>
<td>Central retinal artery occlusion</td>
<td>3%</td>
</tr>
</tbody>
</table>

Table 1 Clinical Presentation

The risk factors for atherosclerosis and stroke in the whole cohort were consistent with previous associations cited earlier (Table 2). Ischaemic heart disease as well as peripheral vascular disease were much more common in the cohort than in the normal population reflecting the underlying generalised vasculopathy in these patients. 26% of patients were still smoking at the time of recruitment. 83% of patients were on aspirin, and the rest were on alternative antiplatelet therapy for example dipyridamole or clopidogrel.
A large number of patients in the study were on concomitant therapy for cardiorespiratory disorders. Table 3 shows a breakdown of other drugs in both groups. There was no statistically significant difference in therapy between both groups.

### Table 2. Characteristics of cohort.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Active</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>28</td>
<td>21</td>
<td>0.23</td>
</tr>
<tr>
<td>Smoking</td>
<td>16</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>15</td>
<td>16</td>
<td>0.83</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4</td>
<td>5</td>
<td>0.74</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>15</td>
<td>18</td>
<td>0.53</td>
</tr>
<tr>
<td>Claudication</td>
<td>10</td>
<td>17</td>
<td>0.12</td>
</tr>
<tr>
<td>Aspirin</td>
<td>41</td>
<td>36</td>
<td>0.36</td>
</tr>
</tbody>
</table>

### Table 3: Comparison of concomitant treatment between active and placebo groups.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Active (n =51)</th>
<th>Control (n = 50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-blocker</td>
<td>7</td>
<td>8</td>
<td>1.00</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>4</td>
<td>4</td>
<td>1.28</td>
</tr>
<tr>
<td>Loop diuretics</td>
<td>8</td>
<td>7</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>15</td>
<td>14</td>
<td>1.00</td>
</tr>
</tbody>
</table>

### Assessment

All patients were assessed at the booking clinic and preoperatively using duplex scans. Angiography was not used routinely except in very difficult cases were it is
not possible to determine conclusively whether an artery had occluded preoperatively. In all three patients had an angiogram: one showed 95% stenosis of the study artery and the patient had an operation, and the other two showed an occluded artery.

There were 41 operations on left sided lesions and 40 operations on right sided lesions. Mean stenosis at preoperative assessment was 79.75% (SD = 8.76%). The distribution of the percentage stenosis is shown graphically in figure 2. Due to different machines being used on the booking and the preoperative scans, four patients were reported as having a stenosis of 60% preoperatively (a common problem when using different scan heads and currently the subject of extensive research in its own right).

**Stenosis in patient subgroups**

![Graph showing stenosis in patient subgroups](image)

Placebo  
Active
Carotid endarterectomy was carried out using the standardised technique described earlier. Patients are transferred to a high dependency unit post-operatively, and transcranial doppler monitoring continued postoperatively till patients were stable. Only one patient suffered a post-operative stroke (I.B), which was found to be haemorrhagic on CT scanning.

5.4 Summary of experimental procedures

The following summarises the experiments carried out on the samples which will be described in detail in the forthcoming chapters.

Determination of levels of doxycycline within the carotid plaque.

These were determined using high performance liquid chromatography following extraction using a commercially available solid phase extraction medium. The serum samples were similarly analysed for levels of doxycycline.

Determination of effect of doxycycline on MMP expression by monocyte derived macrophages.

Monocyte macrophages were isolated from peripheral blood of human volunteers and grown in culture media conditioned with different concentrations of doxycycline and PMA. MMP-9 secretion into the culture medium was determined using gelatin zymography and MMP-1 secretion was determined using enzyme linked immunosorbent assays.

Determination of MMP activity within tissue homogenates

This was determined using gelatin zymography and enzyme linked immunosorbent assays following homogenisation of the tissues in buffer. Gelatin
zymograms were then scanned using an Image Master-1 scanning densitometer to determine average optical density.

**Localisation of MMP-1 within carotid plaques.**

This was identified using a monoclonal antibody to MMP-1 using the ABC technique of immunocytochemical staining.

**Histological analysis of carotid plaques**

This was performed by Dr Louise Jones from the pathology department of the Glenfield Hospital in Leicester, who was also blinded to the medication the patients were receiving.

Each of these techniques will now be described fully in the forthcoming experimental chapters followed by a discussion of the results.
Chapter 6

Doxycycline Assays in Plasma and Carotid Plaques

High Performance Liquid Chromatography
6.0 Introduction.

Doxycycline is a broad spectrum antibiotic, with activity against a wide range of gram positive and gram negative organisms. It is the drug of choice in the treatment of Lyme disease, Brucellosis, and several Rickettsial infections, and it is also utilised in the treatment of sexually transmitted diseases [319, 345]. In recent years there has been increasing interest in its potential therapeutic role as an inhibitor of a group of enzymes called matrix metalloproteinases (MMP’s), which are involved in a number of pathological processes such as periodontitis, degenerative rheumatic diseases, and degenerative vascular disorders. A number of trials are currently underway to evaluate its role in these situations [299, 346, 347].

Several methods have been used to determine doxycycline concentrations in biological tissues. These include microbiological [348, 349] and fluorimetric techniques [350], gas liquid chromatography [351], thin layer chromatography [352], and high performance liquid chromatography techniques [353-355]. Spectrophotometric [356] and combined procedures for example electrospray high-performance liquid chromatography tandem mass spectrometry [357] have also been described.

Microbiologic methods are still used quite extensively, but they are non specific and time consuming, relying on inhibition of a microorganism (usually Bacillus cereus or Bacillus subtilis) in a special culture medium as a means of detection. The inhibition of growth can occur with any antibiotic in the test solution, and so the specificity of the method is very low. Fluorimetric and spectrophotometric techniques are sensitive, but interference from other material cannot always be excluded. In view of this, several methods of chromatographic determination of
tetracycline levels in biological tissues have been described. High performance liquid chromatography has a very high sensitivity, specificity, and speed of assay determinations. This paper describes a simple, quick, and sensitive assay for determination of doxycycline concentrations in biological tissues, requiring only basic HPLC apparatus which is available in most assay laboratories, and making use of a commercially available extraction pack which considerably reduces the variability associated with traditional extraction methods.

6.1 Experimental.

Apparatus

An Iso-chrom liquid chromatography constant flow pump (Spectra Physics, Oxford, UK) was used and was set at a flow rate of 1.25 ml/min. An injection valve with a 200μL loop was fitted between the pump and the column. A Spectra 100 variable wavelength detector (Spectra Physics) set at 350 nm was used for sample detection.

A Hypersil ODS C18 column (Jones Chromatography, Mid Glamorgan, UK) with an internal diameter of 4.6mm and 100mm in length was used. A Hypersil ODS 3cm guard column (Jones Chromatography, UK) with an internal diameter of 4.6mm was fitted in series to protect the separation column. A Shimadzu C-R1B integrator (Shimadzu Corporation, Maryland, US), was used to record the data. All solutions were analysed at room temperature.

Reagents

All chemicals were of analytical grade. Doxycycline, demeclocycline, oxytetracycline, and methacycline were purchased from Sigma (Poole Dorset, UK). Acetonitrile, trifluoroacetic acid, methanol, and 85% phosphoric acid were all
HPLC grade and were also purchased from Sigma. Double distilled water from an Elgastat water purifier was used in the preparation of the stock solutions.

Stock solutions

Solutions of doxycycline, demeclocycline, oxytetracycline, and methacycline were prepared by dissolving in double distilled water and made up to a concentration of 1 mg/ml and stored at 4°C away from light. The solutions were stable under these conditions for 5 days.

Mobile phase

This consisted of a solution of 50% acetonitrile in water v/v, and 0.15% trifluoroacetic acid. The solution was degassed with helium for 10 minutes before use to prevent interference from gas bubbles in the HPLC system.

Calibration

The system was calibrated by preparing serial dilutions of stock solutions from 0.25 to 5.00 μg/ml. 200 μL of the stock solutions were then injected into the chromatography column and the sample detected at 350 nm. A calibration curve was constructed by plotting the peak column height in mV against the relevant doxycycline concentration. An external calibration curve for assays in human plasma was also constructed by spiking 1 ml samples with increasing concentrations of both drugs from 0.25 μg/ml to 5.0 μg/ml. These were extracted using the method described below, and the eluate assayed under the experimental conditions described.
Collection of plasma samples.

2.0ml blood samples from patients taking doxycycline at a dose of 200mgs daily were collected in EDTA bottles. These were centrifuged at 3000g for five minutes and the plasma collected and stored at -85°C till the assays were carried out. Prior to being assayed, the samples were spiked with 2.0μg/ml of internal standard (oxytetracycline) and 1% phosphoric acid. The latter disrupts the drug protein interactions and considerably improves the extraction. The plasma was then vortex mixed for 2 minutes before extraction. Prior studies had shown that the drug was stable under these conditions, and could be stored at -85°C for up to four months with no deterioration in the assays (data not shown).

Tissue samples.

Carotid plaques were collected at operation and snap frozen in liquid nitrogen. The samples were stored at -80°C till the assays were carried out. Prior to extraction the plaques were weighed and homogenised for 10 minutes as described in chapter 8 in homogenising solution using a mechanical homogeniser set at 24000rpm. During this process the samples were kept cold by surrounding the homogenising tubes with ice to prevent any effect of heat degradation on the doxycycline. The homogenised plaques were then spun at 11,000 rpm for one hour and the supernatant used for doxycycline determination. The supernatant was then spiked with internal standard and the drug was then extracted from the solution using the method described below.

6.2 Extraction

A number of techniques have been described for extraction of tetracyclines from biological tissues. At the setting up stage an ethyl acetate extraction technique was
tested as described by Nelis et al. Briefly 200mgs of tissue were added to 2.5mls of 0.1M HCL and 100mL of internal standard and homogenised at 20,000 rpm. This was then transferred to a Teflon lined tube and 3mgs of ascorbic acid were added. The mixture was then extracted twice with 12mls of diethyl ether which was discarded. The pH of the mixture was then adjusted with 200μl of sodium hydroxide (1mol/l) and 1ml of phosphate sulphite buffer (pH 6.1). Extraction of the mixture was then performed with 10ml of ethyl acetate. After centrifugation, the organic layer was transferred to a conical tube and evaporated to dryness under reduced pressure. The resultant residue was then dissolved in 200μl of mobile phase and injected into the chromatographic column.

The technique described above involves multiple extraction steps and a drying up step which make it very time consuming. More importantly, extraction ratios were found to be very low from atheromatous tissue and reproducibility very poor (data not shown). In view of this a commercially available extraction pack (Oasis HLB Extraction cartridge, 1cc/30mgs, Waters Associates, UK), was utilised. This consists of a solid phase extraction medium which retains both polar and non-polar compounds, which are then eluted from the extraction cartridge. After conditioning the extraction cartridge with 1ml of methanol and 1ml of water, the drugs were extracted into the cartridge by injecting 1ml of plasma or tissue homogenate. This was followed by three consecutive washes using 1ml of 5% methanol in water in order to wash off unbound substances in the cartridge and reduce any interfering bands in the chromatograms. The drug was finally eluted off the extraction cartridge using 1ml of 50% acetonitrile in water (the mobile phase) and a 200μL sample was injected into the HPLC system at 1.25mls/min and detected at
During extraction, flow through the extraction cartridges was kept at 1.0ml/min.

Sample to sample variability was determined by repeated assays on five samples from the same batch on the same day. Variability at the limit of quantification was determined in a similar manner using plasma spiked with 0.125 mgs/ml of oxytetracycline and doxycycline. Day to day variability was estimated on the basis of five assays on the same batch of plasma on different days.

**6.3 RESULTS**

**Chromatographic separation**

Chromatographic separation of the doxycycline and the internal standard was completed within four minutes. The retention time for doxycycline was 2.5+/0.12 minutes and for the internal standard 1.7 minutes. The slight variation in the retention time of doxycycline was due to the fact that the integrator identified peaks at the point of reversal of the direction of current. Hence the slightly rounded peaks of the doxycycline curves produce some variation while the perfectly sharp peaks of oxytetracycline produced no variation at all. A chromatogram showing separation of doxycycline and oxytetracycline in stock solutions is shown in Figure 1a. The different substances eluted as clear symmetrical peaks which were well separated with minimal tailing and good separation between adjacent peaks. The lower limit of quantification for both drugs was from plasma samples 125ngs/ml. (Figure 1b)

**Calibration.**

Separate calibration curves were constructed using dilutions of the stock solutions in distilled water and the mobile phase, as well as an external calibration curve...
using human plasma spiked with different concentrations of oxytetracycline and doxycycline (Figure 2). These showed a linear correlation between the peak column height (mV), and the plasma concentration of doxycycline and internal standard, with a correlation coefficient ($r^2$) greater than 0.998 for all curves. The curve from the stock solutions in distilled water had a slope of 7355 +/-42.32 SD for doxycycline and 16410+/-236.3 SD for oxytetracycline, compared with a slope of 7319+/-172.7 SD and 12870+/- 147.2 SD for the solutions in the mobile phase.

**Plasma samples and tissue homogenates**

Figure 3a is a chromatogram of extracted plasma from a patient on a standard dose of doxycycline of 200mgs/day, and figure 3b is a similar chromatogram from a blank sample of serum. The extraction ratio for the sample in figure 1a was 0.78, and corresponds to a plasma concentration of 3.8µg/ml two hours after the last dose. The mean serum concentrations of doxycycline varied from 2.2-5.2 µg/ml (mean 3.7µg/ml) three hours after ingestion and fell to 1.2 to 2.6µg/ml (mean 1.9µg/ml) twelve hours after the last dose. The sensitivity of this method is therefore well within the limits required for estimation of plasma doxycycline.

Doxycycline levels in plaque homogenate from patients on the drug were assayed in a total of 27 patients. Mean concentrations achieved in atherosclerotic tissue varied between 0 µg/ml in one patient to 1.36µg/ml, corresponding to a mean tissue concentration of 6.5µg/g (wet weight) (Figure 4). Assays were also carried out on ten plaques from patients on placebo prior to the trial and no doxycycline at all was detected in these plaques (data not shown). A chromatogram from a sample of carotid plaque homogenate from a patient on a standard dose of 200mgs of
doxycycline daily is shown in figure 3c. The effect of the homogenising process on the stability of doxycycline was tested using stock solutions which were submitted to all the homogenising and quantification process and no significant effect was noted on the drug.

Plasma levels of doxycycline as determined using this assay correspond well with similar levels found in the literature [319, 345]. No work has been carried using HPLC to quantify levels of doxycycline in atherosclerotic tissue, but the levels we found correlate with similar levels of tetracycline obtained by biological assays performed by another group using atherosclerotic tissue in human aorta [322].

**Variability and limit of quantification**

Intra-assay variability was 2.23%. This was estimated on the basis of five different samples from the same batch of fetal calf serum spiked with a known concentration of doxycycline (Table 1). Day to day variation of assays on the same batch was 4.43% (Table 1). The limit of quantification was defined as the lowest concentration producing a reproducible positive deflection with a coefficient of variation less than 15% between estimated levels. Variability at the limit of quantification was 13.23% (Table 1), and was also estimated on the basis of five replicate analyses on the same batch. The limit of detection was 50ngs/ml, producing a positive deflection of around 1000mV on the chromatogram, however at these levels the variability in column height precluded its use for reliable quantification. Extraction ratios from human plasma samples varied between 73% and 93%, with a mean of 82.7% and a coefficient of variation of 7.69% (Table 2). Acidifying the sample prior to extraction considerably improved the extraction ratio.
and reproducibility of the system, presumably because all of the drug was extracted from protein, and all data supplied here is reported was determined using acidified samples as described previously.

6.4 DISCUSSION

The problems associated with tetracycline assays have been reviewed by Ashworth [358]. Several extraction methods have been described, but most involve complex extraction steps [359-361]. The main problem with these procedures is that most are time consuming but most importantly result in a great variability in the extraction ratio between different samples and even in the same sample. In view of this we tested a commercially available extraction pack (Oasis HLB Extraction cartridge, 1cc/30mgs). This consists of a solid phase extraction medium which retains both polar and non-polar compounds, which are then eluted from the extraction pack. Using the method suggested by the manufacturer extraction ratios of 30+/- 5% were obtained. This is because tetracyclines are very polar compounds and attempted extraction with methanol as described resulted in a very poor yield. However different extraction solvents were tested at different concentrations and pH values. These included butanol, acetonitrile, and ethyl acetate. Mean extraction ratios above 80% were obtained using 50% acetonitrile in water (the mobile phase), which was therefore used as described previously. The eluant obtained after the washing up steps with 5% methanol was chromatographed several times at the setting up stage and it was found that no doxycycline was removed by the washing solution (data not shown).
When using one of the above methods, a drying up step is usually involved before a solution of the extracted substance is then made up in the mobile phase and analysed in the HPLC apparatus. By using the mobile phase as the extracting solution, we have been able to avoid a drying up step, thereby simplifying and speeding up the assay procedure. The sensitivity of the system is adequate enough to enable this to be carried out without the need for concentrating the drug in the eluant solution.

Doxycycline is very highly protein bound (82-93%), and both hydrophobic as well as charge transfer mechanisms are involved at physiological pH. [362]. Addition of phosphoric acid to a sample results in dissociation and greatly improves the extraction yield. Other authors have advocated the use of phenylbutazone during the extraction in order to displace the drug from protein and improve extraction yield [360]. Ascorbic acid has also been used to suppress epimerization of tetracyclines and improve the extraction of the internal standard [356], however this was not necessary under our conditions, as long as the samples are injected on the same day as extracted. If the samples are kept at 4°C and protected from light the rate of epimerization is significantly reduced, and they can be injected the next day; provided they are quantitated and compared with a standard prepared at the same time and kept under the same conditions [363].

Tetracyclines are very polar substances, and their dissociation is very highly influenced by pH changes. Hence varying the pH during different phases of extraction and elution has a great influence on the sensitivity of the assays, as discussed fully by De Leenheer et al [364]. In the set up experiments on our system we found that reducing the pH of the mobile phase to 2.0 greatly affected the
sensitivity of the system, and also resulted in symmetrical and well defined peaks.
When calibrating the system, 1% phosphoric acid was added to the stock solutions, 
as in the case of the plasma), as the presence of phosphoric acid in the sample 
resulted in up to a twofold increase in peak height. (sensitivity) per unit 
concentration.

The limits of quantification in the literature vary with the method used for 
detection. Using UV detection, Mulders et al reported a limit of quantification of 
0.5-1μg/g of tissue, while Prevosto et al were able to quantify down to 25ngs/ml of 
plasma. [365] [366]. Studies using fluoroscopic detection report lower limits of 
quantification varying between 5-50ngs/ml [367, 368]. Limits of detection in the 
literature again vary according to method of detection, varying from 50ngs/ml using 
UV detection to 1.38ng/g of doxycycline in porcine muscle using fluoreseence 
detection [367, 369]

Tetracyclines are potent chelators of divalent cations like calcium and magnesium, 
which affect the way the drug elutes. Some authors have suggested the addition of 
EDTA to the mobile phase in order to improve resolution, while others pretreat the 
column with a chelating agent prior to elution [356, 370]. This problem was avoided 
in our case as human plasma were collected in EDTA bottles which chelate any 
divalent cations and prevents interaction with the drug.

The interposition of a 30mm guard column between the injection valve and the 
separating column had the beneficial effect of further sharpening the peaks and 
improving separation as well as its main function of protecting the main column 
from excessive debris. This is because in effect the guard column adds a further 3 
cms to the separating system. We tried to use other tetracyclines including 
demeclocycline and methacycline as internal standards, but the clearest separation
between the peaks was obtained using oxytetracycline as this elutes well before
doxycycline giving clear separation between bands.

The method described is very simple, rapid, and has good reproducibility, and we
have used it to assay doxycycline levels in patients on standard therapeutic doses. A
blood sample can be assayed in less than twenty minutes and the sensitivity of the
system is well within the range of values in the therapeutic range.
6.5 Figures and Tables

Figure 1. Chromatogram showing separation of oxytetracycline (1.7) and doxycycline (2.7) at 2.0μgms/ml (a) and at the limit of quantification (b).
Figure 2. Calibration curves. A is a calibration curve for oxytetracycline in human plasma, B is oxytetracycline in water, C is oxytetracycline in a solution of 50% acetonitrile in water, D and E curves overlap and represent calibration curves for doxycycline in water and 50% acetonitrile in water respectively.
Figure 3. Chromatograms from a patient on 200mgs of doxycycline daily (a), chromatogram of a blank sample of serum (b), chromatogram from tissue homogenate(c), chromatogram from plaque homogenate from a patient on placebo (d). Oxytetracycline peak at 1.7s, doxycycline peak at 2.5s.
Figure 4. Distribution of doxycycline concentrations in carotid plaques. 1mL is equivalent to 100 mgs of tissue homogenate.

Table 2. Extraction ratios from 8 representative plasma samples and carotid plaque homogenates from patients on doxycycline 100mg/day.
<table>
<thead>
<tr>
<th></th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrasample variability</td>
<td>28.3  27.8  27.0  26.8  27.6  2.2%</td>
</tr>
<tr>
<td>Day to day variability</td>
<td>31.6  30.6  28.3  31.3  29.6  4.4%</td>
</tr>
<tr>
<td>Variability at limit of quantification</td>
<td>14.8  15.3  16.9  16.6  11.7  13.7%</td>
</tr>
</tbody>
</table>

Table 1. Data showing coefficients of variation based on five replicate analyses from each batch. Units shown are peak column height of internal standard in volts.

<table>
<thead>
<tr>
<th>Extraction ratios</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.73  0.85  0.87  0.78  0.93  0.8  0.79  0.85  7.6%</td>
</tr>
<tr>
<td>Tissue homogenate</td>
<td>0.64  0.49  0.72  0.48  0.53  0.71  0.58  0.67  15.9%</td>
</tr>
</tbody>
</table>

Table 2. Extraction ratios from 8 consecutive plasma samples and carotid plaque homogenates from patients on doxycycline 100mgs/bd.
The Effect of Doxycycline on MMP-1 and MMP-9 secretion by Monocyte derived Macrophages
7.0 Introduction

The critical role that macrophages play in the inflammatory process of atherosclerosis and their potential role in carotid plaque destabilisation via secretion of cytokines and MMP's have been discussed in previous chapters. Although doxycycline has been shown to inhibit activation of secreted pro-MMP's and to directly inhibit the active enzymes, little work has been done on the effect of doxycycline on MMP expression by human monocyte derived macrophages. When used as an antibiotic, doxycycline achieves its effect by binding to the 30S ribosomal subunit and preventing translation of proteins. Another possible mechanism of action that could be operative in inhibiting protein secretion is inhibition of mRNA expression.

One may therefore hypothesise that a similar effect may be operative to reduce secretion of MMP's by macrophages. Indeed, recent work by Libby et al suggests that it inhibits MMP-2 secretion by inhibiting mRNA expression.

The purpose of this experiment was to investigate the potential effect of doxycycline in inhibiting MMP expression by human monocyte derived macrophages.

Previous work by Shah et al has shown that PMA, a phorbol ester, can activate macrophages and stimulate the secretion of MMP's. Other studies have also shown that when monocytes bind to surfaces they are activated, through a mechanism involving protein kinase C.

In the following experiment, monocytes were isolated from human peripheral blood and stimulated to differentiate into macrophages by PMA. The cells were then incubated with different concentrations of doxycycline, and the amount of MMP-1 and MMP-9 secreted at different concentrations of doxycycline was estimated.
7.1 METHODS

Chemicals and reagents.

Ficoll-Paque was obtained from Sigma (Poole, UK.). RPMI 1640, MEM, fetal calf serum, L-Glutamine, Hepes, penicillin, streptomycin, mercaptoethanol and sodium pyruvate were all obtained from Gibco (US). Mouse monoclonal anti CD68 (PGM1), and rabbit antimouse antibody conjugated to peroxidase were provided by the pathology department of the Leicester Royal Infirmary.

Acrylamide/bis-acrylamide (Protogel) was obtained from National Diagnostics (Atlanta, USA.). TEMED and gelatin were both from Sigma (Poole, UK). Gels were run on a mini Protean II electrophoresis system from Biorad (Hemel Hempstead, UK).

Monocyte isolation and culture.

Heparinised blood was obtained from human volunteers and the leucocytes isolated by density gradient centrifugation at 2000rpm using Ficoll Paque. The white cells were then collected and washed with PBMC wash buffer (500mls Kays MEM, 4mL 0.5M EDTA in phosphate buffered saline, and 20mL foetal calf serum), and resuspended in RPMI 1640 supplemented with 1mmol/L glutamine, 10mls of 1M Hepes, 10mls of penicillin and streptomycin, 0.5mL of 2-mercaptoethanol and 1mL of sodium pyruvate and 10% foetal calf serum. Viability on isolation was >95% as assessed by trypan blue exclusion. The cells were then separated into eight 35mm culture dishes at a density of 1.5 million cells per well. They were then incubated overnight at 37°C and the non adherent cells were then discarded. Fresh culture medium was replaced every 2 days. On day 7, the culture medium was replaced by serum free medium conditioned with 0, 1, 10 and 100μg/mL of doxycycline.
Another set of 4 wells was conditioned as above but in addition the medium was conditioned with phorbol myristate acetate (PMA) at a concentration of 3 ng/ml. The conditioned media were collected after 24 hours of incubation at 37°C.

**Immunocytochemistry.**

Immunocytochemistry was used to confirm the type of cells being cultured. The cells were first fixed in cold 4% paraformaldehyde. They were then incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase. This was followed by a wash with water and phosphate buffered saline, and incubated with normal rabbit serum diluted at 1:20 in PBS to block any non specific binding by the secondary antibody. Mouse monoclonal anti CD68 (PGM1), was then added at a dilution of 1:50 in phosphate buffered saline, and incubated for 1 hour at room temperature. The slides were then washed again and the secondary antibody (rabbit anti-mouse immunoglobulin) was added at a dilution of 1:25 for 30 minutes. After another wash diaminobenzidine was added for five minutes followed by copper sulphate solution. The slides were then washed with tap water and stained with haematoxylin for 30s. The sites of CD68 antigen were then visualised as dark brown/black staining under light microscopy.

**Zymography.**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the supernatant of the monocyte cultures. SDS-PAGE in 10% gels containing 0.1% (wt/vol) gelatin were prepared. The resolving gel consisted of 3ml of double distilled water, 1mg of gelatin, 3.34ml of 30%/0.8%acrylamide bis, 2.5ml of resolving buffer, 100μl of SDS, 50μl of freshly prepared ammonium persulphate, and 5μl of TEMED. The stacking gel consisted of 6.1ml of water, 1.3ml of
30%/0.8% acrylamide/bis, 2.5ml of stacking buffer, 100μl SDS, 50μl ammonium persulphate, and 10μl of TEMED. The supernatant was denatured for 15 minutes in an equal volume of electrophoresis sample buffer (0.5mol/L Tris HCl pH 6.8, 20% glycerol, 10% SDS, and 0.1% bromphenol blue). Twenty five microlitres of sample were loaded onto each well and subjected to electrophoresis at 80V. Following electrophoresis the gels were washed three times with 2.5% Triton X-100, to remove the SDS from the gels and allow the separated proteins within it to renature. The gels were then incubated for 18 hours at 37°C in zymogram buffer (50nM Tris, 10nM calcium chloride, 50nM sodium chloride, 0.05% v/v BRIJ 35S). The gels were then stained for 3 hours with Coomassie blue stain (25% methanol v/v, 10% v/v glacial acetic acid, 0.02% Coomassie blue R-250). The areas of proteolysis of the substrate (gelatin) showed up as clear bands against a dark blue background. Positive controls included supernatant of HT1080 fibrosarcoma cells, a rich source of MMP-2 and 9. A commercially available peptide ladder was also run concomitantly on one of the lanes to allow estimation of the molecular weight of protein products. The zymograms using supernatants from the different groups of cells were analysed using an Image Master 1D scanning densitometer and a value for the average optical density of the bands was generated.

Statistical analysis

All data was analysed using Graphpad statistical analysis software. The data regarding MMP-9 expression is all presented as mean +/- standard error of the mean. The difference in MMP expression was compared with one way ANOVA, with a value of P<0.05 considered significant.
7.2 RESULTS

Immunocytochemistry.

Microscopic examination of the monocytes showed a visible morphologic change after 4-5 days, with increase in size and the appearance of pseudopodia. Viability of the cells was assessed in one group of cells after treatment with doxycycline as described above in order to assess any toxic effect of doxycycline on the cells and showed no change in the number of viable cells. Immunocytochemistry using anti CD68 characterised the cells as monocyte macrophages (Figure 1).

Figure 1. Photograph of monocyte derived macrophages stained with anti CD68 confirming the cultured cells to be macrophages.

Zymography

Zymography of the supernatants obtained from cell cultures showed a clear gelatinolytic band between 92 and 80 KD, consistent with the presence of inactive as well as active forms of MMP-9. There was a dose dependant inhibition of MMP-9 expression which was consistent across all groups of cells cultured.
The results of the average optical density obtained from bands using the supernatants from the cell cultures in the presence and absence of PMA are shown in table 1. There was no difference between PMA stimulated and non PMA stimulated cell groups with regard to MMP expression (Figure 2).

<table>
<thead>
<tr>
<th>Doxycycline concentration in µgs/mL</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro MMP-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No PMA</td>
<td>1.5+/-.27</td>
<td>1.5+/-.25</td>
<td>1.3+/-.33</td>
<td>0.6+/-.28*</td>
</tr>
<tr>
<td>PMA</td>
<td>1.5+/-.44</td>
<td>1.5+/-.46</td>
<td>1.3+/-.26</td>
<td>0.6+/-.34*</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No PMA</td>
<td>0.7+/-.37</td>
<td>0.7+/-.38</td>
<td>0.4+/-.28</td>
<td>0.08+/-.07*</td>
</tr>
<tr>
<td>PMA</td>
<td>0.7+/-.46</td>
<td>0.6+/-.44</td>
<td>0.3+/-.25</td>
<td>0.02+/-.05*</td>
</tr>
</tbody>
</table>

Table 1: Average optical density of gelatinolytic bands on zymography in the presence of different doxycycline concentrations. All values are mean +/- standard deviation. (* = P<0.01, ANOVA)

Figure 2. MMP-9 secretion at 0,1,10, and 100µgs/mL of doxycycline in the presence or absence of PMA stimulation.
The concentration of doxycycline at which there was a 25% reduction in enzymatic activity (IC$_{25}$) is 43µgs/mL of medium, while the IC$_{50}$ is 86µgs/mL. The values in each set of experiments have an $r^2$ value of 0.98 (goodness of fit), and each slope is not significantly non linear.

MMP-1 Elisa
The concentration of MMP-1 produced by monocyte derived macrophages is much lower than that for MMP-9 and is not detectable using zymography. An ELISA kit was therefore used to quantify MMP-1 levels. In contrast to MMP-9, there was very little MMP-1 secreted in the absence of PMA stimulation, whereas PMA caused about a twofold increase in MMP-1 secretion. These different responses to PMA stimulation are probably related to the fact that MMP-9 is more constitutively expressed, whereas MMP-1 secreted upon promoter region activation (chapter 4). The results of both groups are shown in the table 2 below, and depicted graphically in figures 3 and 4.

The concentration of doxycycline at which there was a 25% reduction in enzymatic activity (IC$_{25}$) is 2.4µgs/mL of medium, while the IC$_{50}$ is 64µgs/mL.

<table>
<thead>
<tr>
<th>Doxycycline concentration in µgs/mL</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MMP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No PMA</td>
<td>0.769+/-.027</td>
<td>0.763+/-.077</td>
<td>0.857+/-.06</td>
<td>0.694+/-.03</td>
</tr>
<tr>
<td>PMA</td>
<td>1.42+-.192</td>
<td>0.091+-.076*</td>
<td>0.86+-.052</td>
<td>0.71+-.016*</td>
</tr>
</tbody>
</table>

Table 2. Expression of MMP-1 by monocyte macrophages (mean+/-SD, ngs/mL)
Figure 3. Effect of increasing concentrations of doxycycline on MMP-1 expression by monocyte derived macrophages.

Figure 4. Effect of doxycycline on MMP-1 expression by monocyte derived macrophages: No PMA.
7.3 Conclusion

This experiment has shown that doxycycline inhibits both MMP-1 and MMP-9 expression by monocyte derived macrophages in a dose dependant manner. However, the concentration of doxycycline necessary to inhibit 25% of basal MMP1 expression is much lower than that required to inhibit MMP-9 expression by the same amount (Figure 1 and 3). The concentrations of doxycycline required to produce 25% inhibition were 43μgs/mL and 2.4μgs/mL for MMP-9 and MMP-1 respectively.

On the basis of the doxycycline levels achieved in carotid atherosclerotic plaques from the experiment in chapter 6, one would therefore expect a greater degree inhibition of MMP-1 activity in vivo compared to the level of MMP-9 inhibition. The dose dependant inhibition might be useful in vivo to stabilise a collagenolytic milieu into a more stable microenvironment, and forms the basis of the clinical trial described in the subsequent chapters.

- Doxycycline inhibits MMP-1 and MMP-9 secretion by monocyte derived macrophages in a dose dependant manner.
- The dose at which doxycycline inhibits MMP-1 expression is much lower than the dose at which it inhibits MMP-9 secretion (IC$_{25}$ = 2.4μgs/mL and 43μgs/mL respectively).
- At the concentration of doxycycline achieved in carotid plaques, one would expect a reduction in MMP-1 and 9 expression in vivo, the reduction being higher for MMP-1.
Chapter 8

Zymography and Elisa

Methods and Results
8.0 Methods

Homogenisation

Prior to enzyme quantification all the carotid plaques were homogenised to yield a homogenous suspension. The tissue was first weighed and then cut up into small sections using a scalpel blade to allow better homogenisation. 1mL of homogenising buffer (Urea 2mol/l, Tris HCL 50mmol/l, NaCl 1g/l, EDTA 1g/l, Brij 35 1ml/l, PMSF 0.1 mmol/l, NaOH to a final pH 7.6) was then added per 100mgs of tissue. This was then homogenised mechanically at 20,000rpm for ten minutes, during which time the Dupont tubes were kept in ice to avoid damage to the protein from the heat generated.

Following this the resultant suspension was centrifuged at 11,000rpm for 1 hour at 4C with no brake applied, and the homogenate separated into a cloudy supernatant and a solid precipitate at the bottom. The supernatant was then used for dialysis and the solid precipitate discarded.

Dialysis

Segments of Visking tubing with a molecular weight cut off at 12-14,000 Dalton (Medicell International Ltd, London, U.K,) were boiled in distilled water for 2 minutes, the water was then pressed out and a clip applied to one end. The supernatant was then pipetted into the tubing and the other end closed. This was left in 150mLs of dialysis buffer (Tris HCL 25 mmol/l,CaCl2 10 mmol/l, BRIJ 35 0.1%, PMSF 0.1 mmol/l) per sample for eighteen hours at 4C. The dialysate was then collected and stored at -80C till required.
Protein standardisation

In order to have comparable assays the amount of protein per sample was estimated and then the results of each assay were standardised to 1mg of protein per mL. Standards of 1,2,3 mgs/mL were set up using bovine serum albumin (Sigma Chemicals, Poole, U.K, 10mgs/mL stock solution), in phosphate buffered saline(PBS). Cuvettes were then prepared with 100μL's of PBS (blank), 5 μL's of each standard or sample, and 95 μL's of PBS. 1mL of protein reagent diluted 1:5 (Bio-Rad Protein Assay, Bio-Rad Labs, GmbH), was then added to each cuvette, and left for ten minutes at room temperature. The protein concentrations in each were then prepared using a UV spectrophotometer (Shimadzu UV-1601, Japan). A calibration curve was then constructed using the known standards and the concentration in the unknown samples determined off the standard curve. All samples were standardised at one session and compared to the same controls in order to minimise error.

Zymography

Zymography gels were prepared as described for the monocyte macrophage study in chapter 7. 20 μL's of sample were mixed with 20μL's of non-reducing buffer and 30μL's of the mixture was loaded onto the wells. 15μL's of molecular weight marker was also loaded at one end, and culture medium from HT1080 fibrosarcoma cell lines were loaded onto the gels at the other end to act as controls.

The gels were run for 4 hours at 80mVs and incubated for 18 hours at 37°C. They were then washed 3 times in 2.5% triton and stained with 0.1% Coomassie blue. The resultant bands were then scanned using an Image Master 1D scanning densitometer, and the average optical density of each band determined by comparing to the HT1080 internal control.
Elisa

Principle

The assay is based on a two site enzyme linked immunosorbent assay (ELISA), sandwich format. Standards and samples were incubated in microtitre wells precoated with antibody to the enzyme in question. Any enzyme present in the sample is bound to the wells, the other components of the sample being removed by washing and aspiration. The bound enzyme is then detected by an antibody bound to a peroxidase enzyme, and any excess antibody is removed by washing and aspiration. The amount of antibody present in each well is then determined by the addition of tetramethylbenzidine(TMB) substrate, which forms a bluish insoluble colour on oxidation in the presence of hydrogen peroxide. The reaction is stopped by the addition of an acid solution, and the resultant colour is read at 450nm in a microtitre spectrophotometer. The concentration of enzyme in the sample is determined by interpolation from a standard curve which is constructed with each assay by using several known standards which are supplied by the manufacturer.

There are slight variations in the technique between different assays. The incubation times at different stages vary with different protocols. In the case of MMP-1, an additional step is added after the enzyme is bound to the antibody coated wells. A second antibody is used to bind to the enzyme, and this is then detected by a third antibody conjugated to horseradish peroxidase in the usual way.

Each pack comes with a 96 well plate which allows the construction of a standard curve plus the measurement of 90 samples. In each case a dilution curve was also constructed before the actual sample assays to determine the optimum dilution of the samples for the assay to be within the range of the assay kits. Two samples were
used for each experiment at 1:1,2,5,10,50 serial dilutions. The final concentration used for each experiment is stated in the results section. All samples were loaded onto the assay plate at the appropriate dilution as described above, and the results were then standardised for protein concentration retrospectively, as we found that this produced much less error than doing double dilutions on each sample (for protein standardisation and for optimum dilution for assay).

**Technique**

Again the assay technique was common for all ELISA packs, and a general technique will be described which is common to all. Any changes to a particular assay will be described at the end.

**Preparation of working standards**

Standards were prepared immediately prior to use. The manufacturer supplied vial containing known amounts of working standards with each pack as shown below:

- MMP-1 200ngs/mL
- MMP-2 48ngs/mL
- MMP-3 240ngs/mL
- MMP-9 64ngs/mL
- TIMP-1 100ngs/mL
- TIMP-2 256ngs/mL

Some of the standards were supplied ready mixed and others were reconstituted from the lyophilised protein using the assay buffer.

Known concentrations of each enzyme were then prepared for each assay by serial dilutions of the standard. For example for MMP-2 standards of 24,12,6,3,and 1.5ngs/mL were prepared by serial dilutions of the supplied sample (48ngs/mL) in
assay buffer. The supplied stock solution was not used for the construction of the calibration curve in any of the kits.

Assay protocol

All reagents were equilibrated to 20-27°C before use. The samples were diluted in assay buffer to the optimum level which was determined as described above. The first column was used to construct a calibration curve. 100μL's each of assay buffer (blank control), and 100μL's of each prepared serial dilution of the prepared standard was then added to each well. 100μL's of sample was then added to the rest of the wells. The plate was covered with the lid provided and incubated for 2 hours at room temperature (1 hour at 2-8°C for MMP-3).

All wells were then aspirated and washed 4 times with the wash buffer supplied with the kit ensuring that the plates were completely filled in at each wash. Any excess wash buffer was then removed by tapping the plate upside down onto blotting paper to ensure any residual is removed completely. 100μL's of peroxidase conjugate was then added to all wells and incubated for one or two hours at room temperature as suggested by the manufacturer (2 hours for MMP-1,3,9, and TIMP-1,2 and 1 hour for MMP-2). This was followed by another wash with wash buffer as in the first wash above.

In the final phase of the assay 100μL's of room temperature equilibrated TMB was added to the wells, and the wells are incubated for 30 minutes at room temperature. A blue colour forms from oxidation of the TMB by the peroxidase. The reaction
was stopped by the addition of 100μL's of 1M sulphuric acid to all wells and the resultant yellow colour was read at 630nm.

In the case of MMP-1, a separate step was added following the binding of the enzyme to the wells and the first wash. A polyclonal antibody to MMP-1 was added at this stage and incubated for 2 hours at room temperature. The second antibody bound to the wells was then detected using donkey anti-rabbit antibody conjugated to horseradish peroxidase. After the second wash the amount of bound horseradish peroxidase was detected using the TMB substrate as described above.

**Calibration**

A calibration curve was constructed using the known standards plotted against the resultant absorption spectra at 630nm, and the protein concentration in the samples was then determined using the graph facility on Microsoft Excel®. The resultant values were then divided by the protein concentration ratios obtained from protein standardisation so that the resultant values were all equilibrated to 1mg/mL of protein, and therefore comparable. The resultant value was then multiplied by the dilution value if this was used in the original sample, and again multiplied by 10 to bring the value of the protein concentration to ngs/100mgs of tissue. The latter was necessary because in the original homogenisation the plaque was homogenised using 1mL of homogenising buffer per 100mgs of tissue, so loading 100μL's of sample is equivalent to loading 10mgs of tissue homogenate.
**Statistical analysis**

The data was then analysed using Graphpad Prism statistical analysis software®. Prism tests for deviations from Gaussian distribution using the Kolmogorov-Smirnov test. The P value comes from Dallal and Wilkinson's approximation to Lilliefors' method. The plain Kolmogorov-Smirnov test cannot be used because we don't know the mean and SD of the population. Instead, we estimate these values from the sample being analysed.

Prism reports the Kolmogorov-Smirnov distance, abbreviated KS distance. If the sample followed a Gaussian distribution exactly, this would equal zero. Larger values of the KS distance correspond to larger deviations from an ideal Gaussian distribution.

Prism also reports a P value which answers this question: If the population were really Gaussian, what is the probability that a randomly selected sample of this size would deviate as much from a Gaussian distribution (or more so) than observed here. More precisely, the P value answers this question: If the population were really Gaussian, what is the chance that a randomly selected sample of this size would have a KS distance as large, or larger, than observed?

In practise if the P value is large, for our sample size, it was assumed that the distribution is gaussian and a parametric test was used. On the other hand if the P value was small the chances of the given set of values being selected from a truly gaussian population were highly improbable (P<0.05), and a non parametric test was used. A P value of less than 0.05 in the final statistical analysis was considered significant.
8.1 Results

Zymography

Zymography of carotid plaque homogenate reveals two strong bands at the 92 KD and the 72KD regions corresponding to pro MMP-9 and pro MMP-2. Smaller bands at approximately 80 and 65 KD's below the major bands are visible and correspond to the active forms of the enzymes. A typical zymogram obtained from 7 samples of carotid plaque homogenate is shown in figure 1.

![Pro MMP-9 and Pro MMP-2](image)

Figure 1. Zymogram from 7 carotid plaque homogenates showing major MMP 2 and 9 bands. (P= placebo, A = active).

There was no statistically significant difference on scanning densitometry between the proenzyme forms of MMP-9 and MMP-2, (P= 0.83 and 0.53 respectively) and this data will therefore not be analysed further as it is covered by the Elisa assays.

The zymograms can differentiate between the active and proenzyme forms of MMP's as already described, and therefore the results of the active MMP-9 and MMP-2 assays will be described in detail, as the Elisa Kits measure total enzyme levels. An Elisa kit measuring MMP-9 activity is available and an attempt at
quantifying the active enzymes was made, however the kit was not found to be accurate enough and the data very unreliable, and so was not reported in this thesis.

**Active MMP-9**

The analysis of the data from scanning densitometry of the active MMP bands is shown in table 1 below.

<table>
<thead>
<tr>
<th>Minimum</th>
<th>0.002872</th>
<th>0.0500</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% Percentile</td>
<td>0.1201</td>
<td>0.1300</td>
</tr>
<tr>
<td>Median</td>
<td>0.2457</td>
<td>0.2150</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>0.3243</td>
<td>0.3250</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.5182</td>
<td>0.5300</td>
</tr>
<tr>
<td>Mean</td>
<td>0.2388</td>
<td>0.2434</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>0.1486</td>
<td>0.1422</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.02443</td>
<td>0.02513</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>0.1892</td>
<td>0.1922</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>0.2883</td>
<td>0.2947</td>
</tr>
</tbody>
</table>

**Normality Test**

| KS distance      | 0.09221  | 0.1759 |
| P value          | P > 0.10 | P > 0.10 |
| Passed normality test (*=0.05)? | Yes | Yes |
| P value summary  | ns       | ns     |

Table 1 Analysis of active MMP-9 bands on Zymography. All values are ratios on scanning densitometry

The active MMP-9 bands could not be defined in ten patients that were therefore excluded from the analysis. The mean optical density on scanning densitometry of the group on doxycycline was 0.24 ± 0.02 (Mean +/-SEM), compared to 0.24 ± 0.025 for the placebo group, an absolute difference of 0.0046 ± 0.03 (Relative difference of 2%). The data followed a gaussian distribution and an unpaired t test was used in the analysis. This gave a P value of 0.9, confirming that the difference between the two groups was not statistically significant. The analysis is shown in table 2 and the data is represented graphically in figure 2.
Unpaired t test

**P value** 0.8951

**P value summary** ns

Are means signif. different? (P < 0.05) No

One- or two-tailed P value? Two-tailed

t, df t=0.1324 df=67

How big is the difference?

Mean ± SEM of column A 0.2388 ± 0.02443 N=37

Mean ± SEM of column B 0.2434 ± 0.02513 N=32

Difference between means -0.004654 ± 0.03516

95% confidence interval -0.06558 to 0.07489

R squared 0.0002614

F test to compare variances

F,DFn, Dfd 1.092, 36, 31

P value 0.4037

**P value summary** ns

Are variances significantly different? No

Table 2 Analysis of data from MMP-9 scanning densitometry using an unpaired t test. All values are ratios compared to HT 1080 control (no units)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Average optical density on scanning densitometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>[0.18, 0.22]</td>
</tr>
<tr>
<td>Placebo</td>
<td>[0.17, 0.21]</td>
</tr>
</tbody>
</table>

Figure 2: Active MMP-9 data from Zymography. (Error bars show standard error of mean)
Active MMP-2

The active MMP-2 band on Zymography is the weakest band and could not be identified in 9 gels which were excluded. Therefore there were 37 patients on doxycycline and 33 patients on placebo. A summary of the characteristics of the data is shown in table 3.

<table>
<thead>
<tr>
<th></th>
<th>Number of values</th>
<th>Minimum</th>
<th>25% Percentile</th>
<th>Median</th>
<th>75% Percentile</th>
<th>Maximum</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>37</td>
<td>0.0000</td>
<td>0.03546</td>
<td>0.06500</td>
<td>0.1126</td>
<td>0.3050</td>
<td>0.08898</td>
<td>0.07670</td>
<td>0.01261</td>
<td>0.06340</td>
<td>0.1146</td>
</tr>
<tr>
<td>Minimum</td>
<td>33</td>
<td>0.0100</td>
<td>0.0600</td>
<td>0.0800</td>
<td>0.1400</td>
<td>0.3100</td>
<td>0.1018</td>
<td>0.06574</td>
<td>0.01144</td>
<td>0.07851</td>
<td>0.1251</td>
</tr>
</tbody>
</table>

Normality Test

| KS distance | 0.1894 | 0.1755 |
| P value     | P > 0.10 | P > 0.10 |
| Passed normality test (*=0.05)? | Yes | Yes |
| P value summary | ns | ns |

Table 3 Analysis of data on scanning densitometry of active MMP-2 bands on zymography. All values ratios compared to controls (no units).

The mean optical density of the bands in the active group was 0.09 ± 0.013 (mean±SEM), and 0.10 ± 0.011, and absolute difference of 0.013 ± 0.017. The data followed a normal pattern of distribution and an unpaired t test was used in the analysis, which gave a P value of 0.45 for the difference between the groups. This analysis is shown in table 4 and the results depicted graphically in figure 3.
Unpaired t test

**P value**

| P value summary | 0.4573 |
| Are means signif. different? (P < 0.05) | ns |
| One- or two-tailed P value? | No |
| t, df | Two-tailed |
| 0.75 df=68 |

**How big is the difference?**

| Mean ± SEM of column A | 0.089 ± 0.013 N=37 |
| Mean ± SEM of column B | 0.10 ± 0.011 N=33 |
| Difference between means | -0.013 ± 0.017 |
| 95% confidence interval | -0.021 to 0.047 |
| R squared | 0.0082 |

**F test to compare variances**

| F,DFn, Dfd | 1.4, 36, 32 |
| P value | 0.1894 |
| P value summary | ns |
| Are variances significantly different? | No |

Table 4. Statistical analysis of data from active MMP-2 scanning densitometry.

Figure 3: Optical density of active MMP-2 bands on scanning densitometry. (Error bars show standard error of the mean)
Experience with gelatin zymography

As discussed earlier, zymography was used because of its advantage over ELISA in being able to measure active enzyme concentrations. However, during our experiments the problems discussed below were encountered which greatly hindered any meaningful interpretation of the data obtained.

1. Reproducibility
During the initial experiments a number of gels were run and repeated twice using the same samples. Despite using HT 1080 internal standards to be able to compare different gels, it was found that reproducibility was very poor, and this made any comparison between different gels meaningless (data not available).

2. Identification of bands.
Gelatinolytic bands were identified using the Image Master 1D scanning densitometer. This was initially calibrated to exclude background noise from the image obtained after scanning the gels and then calibrated to recognise areas of lysis on the zymograms. However it was found that as the limits of the bands were not always sharp enough or even linear, there was a large degree of variability in the areas mapped out for densitometric analysis especially on bands with a large element of ‘trailing’ at the edges. Indeed, in some of the gels the densitometer failed to identify some of the bands, while there was considerable overlap between active and proenzyme bands in some other areas, making analysis impossible.

3. Inability to apply gelatin zymography to MMP-1
MMP-1 degrades mainly collagens types I, II and III, and therefore would not produce any visible bands on gelatin zymography. In addition, no data was available on the concentrations of MMP-1 in carotid plaques. In view of this, ELISA kits were used to quantify MMP-1.

4. Gelatin zymography was semiquantitative at best.
In semi quantitative densitometry, the optical densities of bands are compared to an internal standard, and the system is not calibrated to a range of external standards.
Thus, only comparison of different ratios to an internal standard is possible. Elisa kits have an external calibration curve incorporated into the assay, which allows quantitative data on enzyme levels in different specimens to be obtained.

In view of the above limitations, the mainstay of MMP assays in carotid plaques was based on ELISA determinations. The section on gelatin zymography was included merely as it was felt to reflect part of the experience gained from this research project.

**Elisa**

**MMP-1**

The raw data and calculations are shown in appendix 1. The analysis of the data is shown in table 5.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>4.455</td>
<td>5.531</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>5.976</td>
<td>6.887</td>
</tr>
<tr>
<td>Median</td>
<td>7.845</td>
<td>10.09</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>10.06</td>
<td>20.68</td>
</tr>
<tr>
<td>Maximum</td>
<td>58.92</td>
<td>277.2</td>
</tr>
<tr>
<td>Mean</td>
<td>11.52</td>
<td>22.26</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>10.28</td>
<td>45.64</td>
</tr>
<tr>
<td>Std. Error</td>
<td>1.605</td>
<td>7.714</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>8.272</td>
<td>6.579</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>14.76</td>
<td>37.93</td>
</tr>
<tr>
<td>Normality Test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KS distance</td>
<td>0.3064</td>
<td>0.3570</td>
</tr>
<tr>
<td>P value</td>
<td>0.0009</td>
<td>0.0003</td>
</tr>
<tr>
<td>Passed normality test ((*=0.05))? No</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Analysis of data from MMP-1 Elisa. All values in ngs/100mgs.

The mean MMP-1 level for the active group was 11.52ngs/100mgs of tissue wet weight compared with 22.26ngs/g for the placebo group, a relative difference of 50.6%. The P value based on the KS distance is 0.0009, and the data has failed the normality test, which means that the distribution of the data is not Gaussian. A non
parametric analysis was therefore used and the data analysed using a Mann Whitney U test. The results of the test as well as a graphic representation of the data is shown on table 6 and figure 4 respectively. This gave a P value of 0.04, implying a statistically significant decrease in MMP-1 levels between the active and placebo group. The significance of this finding will be discussed fully in chapter 11.

Mann Whitney test

- **P value**: 0.0353
- Exact or approximate P value?: Gaussian Approximation
- P value summary: Significant
- Are medians signif. different? (P < 0.05): Yes
- One- or two-tailed P value?: Two-tailed
- Sum of ranks in column A, B: 1376, 1550
- Mann-Whitney U: 515.0

Table 6: Statistical analysis of data for MMP-1 Elisa. All values in ngs/100mgs.

Figure 4. Graph showing amount of MMP-1 in active and placebo groups in ngs/100mgs of tissue wet weight.
MMP-2

The analysis of the data is shown in table 7.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>9.090</td>
<td>93.56</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>400.0</td>
<td>346.9</td>
</tr>
<tr>
<td>Median</td>
<td>597.5</td>
<td>570.3</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>741.2</td>
<td>752.7</td>
</tr>
<tr>
<td>Maximum</td>
<td>1135</td>
<td>968.8</td>
</tr>
<tr>
<td>Mean</td>
<td>585.7</td>
<td>555.6</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>263.9</td>
<td>243.4</td>
</tr>
<tr>
<td>Std. Error</td>
<td>41.21</td>
<td>40.57</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>502.5</td>
<td>473.2</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>669.0</td>
<td>638.0</td>
</tr>
</tbody>
</table>

Normality Test
- KS distance: 0.08372, 0.1336
- P value: P > 0.10, P > 0.10
- Passed normality test (*=0.05)? Yes, Yes

Table 7. Analysis of data for MMP-2 Elisa. All values in ngs/100mgs.

The mean (+/- SEM) level for the active group was 585.7 ± 41.21 ngs/100mgs of tissue wet weight and the mean for the placebo group was 555.6 ± 40.57 ngs/100mgs of tissue, an absolute difference of 30.14ngs/100mgs more in the placebo compared to the active group (Relative difference of 5%). The data was found to follow a Gaussian distribution on analysis and a parametric test (unpaired t test) was therefore employed. The results of the test as well as the graphic representation of the data is shown in table 8 and figure 5 respectively. This gave a P value of 0.6, implying that there was no statistically significant difference between the two groups of patients.
Unpaired t test

P value summary
Are means signif. different? (P < 0.05) No
One- or two-tailed P value? Two-tailed

How big is the difference?
Mean ± SEM of column A 585.7 ± 41.21 N=41
Mean ± SEM of column B 555.6 ± 40.57 N=36
Difference between means 30.14 ± 58.14
95% confidence interval -146.1 to 85.84
R squared 0.003571

F test to compare variances
F,DFn, Dfd 1.175, 40, 35
P value 0.3150
P value summary ns
Are variances significantly different? No

Table 8. Statistical analysis of data for MMP-2 Elisa. All values in ngs/100mgs.

Figure 5. Graph showing amount of MMP-2 in active and placebo groups in ngs/100mg of tissue wet weight.
MMP-3

The analysis of the data is shown in table 9.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>9.257</td>
<td>7.608</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>21.38</td>
<td>21.76</td>
</tr>
<tr>
<td>Median</td>
<td>29.18</td>
<td>29.04</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>35.74</td>
<td>41.59</td>
</tr>
<tr>
<td>Maximum</td>
<td>70.79</td>
<td>73.28</td>
</tr>
<tr>
<td>Mean</td>
<td>31.03</td>
<td>33.51</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>12.61</td>
<td>17.23</td>
</tr>
<tr>
<td>Std. Error</td>
<td>1.969</td>
<td>2.913</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>27.05</td>
<td>27.59</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>35.01</td>
<td>39.43</td>
</tr>
</tbody>
</table>

**Normality Test**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KS distance</td>
<td>0.1300</td>
</tr>
<tr>
<td>P value</td>
<td>P &gt; 0.10</td>
</tr>
<tr>
<td>Passed normality test (*=0.05)?</td>
<td>Yes</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 9. Analysis of data for MMP-3 Elisa. All values in ngs/100mgs.

The mean (+/-SEM) level for the active group was 31.03 +/-12.61 ngs/100mgs of tissue wet weight, compared to 33.51 +/-17.23 ngs/100mgs for the placebo group, an absolute difference of 2.472 ± 3.432 ngs/100mgs (Relative difference of 7.4%). The data was found to follow a Gaussian distribution on analysis and a parametric test (unpaired t test) was therefore employed. The results of the test as well as the graphic representation of the data is shown in table 10 and figure 6 respectively. This gave a P value of 0.47, implying that there was no statistically significant difference between the two groups of patients.
Unpaired t test

**P value** 0.4735

<table>
<thead>
<tr>
<th>P value summary</th>
<th>ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Are means signif. different? (P &lt; 0.05)</td>
<td>No</td>
</tr>
<tr>
<td>One- or two-tailed P value?</td>
<td>Two-tailed</td>
</tr>
<tr>
<td>t, df t=0.7204 df=74</td>
<td></td>
</tr>
</tbody>
</table>

How big is the difference?

| Mean ± SEM of column A | 31.03 ± 1.969 N=41 |
| Mean ± SEM of column B | 33.51 ± 2.913 N=35 |
| Difference between means | -2.472 ± 3.432 |
| 95% confidence interval  | -4.375 to 9.320   |
| R squared               | 0.006965           |

F test to compare variances

| F, Dan, Dfd | 1.867, 34, 40 |
| P value     | 0.0294        |

Are variances significantly different? Yes

Table 10. Statistical analysis of data for MMP-3 Elisa. All values in ngs/100mgs.

![Graph showing amount of MMP-3 in active and placebo groups in ngs/100mg of tissue wet weight.](image)

Figure 6. Graph showing amount of MMP-3 in active and placebo groups in ngs/100mg of tissue wet weight.
**MMP-9**

The analysis of the data is shown in table 11.

<table>
<thead>
<tr>
<th>Minimum</th>
<th>126.5</th>
<th>121.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% Percentile</td>
<td>162.5</td>
<td>170.5</td>
</tr>
<tr>
<td>Median</td>
<td>253.1</td>
<td>276.9</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>383.3</td>
<td>505.2</td>
</tr>
<tr>
<td>Maximum</td>
<td>1098</td>
<td>1401</td>
</tr>
<tr>
<td>Mean</td>
<td>309.0</td>
<td>396.3</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>198.5</td>
<td>311.9</td>
</tr>
<tr>
<td>Std. Error</td>
<td>31.39</td>
<td>55.13</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>245.5</td>
<td>283.8</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>372.5</td>
<td>508.7</td>
</tr>
</tbody>
</table>

**Normality Test**

| KS distance | 0.1992 | 0.2021 |
| P value     | 0.0838 | P > 0.10 |
| Passed normality test (*=0.05)? | Yes | Yes |
| P value summary | ns | ns. |

Table 11. Analysis of data for MMP-9 Elisa. All values in ngs/100mgs.

The results showed a mean (+/-SEM) of 309.0 ± 31.39ngs/100mgs in the active group compared to 396.3 ± 55 ngs/100mgs in the placebo group (Relative difference of 22%). On the basis of the KS distance the data was consistent with a gaussian distribution and a parametric analysis was used (unpaired t test) This gave a P value of 0.15, implying no statistically significant difference between the two groups. The analysis is summarised in table 12 and depicted graphically in figure 7.
Unpaired t test

P value 0.1536
P value summary ns
Are means significantly different? (P < 0.05) No
One- or two-tailed P value? Two-tailed
t, df t=1.443 df=70

How big is the difference?
Mean ± SEM of column A 309.0 ± 31.39 N=40
Mean ± SEM of column B 396.3 ± 55.13 N=32
Difference between means -87.25 ± 60.48
95% confidence interval -33.51 to 208.0
R squared 0.02887

F test to compare variances
F, DFn, Dfd 2.468, 31, 39
P value 0.0040
Are variances significantly different? Yes

Table 12: Statistical analysis of results of MMP-9 tests.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Active</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>396.3</td>
<td>309.0</td>
<td>-87.25</td>
</tr>
<tr>
<td>SEM</td>
<td>55.13</td>
<td>31.39</td>
<td>60.48</td>
</tr>
<tr>
<td>N</td>
<td>32</td>
<td>40</td>
<td>32</td>
</tr>
</tbody>
</table>

Figure 7: Graph showing amount of MMP-9 in active and placebo groups in ngs/100mg of tissue wet weight.
TIMP-1

The analysis of the data is shown in table 13.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>3078</td>
<td>4669</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>11780</td>
<td>9897</td>
</tr>
<tr>
<td>Median</td>
<td>15300</td>
<td>14430</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>18360</td>
<td>18290</td>
</tr>
<tr>
<td>Maximum</td>
<td>32550</td>
<td>26840</td>
</tr>
<tr>
<td>Mean</td>
<td>15560</td>
<td>14710</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>6316</td>
<td>6169</td>
</tr>
<tr>
<td>Std. Error</td>
<td>986.3</td>
<td>1074</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>13570</td>
<td>12520</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>17560</td>
<td>16890</td>
</tr>
</tbody>
</table>

**Normality Test**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KS distance</td>
<td>0.1039</td>
</tr>
<tr>
<td>P value</td>
<td>P &gt; 0.10</td>
</tr>
<tr>
<td>Passed normality test (*=0.05)?</td>
<td>Yes</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 13. Analysis of data for TIMP-1 Elisa. All values in ngs/100mgs.

The mean (+/- SEM) level of TIMP-1 for the active group was 15.560 ± 0.986.3 mgs/100mls of tissue wet weigh, and the level in the placebo group was 14.710 ± 1.074 mgs/100mls, an absolute difference of 0.8556 ± 1.462 mgs/100mls. The distribution of the data was consistent with a Gaussian pattern and an unpaired t test was used in the analysis. The results are shown in table 14 and a graphic representation of the results is shown in figure 8. The P value for the difference between the two groups was 0.56, implying that the difference between the groups was not significant.
Unpaired t test

P value summary
P value
Are means signif. different? (P < 0.05)
One- or two-tailed P value?
t, df t=0.5853 df=72

How big is the difference?
Mean ± SEM of column A
Mean ± SEM of column B
Difference between means
95% confidence interval
R squared

F test to compare variances
F,DFn, Dfd
P value
P value summary
Are variances significantly different?

Table 14. Statistical analysis of results of TIMP-1 results.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Mean ± SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>15560 ± 986.3</td>
<td>41</td>
</tr>
<tr>
<td>Placebo</td>
<td>14710 ± 1074</td>
<td>33</td>
</tr>
</tbody>
</table>

Figure 8: Graph showing amount of TIMP-1 in active and placebo groups in ngs/100mg of tissue wet weight.
TIMP-2

The analysis of the data is shown in table 15.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>18.23</td>
<td>213.6</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>353.2</td>
<td>383.3</td>
</tr>
<tr>
<td>Median</td>
<td>540.1</td>
<td>548.6</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>641.1</td>
<td>642.2</td>
</tr>
<tr>
<td>Maximum</td>
<td>901.3</td>
<td>938.1</td>
</tr>
<tr>
<td>Mean</td>
<td>490.2</td>
<td>533.7</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>216.0</td>
<td>172.5</td>
</tr>
<tr>
<td>Std. Error</td>
<td>34.16</td>
<td>30.50</td>
</tr>
<tr>
<td>Lower 95% CI</td>
<td>421.1</td>
<td>471.5</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>559.3</td>
<td>595.9</td>
</tr>
</tbody>
</table>

**Normalitv Test**

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS distance</td>
<td>0.1299</td>
<td>0.1041</td>
</tr>
<tr>
<td>P value</td>
<td>P &gt; 0.10</td>
<td>P &gt; 0.10</td>
</tr>
<tr>
<td>Passed normality test (*=0.05)?</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 15. Analysis of data for TIMP-2 Elisa. All values in ngs/100mgs.

The assays were carried out on neat samples, and the mean (+/- SEM) the active group was 490.2 ± 34ngs/100mgs compared to 533.7 ± 30.50 ngs/100mgs in the placebo group, an absolute difference of 43.48 ± 46.94 ngs/100mgs (Relative difference 8.1%). The data was consistent with a normal distribution and an unpaired t test resulted in a P value of 0.35, which was not consistent with a statistically significant difference between the two groups. The statistical analysis as well as a graphic representation of the results is shown in table 16 and figure 9 respectively.
Unpaired t test

P value
0.3576
P value summary
ns
Are means signif. different? (P < 0.05)
No
One- or two-tailed P value?
Two-tailed
t, df  t=0.9261 df=70

How big is the difference?
Mean ± SEM of column A 490.2 ± 34.16 N=40
Mean ± SEM of column B 533.7 ± 30.50 N=32
Difference between means -43.48 ± 46.94
95% confidence interval -50.26 to 137.2
R squared 0.01210

F test to compare variances
F,DFn, Dfd 1.568, 39, 31
P value 0.0997
P value summary ns
Are variances significantly different? No

Table 16. Statistical analysis of TIMP-2 Elisa results. All values in ngs/100mgs.

Figure 9: Graph showing amount of TIMP-2 in active and placebo groups in ngs/100mg of tissue wet weight.
TIMP/MMP Ratios

As discussed in chapter 4, the tissue inhibitors of metalloproteinases (TIMPs) are the natural inhibitors of the MMP's and serve to regulate the lytic activity if the metalloproteinases. The TIMPs bind in a 1:1 molar ratio to the proenzymes and to a lesser extent the activated enzymes inhibiting their activity. TIMP-1 binds most avidly to MMP-9 and MMP-1, and TIMP-2 mainly binds MMP-2, although some less specific binding probably occurs with other enzymes by both TIMP1 and 2.

The TIMP/MMP ratios within the carotid plaques were also calculated using the data presented earlier. The ratios of TIMP-2 to MMP-2 and the ratio of TIMP-1 to MMP-9 are depicted graphically in figures 10 and 11 below. There was no difference in the mean TIMP-2/MMP-2 ratios between active and placebo groups (1.04+/-0.8 [mean+/-SD] and 1.05+/-0.6 respectively, P=0.67, Mann Whitney). Although the TIMP-1/MMP-9 ratio was higher in the active than in the placebo group, reflecting the lower MMP-9 in the active group, this failed to reach statistical significance on analysis using an unpaired t test (63.08 ± 5.65[mean +/- SD] and 55.32 ± 7.21 respectively: P=0.4, unpaired t test).

Figure 10. TIMP-2/MMP-2 ratio in active and placebo groups.

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Analysis of the TIMP-1/MMP-1 ratio showed a much higher level of TIMP-1 to MMP-1 in the active group compared to control (1938 ± 207.4 [mean +/-SEM] in active and 1410 ± 183.0 in placebo)(Figure 12). The higher ratio in the active group was due to a lower MMP-1 level in this cohort, as there was no difference in TIMP-1 levels when analysed independently. This difference approached statistical significance (P=0.06), potentially implying a lower collagenolytic activity in the active group compared to controls due to a shift in the equilibrium between the lytic enzymes and their natural inhibitors.
One other interesting point that emerged from the latter analysis is the large difference in the TIMP/MMP ratios. The TIMP-2/MMP-2 and TIMP-1/MMP-9 ratios having a means of 1.1 and 59.6 respectively compared to a mean of 1706 for the TIMP-1 /MMP-1 ratio. This is consistent with a much tighter inhibition of MMP-1 than the gelatinases, which may be a reflection of its role early on in the activation cascade of MMP's as will be discussed in chapter 11.
Localisation of MMP-1 Expression within the Carotid Plaque

Immunocytochemistry
9.0 Introduction

In order to identify the sites of MMP-1 secretion within carotid plaques, and to identify the cell types secreting MMP-1, immunohistochemistry using an antibody to MMP-1 was carried out using a standard ABC technique on tissue sections.

9.1 Technique

Tissue samples had been previously fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin wax. Sections were then cut to 10μm thickness and fixed to saline coated slides. Slides were then dewaxed by immersion in serial concentrations of alcohols for 2 minutes each (Xylene x 3.99%, alcohol x 2.95%, alcohol x1), and finally washed in tris buffered saline. Following this they were incubated with normal goat serum for 10 minutes at room temperature.

The excess serum was then drained away and the primary antibody was added to the slides (Chemicon International Ltd, UK Cat. No MAB 1346). This was diluted at 1:50 in tris buffered saline, and the slides incubated overnight at 4°C.

This was followed by a wash in phosphate buffered saline for 20 minutes at room temperature. The secondary antibody was then applied, (goat anti-mouse/rabbit biotinylated immunoglobulin, Dako Duet Kit, K0492) and the slides incubated at room temperature for 30 minutes. This was followed by wash in PBS for 20 minutes, and then and the slides incubated at room temperature for 30 minutes with preformed avidin biotin complex (Dako Duet Kit, K0492). The chromogen was then prepared (0.05%DAB + Hydrogen peroxide) and filtered onto the slide and left for five minutes. The slides were washed in water again and 1% copper sulphate
was added for 5 minutes, followed by another wash in water for 5 minutes. Finally the haematoxylin and eosin counterstain was applied for 30 seconds, and followed by a final wash with tap water for 2 minutes.

A negative control without the primary antibody was run in parallel with the rest of the histological sections.

The slides were then examined by Dr Louise Jones, (Consultant Histopathologist at the Glenfield hospital, Leicester), who was blinded to the trial. Unstable regions of plaques were identified using criteria previously described in by Carr et al [189], namely the cap and shoulder regions, and areas of plaque rupture.

9.2 Results

A few representative areas from the stained slides are shown in the following pages. As has been shown in other studies, there was an intense inflammatory infiltrate of macrophages in the unstable regions of the carotid plaque. Other areas of the plaque lacked this inflammatory infiltrate and consisted mainly of smooth muscle cells. The MMP-1 antibody appeared as brownish stained areas, and there was intense staining in the foamy cytoplasm of macrophages in unstable regions of the plaque. No MMP-1 staining was visualised in the smooth muscle cells of the plaque, although other studies have documented staining of MMP-1 in endothelial and smooth muscle cells [275].

Figures 7 is a control slide without the primary antibody, and shows a total lack of the characteristic brown staining seen in an identical area of the same slide shown in figure 8.
Figure 1. Unstable region of carotid plaque showing intense brown staining for MMP-1. (x100)

Figure 2. High power view of section in figure 1 showing MMP-1 staining colocalising with areas of macrophage infiltration. (x200)
Figure 3. Area of heavy MMP-1 staining at site of plaque rupture (x 25)

Figure 4. High power view of shoulder region from plaque shown in figure 3 showing heavy MMP-1 staining at site of macrophages (x 100)
Figure 5. Another section from the shoulder region of an unstable plaque showing macrophage infiltration and MMP-1 staining. (x 25)

Figure 6. High power view of cells in 5 showing foamy cytoplasm and intense staining for MMP-1 (x 100)
Figure 7. Negative control slide without primary antibody showing absence of brown staining in areas of macrophages. (x 200)

Figure 8. Central area of slide 7 showing staining for MMP-1 when primary is used. (adjacent section) (x 200)
9.3 Conclusion

This experiment has provided additional evidence about the localisation of MMP-1 within carotid plaques. The slides clearly show intense MMP-1 staining in unstable plaques, which colocalises with areas of macrophage infiltration. The significance of this observation in relation to this study is discussed in chapter 11.
Chapter 10

Histological Analysis

of the

Carotid Plaques and Clinical Outcome
Carotid plaques were collected at the time of surgery and divided into two longitudinal sections. One section was fresh frozen in liquid nitrogen and the other half was stored in 4% paraformaldehyde and subsequently processed within twenty-four hours as described below for further histological analysis.

10.0 Preparation of slides.

The specimens were mounted onto paraffin blocks using an automated tissue processor (Shandon Hypercentre) following the protocol described in Figure 1:

<table>
<thead>
<tr>
<th>FLUID</th>
<th>TEMPERATURE</th>
<th>VACUUM?</th>
<th>TIME</th>
<th>DRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10% FORMALIN (OPTIONAL)</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>1min</td>
</tr>
<tr>
<td>2. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>15sec</td>
</tr>
<tr>
<td>3. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>15sec</td>
</tr>
<tr>
<td>4. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>15sec</td>
</tr>
<tr>
<td>5. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>15sec</td>
</tr>
<tr>
<td>6. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>15sec</td>
</tr>
<tr>
<td>7. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>15sec</td>
</tr>
<tr>
<td>8. 99% ETHANOL</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr</td>
<td>1min</td>
</tr>
<tr>
<td>9. XYLENE</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr 30mins</td>
<td>15sec</td>
</tr>
<tr>
<td>10. XYLENE</td>
<td>Ambient</td>
<td>Y</td>
<td>1hr 30min</td>
<td>1min</td>
</tr>
<tr>
<td>11. WAX</td>
<td>60C</td>
<td>Y</td>
<td>2 hrs</td>
<td>1min</td>
</tr>
<tr>
<td>12. WAX</td>
<td>60C</td>
<td>Y</td>
<td>2 hrs</td>
<td>30sec</td>
</tr>
</tbody>
</table>

Figure 1: Tissue processing schedule for Hypercentre XP

The paraffin blocks were then cut into sections 4µm thick and mounted onto glass slides for haematoxylin and eosin staining. This was carried out using an automated stainer (Jung Autostainer), using the protocol described in figure 2.
<table>
<thead>
<tr>
<th>Process</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven at 65°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>30 sec</td>
</tr>
<tr>
<td>99% Ethanol</td>
<td>20 sec</td>
</tr>
<tr>
<td>99% Ethanol</td>
<td>20 sec</td>
</tr>
<tr>
<td>Mayers Haematoxylin</td>
<td>2 min 15 sec</td>
</tr>
<tr>
<td>Mayers Haematoxylin</td>
<td>2 min 15 sec</td>
</tr>
<tr>
<td>Running tap water</td>
<td>2 min</td>
</tr>
<tr>
<td>0.5% eosin</td>
<td>40 sec</td>
</tr>
<tr>
<td>Running tap water</td>
<td>30 sec</td>
</tr>
<tr>
<td>99% Ethanol</td>
<td>20 sec</td>
</tr>
<tr>
<td>99% Ethanol</td>
<td>20 sec</td>
</tr>
<tr>
<td>99% Ethanol</td>
<td>20 sec</td>
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<tr>
<td>99% Ethanol</td>
<td>20 sec</td>
</tr>
<tr>
<td>Xylene</td>
<td>10 sec</td>
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<tr>
<td>Xylene</td>
<td>10 sec</td>
</tr>
<tr>
<td>Xylene</td>
<td>10 sec</td>
</tr>
<tr>
<td>Xylene</td>
<td>10 sec</td>
</tr>
<tr>
<td>Xylene</td>
<td>10 or longer until coverslipped</td>
</tr>
</tbody>
</table>

Figure 2. Haematoxylin and eosin staining schedule for the Leica Stainer

An independent histopathologist (Dr Louise Jones, Glenfield Hospital, Leicester) who was blinded to the trial subsequently reviewed the slides. The slides were graded for histological criteria as described in the paper by Carr et al[189]. Plaque rupture, necrosis, cap thinning, extent of foam cell infiltration of the fibrous cap and calcification of the plaque were all examined. Cap foam cell infiltration was graded into four groups from 0 to 3, depending on whether foam cells were absent, or whether infiltration was mild, moderate or severe respectively.

Representative sections showing intraplaque haemorrhage, foam cell infiltration, calcification, and plaque rupture are shown in the following sections (Figure 3-6)
Figure 3: Area of intraplaque haemorrhage showing free red blood cells within the plaque.

Figure 4: Area of macrophage infiltration showing foamy cytoplasm of cholesterol laden cells.
Figure 5: Area of intraplaque calcification close to fibrous cap.

Figure 6: Rupture of fibrous cap of plaque.
Statistical analysis.

Statistical analysis was carried out using the graphpad statistical package. Data from both patient groups was compared using a Fisher's exact test for non-continuous variables, whereas data regarding cap foam cell infiltration was graded from 0 to 4 and was compared using a Mann Whitney U test. The results are expressed as a P value with a value of less than 0.05 considered significant.

10.1 Results.

Cap foam cell infiltration

The analysis of the data for cap foam cell infiltration is shown in figure 7. Minimum values were 0 in both groups (no cap foam cell infiltration) and 4 in the active and 3 in the placebo group. The data failed the normality test with a KS distance of 0.3 for both groups and a Mann Whitney U test was therefore used for analysis. The P value for the difference between both groups is 0.274, signifying no statistically significant difference between both groups. The result is shown graphically in figure 8.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>41</td>
<td>36</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Median</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>2.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Maximum</td>
<td>4.000</td>
<td>3.000</td>
</tr>
<tr>
<td>Mean</td>
<td>1.659</td>
<td>1.361</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>1.015</td>
<td>0.8333</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.1585</td>
<td>0.1389</td>
</tr>
</tbody>
</table>
Lower 95% CI 1.338 1.079  
Upper 95% CI 1.979 1.643  

Normality Test

KS distance 0.3027 0.3065  
P value 0.0011 0.0023  
Passed normality test (*=0.05)? No No  
P value summary ** **

Figure 7: Analysis of presence or absence of intensity of cap foam cell infiltration between both groups.

Cap foam cells

![Graph showing intensity of cap foam cell infiltration between active and placebo groups.]

Figure 8: Intensity of cap foam cell infiltration between active and placebo groups.

Cap thinning

The presence or absence of cap thinning between the active and placebo groups was compared between the active and the placebo groups using the Fisher's exact test. The results are shown in figure 9 and depicted graphically in figure 10. The p value
for the difference between both groups was 0.2, implying no statistically significant difference in the presence of cap thinning between both groups.

**Fisher's exact test**

<table>
<thead>
<tr>
<th>P value</th>
<th>0.2103</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value summary</td>
<td>Ns</td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
</tr>
<tr>
<td>Statistically significant? (alpha-)</td>
<td>No</td>
</tr>
<tr>
<td>Strength of association</td>
<td></td>
</tr>
<tr>
<td>Relative Risk</td>
<td>1.222</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.9100 to 1.640</td>
</tr>
</tbody>
</table>

Figure 9: Results of Fisher's exact test comparing the presence of cap thinning between active and placebo groups.

**Cap thinning**

![Cap thinning](image)

Figure 10: Comparison of plaques demonstrating cap thinning between active and placebo groups.
Plaque haemorrhage

Plaques were also examined for the presence of intraplaque haemorrhage. Presence or absence of haemorrhage between both groups was compared using a Fisher’s exact test. The results are shown in figure 11, and depicted graphically in figure 12. The p value for the difference between both groups was 0.8, implying no statistically significant difference between both groups with regard to intraplaque haemorrhage.

Fisher’s exact test

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.8204</td>
</tr>
<tr>
<td>P value summary</td>
<td>Ns</td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
</tr>
<tr>
<td>Statistically significant?</td>
<td>(alpha- ) No</td>
</tr>
</tbody>
</table>

Strength of association

- Relative Risk: 0.9048
- 95% confidence interval: 0.5675 to 1.442

Figure 11: Comparison of intraplaque haemorrhage between active and placebo groups using Fisher’s exact test.

Haemorrhage

- Haemorrhage
- No Haemorrhage

Figure 12: Presence of intraplaque haemorrhage between active and placebo groups.
**Plaque necrosis.**

Analysis of plaques for differences in areas of necrosis using the Fisher's exact test is shown in figure 13, and depicted in figure 14. There was no difference in the presence of necrotic areas between the active and placebo groups as confirmed by a p value of 1.0 on statistical analysis.

<table>
<thead>
<tr>
<th>Fisher's exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
</tr>
<tr>
<td>P value summary</td>
</tr>
<tr>
<td>One- or two-sided</td>
</tr>
<tr>
<td>Statistically significant?</td>
</tr>
</tbody>
</table>

**Strength of association**

| Relative Risk | 1.020 |
| 95% confidence interval | 0.7033 to 1.480 |

Figure 13: Comparison of the presence of haemorrhage between active and placebo groups using Fisher's exact test.

Figure 14: Presence of necrosis between active and placebo groups.

![Necrosis Bar Chart](image)

**Figure 14: Presence of necrosis between active and placebo groups**
**Plaque rupture.**

Microscopic examination for plaque rupture showed no statistically significant difference between both groups. The results of the Fisher's exact test are shown in figure 15 and graphically in figure 16. The p value for the difference between both groups was 0.59.

<table>
<thead>
<tr>
<th>Fisher's exact test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.5935</td>
</tr>
<tr>
<td>P value summary</td>
<td>Ns</td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
</tr>
<tr>
<td>Statistically significant? (alpha-)</td>
<td>No</td>
</tr>
</tbody>
</table>

Strength of association:
- Relative Risk: 1.289
- 95% confidence interval: 0.5653 to 2.941

Figure 15: Results of Fisher's exact test comparing rupture between active and placebo groups.

![Rupture](image)

Figure 16. Comparison of number of plaques with features of rupture between active and placebo groups.
Calcification

The last histological feature assessed was the presence of calcification within the plaque, which as discussed in an earlier chapter is associated with plaque stability. Presence or absence of calcification between both groups was analysed using a Fisher's exact test, the results of which are shown in figure 17 and depicted in figure 18. A p value of 0.3 was obtained for the differences between both groups, implying no significant difference between these groups with regard to the presence of calcification.

Fisher's exact test

| P value | 0.3186 |
| P value summary | Ns |
| One- or two-sided | Two-sided |
| Statistically significant? (alpha-) | No |

Strength of association

- Relative Risk: 1.193
- 95% confidence interval: 0.8740 to 1.629

Figure 17: Analysis data for presence of calcification between active and placebo groups.

Figure 18: Presence of calcification between active and placebo groups.
10.2 Clinical Outcome

Clinical end points occurring prior to surgery were stroke and carotid artery occlusion. A stroke was defined as a focal neurological deficit lasting more than 24 hours. There were a total of five events prior to surgery which resulted in exclusion from the rest of the trial as planned. Four events occurred in the placebo group and one event occurred in the treatment group Figure 19.

Events Prior to Surgery

![Diagram showing events prior to surgery with active n=1 and placebo n=4 with occlusion as the common occurrence]

Figure 19. Clinical events prior to surgery

The events in both groups were compared using a Fisher’s exact test and the contingency table 1 shown below. There was no statistically significant difference between both groups (P= 0.2), however an interesting trend towards clinical benefit was obtained which warrants further investigation in a larger series of patients.
<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Placebo</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Event</td>
<td>50</td>
<td>43</td>
<td>93</td>
</tr>
<tr>
<td>Event</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1: Contingency table analysing the number of events in each group.

**Summary.**

In summary, no histological difference was demonstrated between both groups with regard to the histological criteria analysed. As the plaques were analysed using standard light microscopy, one cannot exclude an early change at the molecular level which was not observed by the methods we used. Also more time might possibly be required on treatment in order to observe changes in plaque morphology using light microscopy. Ultimately, no change may actually ever be apparent histologically because inhibition of MMP-1 may only result in a shift of a mainly collagenolytic milieu to a more stable environment where collagen degradation is equal to collagen deposition and results in a more stable plaque.
Chapter 11

Conclusion and Future Studies.
This chapter aims to discuss the results of the experimental studies described in the preceding chapters and to deliberate potential future studies. The different aspects relating to the quantification of doxycycline in carotid plaques have been fully analysed in chapter 6, and will not be elaborated further here.

11.0 Monocyte macrophage study.

To our knowledge, at the time of writing of this thesis, this is the only experiment that investigated the effect of doxycycline on MMP secretion by *human* monocyte derived macrophages *in vitro*.

**Findings**

Our experiments have shown that basal MMP-1 secretion by monocyte derived macrophages is much lower than MMP-9 secretion. Doxycycline was found to inhibit the secretion of both enzymes in a dose dependent manner. However, the effect of doxycycline on MMP-1 secretion by macrophages is much more potent than that on MMP-9 secretion. The concentration of doxycycline required to inhibit 25% of the basal MMP-1 secretion was 2.4μg/mL, whereas the concentration of doxycycline to inhibit MMP-9 secretion to a similar level was found to be 43 μg/mL. This marked difference in the *in vitro* inhibitory activity was reflected in our *in vivo* findings to be discussed later. The levels of doxycycline in carotid plaques were found to be around 6.5 μg/g of tissue, and at these levels one would expect around 50% inhibition of MMP-1 activity based on these *in vitro* experiments. Indeed, this has been reflected in our findings *in vivo*.

Levels of MMP-9 secretion did not differ significantly between PMA stimulated and non-PMA stimulated groups of cells in our experiments. PMA is a phorbol ester that has been shown to be a strong stimulator of MMP transcription [242, 372-]
PMA activates protein kinase C, and triggers many phosphorylation events and signal pathways that are stimulated by inflammatory cytokines [242, 372, 374]. In addition, several studies have linked phorbol induction to stimulation of the AP-1 binding site discussed below [373].

However, in the case MMP-1, there was no detectable secretion of MMP-1 in the absence of PMA stimulation. A possible explanation for this observation may lie in the way MMP-1 and MMP-9 are expressed.

The regulation of MMP-1 secretion and activity has been extensively reviewed by Vincenti et al [375]. MMP-1 is secreted from cells as soon as it is synthesised. In contrast MMP-9 is stored in granules within the cell which are released from the cell in response to specific stimuli [376]. Although both MMP-1 and MMP-9 promoters have AP-1 binding sites which are responsive to products of Fos and Jun activation and may serve as final pathways of MMP gene expression, several differences in the promoter sequence have been described and these may be responsible for the different responses to varying levels of doxycycline in vivo [373, 377, 378].

Furthermore, recent studies have shown that human monocyte/macrophages are mechanically responsive, and that adhesion to extracellular matrix provides structural anchors that induces the expression of the early response genes c-fos and c-jun with resultant differential expression of MMP's [379]. In particular, the secretion of MMP-1 and MMP-9 have been shown to be markedly different in response to mechanical deformation. In addition, the secretory response to PMA has also been shown to be different in this situation with PMA stimulating MMP-1
expression to a much higher degree than MMP-9, as we have observed in our experiment [379].

An alternative explanation may be that although doxycycline inhibits expression to the same degree, the basal secretion of MMP-9 is so much higher than MMP-1 that the effect on MMP-1 secretion is much more noticeable with current methods of enzyme quantification.

Recent work investigating the effect of doxycycline on MMP-1 expression by corneal epithelial cells has shown that doxycycline seems to reduce IL-1 production, and this in turn reduces MMP-1 expression via reduced IL-1 stimulation of transcription [380, 381]. This work lends support to the findings we have observed in this thesis and provides a plausible explanation whereby the anti-inflammatory effect of doxycycline also indirectly reduces the levels of MMP-1 with its potential for plaque destabilisation.

This experiment is however limited to a single cell type which has been unphysiologically stimulated by PMA. In the in vivo situation, stimulation is the sum total of a very complex myriad of inhibitory/stimulatory cytokines e.g. IL-1, TNFα, PDGF, TGF ect, as described in detail in chapter 4. In addition several different cell types (macrophages, smooth muscle cells, endothelial cells) produce MMP’s, all with differing responses and the varying sensitivities to the above regulatory cytokines and doxycycline, which will then have an effect on the overall enzyme levels and activity observed in the in vivo situation.

This is a very interesting area of doxycycline/MMP-1 interaction and further work is required to clarify the underlying inhibitory pathways in order to allow better exploitation of these useful effects at a clinical level.
11.1 MMP levels in carotid plaques.

This forms the essential question that was originally set out at the beginning of this study, and forms the basis of this thesis. The results of different MMP levels and TIMPS in carotid plaques are summarised in the table 1 below:

<table>
<thead>
<tr>
<th></th>
<th>Active ngs/g</th>
<th>Placebo ngs/g</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>11.52</td>
<td>22.26</td>
<td>0.035*</td>
</tr>
<tr>
<td>MMP-2</td>
<td>585.7</td>
<td>555.6</td>
<td>0.61</td>
</tr>
<tr>
<td>MMP-3</td>
<td>31.03</td>
<td>33.51</td>
<td>0.47</td>
</tr>
<tr>
<td>MMP-9</td>
<td>309.0</td>
<td>396.3</td>
<td>0.15</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>15560</td>
<td>14710</td>
<td>0.56</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>490.2</td>
<td>533.7</td>
<td>0.357</td>
</tr>
</tbody>
</table>

Table 1: Levels of MMP's and TIMPS in carotid plaques.

There was no statistically significant difference in the levels of MMP-2, 3, 9, and TIMP 1,2, between the active or placebo groups. MMP-9 levels were reduced by a total of 22%, however this did not reach statistical significance due to wide standard deviation and due to a small numbers of patients. However, this finding is very interesting as it reflects the reduction in MMP-9 levels one would have expected based on our in vitro experiments and the levels of doxycycline achieved in carotid plaques. Although not statistically significant, if this level of enzymatic reduction was reproducible and sustainable, it might still be a very important observation as it represents a shift in the microenvironment of the plaque towards a more stable state. One would not require complete inhibition of MMP-9 production for clinical benefit but merely to shift the equilibrium from a mainly collagenolytic to a more stable milieu.
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<th>Placebo ngs/g</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>11.52</td>
<td>22.26</td>
<td>0.035*</td>
</tr>
<tr>
<td>MMP-2</td>
<td>585.7</td>
<td>555.6</td>
<td>0.61</td>
</tr>
<tr>
<td>MMP-3</td>
<td>31.03</td>
<td>33.51</td>
<td>0.47</td>
</tr>
<tr>
<td>MMP-9</td>
<td>309.0</td>
<td>396.3</td>
<td>0.15</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>15560</td>
<td>14710</td>
<td>0.56</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>490.2</td>
<td>533.7</td>
<td>0.357</td>
</tr>
</tbody>
</table>

Table 1: Levels of MMP's and TIMPS in carotid plaques.

There was no statistically significant difference in the levels of MMP-2, 3, 9, and TIMP 1,2, between the active or placebo groups. MMP-9 levels were reduced by a total of 22%, however this did not reach statistical significance due to wide standard deviation and due to a small numbers of patients. However, this finding is very interesting as it reflects the reduction in MMP-9 levels one would have expected based on our in vitro experiments and the levels of doxycycline achieved in carotid plaques. Although not statistically significant, if this level of enzymatic reduction was reproducible and sustainable, it might still be a very important observation as it represents a shift in the microenvironment of the plaque towards a more stable state.

One would not require complete inhibition of MMP-9 production for clinical benefit but merely to shift the equilibrium from a mainly collagenolytic to a more stable milieu.
The most important finding emerging from this study is the reduction of MMP-1 levels in the carotid plaques of patients on doxycycline. Our data has shown that MMP-1 levels in patients on doxycycline were 11.52ngs/g, compared to 22.26ngs/g the placebo group, a relative difference of 50.6% between both groups. Based on the mean levels of doxycycline observed within carotid plaques and the reduction of MMP-1 secretion by monocyte/macrophage in the presence of doxycycline in vitro, this is the level of MMP-1 reduction one would expect in carotid plaques in vivo.

The levels of MMP-1 in carotid plaques were found to be much lower than the levels of the gelatinases (MMP-2, 9)(Table 1). These levels correlate with similar levels of MMP's found in carotid plaques by Loftus et al, also from this unit. The lower levels of MMP-1 are very close to levels of stromelysin (MMP-3), and the reason for this difference may lie in the fact that both these enzymes are very high in the MMP cascade, and they provide substrate and possibly activate enzymes lower down in the cascade such as the gelatinases.

One of the potential pitfalls of using ELISA is that the available kits only measure the total MMP-1, as opposed to the active and proenzyme. Zymography differentiates between active and proenzyme levels, however it could not be used in this situation because the MMP-1 levels in carotid plaques are too low to allow quantification using this method. Indeed, no MMP-1 bands at all were detected in the zymograms performed for MMP-2 and 9 on carotid plaque homogenate. However, even measurement of active and proenzyme subfractions would not have been conclusive of increased collagenolytic activity in plaques because enzymatic activity depends on a balance between MMP's and their natural inhibitors such as the TIMPS in the plaque microenvironment as discussed earlier.
The reduced MMP-1 levels in plaques of patients on doxycycline represents a very interesting observation with a very important potential for clinical benefit that warrants further investigation. The findings tie in very well with a study published by Sukhova et al showing increased levels of MMP-1 in unstable atheromatous plaques that are associated with increased collagenolysis and consequent plaque instability [282]. It would be interesting to investigate whether plaques from patients on doxycycline show a reduction in collagenolytic activity on in situ zymography as compared to plaques from patients on placebo.

Interestingly, since our data was collected Solomon et al have published data which lends support to the observations in this study [380]. They investigated the effect of doxycycline on interleukin-1 secretion by corneal epithelial cells. It emerged that doxycycline at a concentration of 10μg/ml inhibited IL-1 expression by inhibiting transcription of IL-1 genes. Furthermore they showed that IL-1 secreted into the conditioned media of the corneal epithelial cells was functionally active in inducing MMP-1 secretion by corneal epithelial cells, and that this effect was not seen in media from cells grown in the presence of doxycycline. They concluded that doxycycline may inhibit MMP-1 secretion indirectly by inhibiting IL-1 secretion by the corneal epithelial cells, a mechanism which may explain the findings in our study [380, 381]. It is interesting to note in this regard that MMP-1 is not stored in granules within cells, and is secreted as soon as it is produced. Hence decrease in levels of MMP-1 mRNA would be accompanied by an immediate decrease in enzyme levels. This would not be observed in enzymes which are stored in granules within cells (such as MMP-9) as a more constant rate of secretion is sustainable till intracellular stores are depleted.
11.2 Immunocytochemistry

This part of the study was carried out as a correlation with previously published observations. MMP-1 staining colocalised with areas of macrophage accumulation, and both were in turn highly concentrated in areas of plaque instability. This lends support to the acute inflammatory theory of plaque instability, and gives the findings of this study a stronger significance as they emphasise the role of MMP-1 in plaque instability.

11.3 Histology

The carotid plaques were analysed histologically according to the criteria described by Carr et al, as outlined in chapter 11 [189]. The results are summarised in table 2 below. There was no statistically significant difference between the two groups for any of the histological criteria analysed. Several factors may account for the lack of noticeable histological differences.

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Table 2: Histological analysis of carotid plaques. * Values for cap foam cells are mean values for from 0 to 4.

Firstly, no histological difference may indeed be present between both groups. The inhibition of MMP-1 by doxycycline may simply alter the balance between a mainly collagenolytic milieu present in the plaque to one where collagenolysis and
deposition are more closely balanced. This would result in a more stable plaque with a lower incidence of fibrous plaque surface disruption that may not be detectable histologically.

Another possible reason may be the fact that the plaques were examined using a standard light microscope. This method would possibly fail to detect fine structural alterations such as increased collagen deposition over a six-week period. Analysis was also very limited by the number of sections examined. The sections were empirically taken from the centre of the plaque, and only one section per plaque was analysed. This obviously leaves a great potential for missing structural changes between both groups. However complete analysis of multiple sections from all plaques is practically impossible and indeed would constitute a separate thesis in its own right.

11.4 Clinical outcome

The present study was powered to investigate MMP concentrations in atherosclerotic tissue, and so a reduction in clinical events was not expected. However, there were several clinical findings that warrant comment. Overall, 4 patients in the trial developed a carotid occlusion between randomisation and surgery, 3 in the placebo group and one in the doxycycline cohort. This was an unexpectedly high number, (the usual rate in Leicester is less than 1.5%), but did represent a tendency towards a lower occlusion rate in patients treated with doxycycline. Clearly, a larger cohort would be required to investigate the clinical effects of MMP inhibition on clinical outcome, but these initial data show some promise.
There are a number of limitations to our trial. The primary concern was the number of patients that were withdrawn prior to carotid endarterectomy. Some of these had a clinical event that served as a definitive end point, but the majority were withdrawn as they required specialist medical treatment prior to endarterectomy. In retrospect, it may have been more appropriate not to randomise these patients until their medical co-morbidity had been evaluated and treated. Patients also received doxycycline for variable times prior to surgery. Ideally, all patients would be medicated for identical periods of time. Unfortunately, this was not possible in our centre due to the waiting times and unavoidable operative cancellations.

11.5 Future studies

Atherosclerotic plaque biology and factors affecting plaque stability and progression is an area of intensive research at the time of writing of this thesis. To our knowledge, this is the first study of its kind demonstrating a pharmacologic reduction of a collagenolytic destabilising enzyme in an atherosclerotic plaque in vivo with the potential for plaque stabilisation. As discussed in chapter 4, plaque stability is the result of a vast array of biochemical processes which all interact to affect plaque behaviour. It is simplistic to assume that alteration of one pathway will result in a dramatic change and resultant stabilisation of an atherosclerotic plaque. However, the multiple convergent pathways provide different routes of possibly interacting with the pathological process and altering its progression.

Other studies similar to the above involving other types of MMP inhibitors are necessary. In particular, the statins, which have already been shown to have a proven clinical benefit in certain clinical situations may be particularly useful as
they are both MMP inhibitors and have a positive effect on lipid metabolism that in turn affects MMP activity indirectly. Inhibition of areas of MMP activation such as antioxidant therapy also provides a further area which might yield interesting data. Studies with other tetracycline antibiotics with different pharmacokinetics such as minocycline also provide another avenue which could be explored.

The above study involved only limited numbers of patients and the data obtained has limitations as discussed earlier. However it provides as a very interesting and potentially very important insight into the possibility of atherosclerotic plaque stabilisation using pharmacotherapy. A much larger cohort of patients are needed with much longer follow up; however should the results be reproducible in a larger study and eventually make it to clinical practise one of the most common and seriously debilitating human conditions may be prevented.
### Appendix 1

**Raw data for MMP-1**

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Notes.

1. n/c = not collected
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3. Column 2 = Protein concentration on Eliza kit
4. Column 3 = Protein concentration of raw sample after standardisation
   Column 4 = corrected value for protein concentration
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