The Role of Collagenases in Atherosclerotic Plaque Instability

Thesis submitted for the degree of
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by

Kevin John Molloy
University of Leicester

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Abstract:

Unstable carotid plaques are characterised by rupture of the collagen cap leading to thromboembolism and stroke. The proteolytic mechanisms causing plaque disruption are poorly defined, but the collagenases (MMP-1,-8 and-13) may be involved. The primary aim of this thesis was to quantify the collagenase concentrations in carotid plaques and to relate them to markers of plaque instability.

Recent studies have shown that statin therapy decreases cardiovascular risk, even in patients with normal cholesterol levels. A further aim of this thesis was to observe the effects of statins on clinical and biochemical indicators of plaque instability.

Atherosclerotic plaques were collected from 159 patients undergoing carotid endarterectomy. The presence and timing of carotid territory symptoms was ascertained. Pre-operative embolisation was recorded by transcranial Doppler. Each plaque was assessed for histological features of instability. Plaque MMP and cytokine concentrations were quantified using ELISA.

Significantly higher concentrations of active MMP-8 were observed in the plaques of symptomatic patients (p=0.0002), emboli-positive patients (p=0.0037) and in those plaques demonstrating histological evidence of rupture (p=0.0036). No differences were seen in the levels of MMP-1 and MMP-13. Immunohistochemistry, in situ hybridisation and colocalisation studies confirmed the presence of MMP-8 protein and mRNA within the plaque, which colocalised with macrophages. These data suggest that the active form of MMP-8 may be partly responsible for degradation of the collagen cap of atherosclerotic plaques. This enzyme represents an attractive target for drug therapy aimed at stabilising vulnerable plaques.

Patients on statins were less likely to have suffered symptoms in the month prior to surgery (p=0.0049) and less likely to have cerebral embolisation detected (p=0.0459). Carotid plaques retrieved from statin-taking patients, revealed significantly lower concentrations of MMP-9 (p=0.0018) and IL-6 (p=0.0005). These data suggest that statins may stabilise plaques by lowering MMP and cytokine levels, resulting in decreased embolisation and symptoms.
Statement of Originality

The material on which this thesis is based is my own independent work except where acknowledged

Kevin Molloy
December 2004
Acknowledgements

I would like to dedicate this thesis to the memory of my Dad, John Molloy, who died at the age of 47 of a myocardial infarction. This was probably caused by rupture of a coronary artery atherosclerotic plaque.

I am extremely grateful for the help and opportunities given to me by Mr Ian Loftus, Professor Matt Thompson and Professor Sir Peter Bell. In an analogy with the world of football, Prof Bell would be the club chairman (although he obviously knows much more about surgery than most chairmen do about football!), Matt Thompson the manager (responsible for my selection and guidance over the following 2 “seasons”) and Ian Loftus the captain (constantly alongside me, always encouraging).

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Publications & presentations arising from this thesis

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- Molloy KJ, Thompson MM, Schwalbe EC, Bell PRF, Naylor AR, Loftus IM. Elevation in plasma MMP-9 following carotid endarterectomy is associated with particulate cerebral embolisation. *Eur J Vasc Endovasc Surg* 2004;27:409-413

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- American Heart Association (Chicago 2002)
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- Society of University Surgeons Tripartite Meeting (Houston 2003)
- European Society for Vascular Surgery (Dublin 2003)
- European Society of Cardiology (Vienna 2003)
## Contents

<table>
<thead>
<tr>
<th>Section I: Introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1: The burden of cardiovascular disease</td>
<td>6</td>
</tr>
<tr>
<td>Chapter 2: Atherosclerosis &amp; mechanisms of plaque rupture</td>
<td>7</td>
</tr>
<tr>
<td>Chapter 3: The role of collagenases &amp; other MMPs in plaque instability</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 4: The role of COX-2, cytokines &amp; inflammatory markers</td>
<td>32</td>
</tr>
<tr>
<td>Chapter 5: Pharmacotherapy aimed at plaque stabilisation</td>
<td>51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section II: Methods</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 6: Patients &amp; processing of samples</td>
<td>66</td>
</tr>
<tr>
<td>Chapter 7: Laboratory methodology</td>
<td>67</td>
</tr>
<tr>
<td>Chapter 8: Statistical analysis</td>
<td>74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section III: Results</th>
<th>91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 9: Unstable carotid plaques exhibit raised MMP-8 activity</td>
<td>95</td>
</tr>
<tr>
<td>Chapter 10: COX-2 activity in atherosclerotic plaques</td>
<td>96</td>
</tr>
<tr>
<td>Chapter 11: Cytokines, tissue factor &amp; inflammatory markers</td>
<td>112</td>
</tr>
<tr>
<td>Chapter 12: HMG-CoA reductase inhibitors stabilise carotid atheroma by reducing plaque concentrations of MMPs &amp; cytokines</td>
<td>119</td>
</tr>
<tr>
<td>Chapter 13: Elevation in plasma MMP-9 following carotid endarterectomy is associated with particulate cerebral embolisation</td>
<td>123</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section IV: Conclusion</th>
<th>129</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 14: Discussion</td>
<td>136</td>
</tr>
<tr>
<td>Chapter 15: Future work</td>
<td>137</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section V: Appendices &amp; bibliography</th>
<th>148</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix A: Reagents &amp; solutions</td>
<td>152</td>
</tr>
<tr>
<td>Appendix B: Protein-standardised results</td>
<td>153</td>
</tr>
<tr>
<td>Appendix C: Abbreviations</td>
<td>156</td>
</tr>
<tr>
<td>Bibliography</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>165</td>
</tr>
</tbody>
</table>
Section I: Introduction
Chapter 1: The burden of cardiovascular disease

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Definitions</td>
<td>8</td>
</tr>
<tr>
<td>1.2 Mortality</td>
<td>8</td>
</tr>
<tr>
<td>1.3 Morbidity</td>
<td>9</td>
</tr>
<tr>
<td>1.3.1 Incidence</td>
<td>9</td>
</tr>
<tr>
<td>1.3.2 Prevalence</td>
<td>9</td>
</tr>
<tr>
<td>1.4 Cost</td>
<td>9</td>
</tr>
</tbody>
</table>
1.1 Definitions

- “Cardiovascular disease” (CVD) is a broad term that refers to coronary heart disease, stroke and all other diseases of the circulatory system (e.g., peripheral vascular disease, abdominal aortic aneurysm, congenital heart disease, heart failure).

- “Coronary heart disease” (CHD), also known as ischaemic heart disease (IHD), refers to coronary artery narrowing by atheroma.

- “Acute coronary syndrome” is a single term used to describe myocardial infarction or unstable angina, suggesting a common pathological process.

- “Stroke” is an acute loss of focal cerebral function (occasionally global, when applied to cases of coma) with symptoms >24 hours (or leading to death) and no apparent cause other than one of vascular origin.

- “Transient ischaemic attack” (TIA) is an acute loss of focal cerebral function, or monocular visual loss, with symptoms <24 hours in duration and no apparent cause other than one of vascular origin.

1.2 Mortality

CVD accounts for 40% of all deaths in the UK (Compendium of Health Statistics, 14th edition) - 240,000 deaths in 2001 (Office for National Statistics, 2002). Half of these deaths (120,000) were due to CHD - the most common single cause of death in the UK, and also the most common cause of premature death, before the age of 75 (British Heart Foundation definition). A quarter of these deaths (60,000) were caused by stroke (the third most common cause of death, behind CHD and cancer). Figures 1 & 2 illustrate the relative frequencies of UK male and female causes of death (adapted from the British Heart Foundation statistics website, www.heartstats.org).
Figure 1.1 Male deaths by cause, United Kingdom (2001)

Figure 1.2 Female deaths by cause, United Kingdom (2001)
1.3 Morbidity

1.3.1 Incidence

It is estimated that the incidence of myocardial infarction is 2 to 2.5 times its mortality rate (Volmink et al, 1998; Tunstall-Pedoe et al, 1999; Lampe et al, 2000). Therefore, each year 275,000 UK people will have an MI (97,000 of these will be <75 years old). In addition, 140,000 will have a stroke (National Service Framework for Older People, 2001).

1.3.2 Prevalence

Currently in the UK, it is estimated that the prevalence of CHD is 2.65 million, 1.2 million of whom have had a heart attack (British Heart Foundation Statistics Website, www.heartstats.org). In addition, there are 300,000 people affected by stroke at any one time, which makes it the largest single cause of severe disability.

1.4 Cost

The cost of CHD to the UK healthcare system is estimated to be £1.8 billion per year. Looking only at this healthcare cost will result in a gross underestimation of the total burden on the UK economy. To illustrate this, a recent study (Liu et al, 2002) estimated the cost of productivity losses and informal care due to CHD to be £5.3 billion.

Despite having a significantly lower prevalence than CHD, the yearly healthcare cost of stroke was £2.3 billion, at 1996 prices (Department of Health, 1996).
# Chapter 2: Atherosclerosis & mechanisms of plaque rupture

## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Introduction</td>
<td>12</td>
</tr>
<tr>
<td>2.2 Evidence for the “plaque rupture” theory</td>
<td></td>
</tr>
<tr>
<td>2.2.1 Coronary circulation</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2 Cerebral circulation</td>
<td>14</td>
</tr>
<tr>
<td>2.3 The role of individual components of the arterial wall</td>
<td>15</td>
</tr>
<tr>
<td>2.3.1 The endothelium</td>
<td>17</td>
</tr>
<tr>
<td>2.3.2 The lipid core</td>
<td>19</td>
</tr>
<tr>
<td>2.3.3 The cap of the plaque</td>
<td>22</td>
</tr>
<tr>
<td>2.3.3.1 Smooth muscle cells &amp; collagen production</td>
<td>23</td>
</tr>
<tr>
<td>2.3.3.2 Macrophages &amp; collagen degradation</td>
<td>25</td>
</tr>
<tr>
<td>2.3.4 The vessel lumen</td>
<td>25</td>
</tr>
<tr>
<td>2.4 The role of angiogenesis</td>
<td>27</td>
</tr>
<tr>
<td>2.5 The role of infectious agents</td>
<td>28</td>
</tr>
<tr>
<td>2.6 Risk prediction of plaque instability</td>
<td>29</td>
</tr>
<tr>
<td>2.6.1 Imaging</td>
<td>29</td>
</tr>
<tr>
<td>2.6.2 Blood markers</td>
<td>30</td>
</tr>
<tr>
<td>2.7 Therapy aimed at plaque stabilisation</td>
<td>31</td>
</tr>
<tr>
<td>2.8 Summary</td>
<td>31</td>
</tr>
</tbody>
</table>
2.1 Introduction

Cardiovascular disease is the leading cause of mortality and morbidity in the developed world (Yusuf et al, 2001). While risk factors have been clearly identified, their precise roles in early atherogenesis are complex. Early atherosclerotic changes are dependent upon interactions between damaged endothelial cells, vessel wall smooth muscle cells and circulating inflammatory cells (Fan & Watanabe, 2003). These changes are mediated by the release of cytokines, growth factors and cell adhesion molecules (Ito & Ikeda, 2003). Plaque formation may represent a cell-mediated immune phenomenon, with a variety of potential antigenic agents identified. Shear stress and flow considerations also play a part.

Atherosclerosis begins in childhood with the appearance of fatty streaks, but the evolution of these initial lesions into the mature plaques responsible for the onset of ischaemic symptoms, takes place over many decades. Whilst plaque growth due to smooth muscle cell proliferation, matrix synthesis and lipid accumulation may narrow the arterial lumen and ultimately limit blood flow, uncomplicated atherosclerosis is essentially a benign disease. The final clinical outcome depends on whether a plaque becomes unstable, leading to acute disruption of its surface and exposure of its thrombogenic core to the luminal blood flow. The concept of a “vulnerable plaque” was initially described by Little (1990) and is now largely accepted (Casscells et al, 2003).

The mature atherosclerotic plaque is composed of a lipid core that is separated from the vessel lumen by a cap composed of fibrillar collagen. Disruption of this cap exposes the plaque’s underlying thrombogenic core to the bloodstream, resulting in thromboembolism. This process of “plaque rupture” is responsible for the majority of acute coronary syndromes (Falk, 1985; Falk et al, 1995; Libby, 2001a; Shah, 2000) and ischaemic cerebral events (Carr et al, 1996; Loftus et al, 2000; Sitzer et al, 1995).
Unravelling the complex biochemical and haemodynamic factors leading to plaque rupture is one of the greatest challenges facing contemporary medical research. The vital question in plaque pathogenesis is why life-threatening disruption and subsequent thrombosis should suddenly occur, after years of indolent growth. Plaque stabilisation may prove to be an important clinical strategy for preventing the development of complications (Shah, 2000). By identifying “vulnerable” plaques (ie, those most at risk of rupture) we can more effectively direct pharmacotherapy to those most likely to benefit. Also, by understanding the mechanisms of plaque rupture we can strive to develop new treatments aimed at prevention.

2.2 Evidence for the “plaque rupture” theory

2.2.1 Coronary circulation

Evidence that plaque rupture leads to acute coronary syndromes has been provided from a number of sources. Early pathological studies using post-mortem specimens from fatal cases of acute myocardial infarction have revealed that virtually all cases of coronary thrombosis are related to rupture or fissuring of atheromatous plaques, along with evidence of distal embolisation (Davies & Thomas, 1984; Falk, 1983; Falk, 1985; Friedman, 1970). Angioscopic findings in patients with stable angina have identified smooth atheroma within their coronary arteries, but disrupted irregular atheroma in the arteries of those with unstable angina (Forrester et al, 1987; Sherman et al, 1986).

Radiological and histological studies have demonstrated that patients with a plaque morphology consisting of large lipid cores and thin fibrous caps are at increased risk of cardiovascular events (Davies, 2000; Felton et al, 1997; Kolodgie et al, 2001). In addition, these “unstable” plaques are not necessarily the ones causing severely stenotic lesions (Ambrose et al, 1985; Giroud et al, 1992; Hackett et al, 1988).
2.2.2 Cerebral circulation

A similar association between carotid plaque rupture and cerebrovascular events has been shown. In patients undergoing multiple TIAs or stroke progression, microemboli can be detected in the middle cerebral artery by transcranial Doppler (Markus et al, 1995; Sitzer et al, 1995), while surface ulceration of carotid plaques seen on ultrasound imaging correlates well with symptoms (Golledge et al, 1997).

Early work utilising carotid plaques retrieved at carotid endarterectomy, highlighted the relationship between the presence of thrombus and the clinical status of patients (Gunning et al, 1964; Harrison & Marshall, 1977). This supported the theory that ischaemic attacks resulted from embolism rather than reduction in cerebral blood flow, particularly as few strokes occur in watershed areas (Bogousslavsky et al, 1988).

The most compelling evidence for an association between carotid plaque rupture and ischaemic cerebral events, is that carotid endarterectomy specimens removed from symptomatic patients are more likely to show histological evidence of rupture, compared to those from asymptomatic patients (Carr et al, 1996; Loftus et al, 2000). Van Damme & Vivario (1993) showed that 53% of complicated carotid plaques (intraplaque haemorrhage, haematoma, thrombus or ulceration) were symptomatic with a corresponding neurological deficit, compared to 21% of simple uncomplicated plaques.

The vast majority of acute coronary syndromes are due to medium and large artery atheroma (Fuster et al, 1992), whereas stroke is a more heterogenous disorder. Cerebral infarction accounts for 80% of strokes, with 20% caused by haemorrhage (Dennis & Warlow, 1987). Of the ischaemic strokes, large artery thromboembolism (from the internal carotid and middle cerebral arteries) and small vessel occlusion together account for the majority (Dennis et al, 1989; Schulz & Rothwell, 2003). Plaque rupture is responsible for these events and is therefore the underlying pathology in a large proportion of, but not all, strokes. The aetiology of stroke is illustrated in figure 2.1.
2.3 The role of individual components of the arterial wall

A number of intrinsic and extrinsic factors have been identified that determine plaque vulnerability: the size and consistency of the plaque core, the thickness and collagen content of the fibrous cap, and inflammation within the plaque. Further factors such as haemodynamic stress upon the plaque may ultimately contribute to cap disruption.

The evolution of a stable to an unstable plaque, with cap rupture and thrombosis, is outlined in simplistic terms in figure 2.2.

Each of the components contributing to plaque rupture will be discussed in further detail. The relevant processes occur in the endothelium, the lipid core, the fibrous cap and the vessel lumen.
Various factors (see text) cause endothelial activation. Dysfunctional endothelium allows inflammatory cells (ө) and LDL (庄村) to pass into the subendothelial space and lipid core. Free radicals (═) oxidise LDL to form a reactive and cytotoxic compound. Oxidised-LDL stimulates inflammatory cells to produce proteases, such as MMPs and cytokines (CYTO). MMPs degrade plaque cap exposing underlying thrombogenic core to luminal blood. Thrombus forms at site of rupture which may lead to embolism depending on local coagulation and fibrinolytic mechanisms.

**Figure 2.2** The stages of plaque rupture. Endothelial damage allows passage of inflammatory cells and LDL into the vessel intima; free radicals are responsible for oxidation of the deposited LDL; oxidised-LDL promotes cytokine and protease release from macrophages; proteases degrade the fibrous cap causing disruption; exposure of the thrombogenic core to the blood results in clot formation; local thrombotic and fibrinolytic activity determine the degree of thrombus progression or dissolution.
2.3.1 The endothelium

The origin of plaque destabilisation can be traced back to endothelial dysfunction, or "activation". The endothelium is a single layer of highly specialised cells lining the vessel wall/lumen interface. It plays a vital role in modulating vascular permeability, perfusion, contraction and haemostasis. Leukocytes do not bind to normal endothelium. However, endothelial activation leads to the early surface expression of cell adhesion molecules, including VCAM-1, ICAM-1, E-selectin and P-selectin, which permit leukocyte binding. Many of the known atherosclerosis risk factors (eg, smoking, hyperlipidaemia, hyperglycaemia, hypertension, hyperhomocysteinaemia) exert their damaging effects by causing endothelial activation (Barua et al, 2002; Barua et al, 2003; Hanratty et al, 2001; Luscher et al, 1996; Salt et al, 2003; Taddei et al, 2001).

Activated endothelial cells express chemo-attractant cytokines such as MCP-1, M-CSF, IL-1, IL-6 and TNF-α, as well as cell adhesion molecules. This pro-inflammatory environment, in conjunction with the altered permeability of the dysfunctional endothelium, mediates the migration and entry of leukocytes (mainly monocytes and lymphocytes) into the intima (Cybulsky & Gimbrone, 1991; Van der Wal et al, 1992; Vanhoutte, 1997).

The degree of endothelial dysfunction depends upon the balance between endothelial activation and endothelial "passivation" (see figure 2.3). Nitric oxide is the predominant molecule responsible for passivation, and the endothelium acts as an autocrine organ in its production (De Caterina et al, 1995). Nitric oxide is an antioxidant, but has other plaque-stabilising properties including reducing cell adhesion molecule expression (Moncada & Higgs, 1993), platelet aggregation and SMC proliferation. Endothelial nitric oxide synthase, the enzyme responsible for nitric oxide production, is increased in people undergoing regular physical exertion, which may partly explain the benefits of exercise in atherosclerosis prevention (Kingwell et al, 1997).
Endothelial cells are exposed to 3 different types of mechanical force. Hydrostatic forces (generated by the blood) and circumferential stress (generated by the vessel wall) are responsible for endothelial injury and activation. The third force is haemodynamic shear stress (generated by the flow of blood), which is inversely related to atherosclerosis formation – areas of high shear stress being relatively protected (Malek et al, 1999).

Despite the systemic nature of atherosclerosis, it is an anatomically focal disease with certain sites having a propensity for plaque formation. Arterial bifurcations exhibit slow blood flow, sometimes even bi-directional flow, resulting in decreased shear stress. The activity of endothelial nitric oxide synthase is decreased in these areas of non-laminar blood flow (Gimbrone et al, 1997; Nadaud et al, 1996). In addition, there is increased oscillatory and turbulent shear stress at bifurcations, associated with augmented oxygen free radical production (De Keulenaer et al, 1998) and monocyte adhesion (Chappell et al, 1998).
According to Laplace’s law, the higher the blood pressure and the larger the luminal diameter, the more circumferential tension develops in the wall (Lee & Kamm, 1994). This phenomenon combined with a radial compression of the vessel wall may lead to unbearable stress in vulnerable regions of the plaque, particularly the cap and shoulder (Cheng et al, 1993). For fibrous caps of the same tensile strength, those caps covering moderately stenotic plaques are more prone to rupture than those covering severely stenotic plaques, because the former have to bear a greater circumferential tension (Loree et al, 1992).

The propagating pulse wave causes cyclic changes in lumen size and shape with deformation and bending of plaques, particularly those with a large soft plaque core. Eccentric plaques typically bend at the junction between the relatively stiff plaque and the compliant vessel wall (Maclsaac et al, 1993). The force applied to this region is accentuated by changes in vascular tone.

High blood velocity within stenotic lesions may shear the endothelium away, but whether high wall stress alone may disrupt a stenotic plaque is questionable (Falk et al, 1995). The absolute stresses induced by wall shear are usually much smaller than the mechanical stresses imposed by blood and pulse pressure (Gronholdt et al, 1998).

It is clear that the endothelium is much more than an inert arterial wall lining. It is, in fact, a dynamic autocrine and paracrine organ responsible for the functional regulation of local haemodynamics. Factors that disturb this delicate balance are responsible for the initiation of a cascade of events eventually leading to plaque rupture.

2.3.2 The lipid core

The size and consistency of the atheromatous core is variable and critical to the stability of individual lesions – a large volume lipid core (figure 2.4) being one of the constituents of the vulnerable plaque. It appears that the accumulation of lipids in the intima renders the plaque inherently unstable.
Although extremely variable, the “average” coronary plaque is predominantly sclerotic with the lipid core making up <30% of the plaque volume (Kragel et al, 1989). The variability in plaque composition is poorly understood, and appears to bear no relationship to any of the identified risk factors for atherosclerosis.

Gertz & Roberts (1990) examined the histological composition of post-mortem plaques from 17 infarct-related coronary arteries. They found much larger proportions of the disrupted plaques to be occupied by atheromatous gruel in comparison to the intact plaques. Davies et al (1993) found a similar relationship in aortic lesions, with 91% of thrombosing plaques versus 11% of intact plaques exhibiting a lipid core that occupied >40% of the total plaque volume.

Histological data regarding the necrotic core of carotid plaques is limited. Feeley et al (1991) demonstrated a significant increase in the proportion of symptomatic carotid plaques occupied by amorphous material compared to asymptomatic plaques, though other studies have failed to show such a relationship (Carr et al, 1996). There is, however, considerable evidence to link ultrasound-detected echolucent plaques (deemed to contain more soft or amorphous tissue) with symptomatology (El-Barghouti et al, 1996; Reilly et al, 1983).
LDL plays a more complex role in plaque instability than can be explained simply by the “space-occupying” effect of accumulated lipid. A large core may produce a greater luminal narrowing, but plaque rupture sites are often characterized by “outward remodelling” whereas those stenoses causing stable angina are more likely to be associated with “inward remodelling” (Takano et al, 2001). Indeed, it has been shown that in patients suffering acute coronary syndromes who had undergone angiography in the preceding months, the responsible lesion was recorded as causing a <70% stenosis in the majority of cases (Ambrose et al, 1985; Giroud et al, 1992; Takano et al, 2001). This is perhaps not surprising since, as mentioned earlier, a larger lumen places increased circumferential stress on the plaque, predisposing it to rupture.

As inflammatory cells cross the dysfunctional endothelium, cholesterol also enters in the form of LDL, and becomes trapped in the subendothelial space. This LDL is oxidized by free radicals creating a pro-inflammatory compound (Steinberg, 1997). Oxidized-LDL is taken up by intimal macrophages – the process being mediated via receptors expressed on the macrophage surface (Nicholson et al, 2001), although endocytosis of native LDL has also been demonstrated (Kruth et al, 2002). This process initially protects the surrounding smooth muscle and endothelial cells from the direct cytotoxic effects of oxidised-LDL, but leads to the formation of “foam cells” (lipid-laden macrophages). Uptake of oxidized-LDL stimulates the expression of cytokines and proteolytic enzymes, propagating the cycle of inflammation.

The formation of a lipid core is a balance between LDL deposition of cholesterol in the damaged intima and removal by HDL (figure 2.5). HDL and its carrier, apolipoprotein A-I, are responsible for so-called “reverse cholesterol transport” – moving cholesterol from cells to the blood, from where it can be transferred to the liver for excretion in the bile (De la Llera Moya et al, 1994). It may also be capable of effecting lipid removal directly from the plaque, one of the possible explanations for plaque regression seen with increased HDL levels (Badimon et al, 1990). HDL may have other beneficial effects also, such as improving endothelial function (Spieker et al, 2002), decreasing cell adhesion molecule expression (Barter, 1997) and inhibiting oxidation of LDL (Lin et al, 2002).
2.3.3 The cap of the plaque

The cap of the atherosclerotic plaque plays a vital role in isolating the plaque’s thrombogenic core from the bloodstream. The thickness and collagen content of the cap are important determinants of its strength, and therefore the overall stability of the plaque (Loree et al., 1992). The cap is composed predominantly of fibrillar collagens, type I and type III (Katsuda et al., 1992). The fibrillar collagens present in the cap have a lower thrombogenicity than the underlying core but, nevertheless, their exposure can be responsible for thrombus formation following erosion of the overlying endothelium (Van der Wal et al., 1994; Farb et al., 1996). This phenomenon accounts for one-third of acute coronary syndromes (Virmani et al., 2000), and the subsequent healing process of erosions can account for rapid and step-wise progression in plaque growth leading to sudden increases in stenosis, or occlusion (Burke et al., 2001).

The most vulnerable area of the plaque is the shoulder region, where the cap is often at its thinnest (Falk, 1985). Studies have shown a reduction in the collagen content of the cap around areas of plaque disruption, as well as steep transverse gradients of connective tissue constituents across ulcerated plaques (Burleigh et al., 1992). This may result from a reduction in extracellular matrix (ECM) production by smooth muscle cells, which exhibit
diminished numbers in areas of plaque disruption (Davies et al, 1993), or from increased degradation of matrix by proteolytic enzymes. It is most likely, however, that a combination of excessive matrix degradation and reduced matrix production are responsible for cap thinning (figure 2.6). A reduction in SMCs within the fibrous cap would certainly undermine its strength (Davies, 1996). Recently there has been interest in the role of smooth muscle cell apoptosis in plaque cap weakening, caused by a combination of intrinsic and extrinsic factors, particularly macrophage and lipid-derived products (Kockx et al, 1998; Kockx 1999).

![Figure 2.6 Matrix degradation vs matrix production](image)

**Figure 2.6 Matrix degradation vs matrix production**

### 2.3.3.1 Smooth muscle cells & collagen production

The SMC has a paradoxical role in atherosclerosis. On the one hand, it produces the ECM constituents that comprise the atherosclerotic plaque, and so is responsible for the plaque’s progression. Some of these ECM components however are the collagens, and so, on the other hand, it confers stability upon the plaque. Therefore, SMC inhibition potentially has
both beneficial (preventing plaque progression) and detrimental (plaque-destabilising) effects.

In the normal arterial wall, SMCs are present in the media and express a differentiated phenotype. They are contractile and do not divide or migrate (Barnes & Farndale, 1999). In atherosclerosis, when stimulated by the milieu of growth factors and cytokines, they "dedifferentiate" and express a synthetic phenotype (Dilley et al, 1987). In the media, SMCs are surrounded by a basal lamina consisting of type IV collagen. Proteolytic enzymes secreted by macrophages are responsible for digestion of this supporting framework. The released SMCs are then able to migrate to the intima, where they secrete new extracellular matrix (Newby, 1997). SMCs play a crucial role in stabilising atherosclerotic plaques, as they are responsible for the production of the cap fibrillar collagens (Barnes & Farndale, 1999). Certain platelet factors, including PDGF and TGF-β, are felt to be particularly important in stimulating collagen synthesis by SMCs, whereas γ-interferon (from activated T-cells) has an inhibitory effect (Amento et al, 1991).

SMC apoptosis may also be responsible for decreased plaque collagen (Geng & Libby, 1995; Geng 2001). A small study has demonstrated that the proportion of SMCs undergoing apoptosis and the frequency of cytoplasmic remnants of apoptotic cells were significantly increased in unstable versus stable angina atherectomy specimens (Bauriedel et al, 1999). Apoptotic SMCs and macrophages have been identified within plaques, but only in advanced disease with dense macrophage infiltration. Apoptosis is characterised by a series of morphological changes, starting with shrinkage of the cell membrane and leading on to condensation of nuclear chromatin, cellular fragmentation and eventually engulfment of apoptotic bodies by surrounding cells (Kockx, 1999).

Pro-apoptotic proteins are present in advanced plaques, and it has been observed that cells derived from the plaque, but not the adjacent media, die when brought into culture (Bennett et al, 1995; Kockx et al, 1998). Intimal cell apoptosis may account for the low density of smooth muscle cells in unstable plaques, and may contribute to the events leading up to plaque disruption. However, this phenomenon should also result in decreased plaque progression, and the precise role of cellular apoptosis remains unclear.
2.3.3.2 Macrophages & collagen degradation

It is now known that inflammation plays a major role in plaque progression and especially in the period just prior to its rupture (Buja & Willerson, 1994). Macrophages control many of the inflammatory processes within the plaque (Libby & Simon, 2001) and are responsible for the production of proteolytic enzymes capable of degrading the extracellular matrix (Moreno et al, 1994; Shah et al, 1995). The predominant proteolytic enzymes involved in plaque disruption are the matrix metalloproteinases or MMPs (Loftus et al, 2002).

The MMPs are a family of proteolytic enzymes characterised by the presence of zinc ions at their active sites. They are essential in normal healthy individuals, playing a key role in processes such as wound healing (Wysocki et al, 1993; Agren et al, 1998). However, there is growing interest in their role in disease states where ECM breakdown is a dominant feature (Krane, 1994). Early interest focused on a pathological role for MMPs in the resorption of structures in periodontal disease (Page, 1991), the destruction of joints in rheumatoid arthritis (Harris, 1990) and the invasive behaviour of malignancies (Parsons et al, 1997). In vascular disease, they have been implicated in many of the stages of atherosclerosis but most particularly in acute plaque disruption (Dollery et al, 1995). The site of rupture is characterised by an intense inflammatory infiltrate consisting predominantly of macrophages (Moreno et al, 1994) that undergoes activation resulting in increased MMP expression. This shifts the delicate equilibrium towards proteolysis and away from matrix accumulation, making plaque disruption more likely (figure 2.6).

The role of MMPs in atherosclerotic plaque instability will be discussed in more detail in chapter 3.

2.3.4 The vessel lumen

Disruption alone would not precipitate ischaemic syndromes without thrombus formation on the plaque surface, so plaque instability and thrombogenicity in tandem predispose to acute clinical events. Platelet adherence to the subendothelium after surface disruption leads to activation, with ADP and serotonin release stimulating further platelet recruitment and activation.
Once formed, thrombus can behave in three ways, dependent on the physical nature of the rupture and the balance between local fibrinolytic and coagulation processes (figure 2.7). Firstly, the initial thrombus may progress to cause occlusion of the vessel. Secondly, the thrombus may disintegrate resulting in distal embolisation. Thirdly, the clot can undergo rapid dissolution, with the healed rupture resulting in a variable decrease in vessel lumen diameter (Burke et al, 2001).

![Diagram of Fibrinolysis vs Coagulation](image)

**Figure 2.7 Fibrinolysis vs coagulation**

Tissue factor is a major regulator of haemostasis (Nemerson, 1988). It is the most thrombogenic component of atherosclerotic plaques (Fernandez-Ortiz et al, 1994) and is expressed by numerous cell types, including endothelial cells. The level of tissue factor in coronary plaques from patients with unstable angina is more than twice the value observed in those plaques from stable angina patients (Moreno et al, 1996). Positive immunostaining for tissue factor correlates with areas of intense macrophage infiltration and SMCs, suggesting a cell-mediated increased thrombogenicity in unstable plaques (Jander et al, 2001).
The increase in tissue factor levels seems to be linked to expression of the CD-40 receptor on the macrophage cell surface. The CD-40 ligand is expressed on activated T-lymphocytes, and other atheroma-associated cells (Mach et al, 1998), which can therefore induce tissue factor production by macrophages via this signalling system. Expression is also regulated by cytokines and oxidised-LDL (Aikawa et al, 1999; Bevilacqua et al, 1986). It has been reported that a blood-borne pool of tissue factor exists (Giesen et al, 1999), though in the context of plaque disruption, macrophage production of tissue factor is predominantly responsible for plaque thrombogenicity (Moreno et al, 1996; Meisel et al, 2002; Toschi et al, 1997). It is interesting to note that many of the recognised cardiovascular risk factors increase the expression of tissue factor (Matetzky et al, 2000; Sambola et al, 2003).

2.4 The role of angiogenesis

Angiogenesis is essential for normal growth and development. Neovascularisation has been observed in plaques (Barger et al, 1984) and it is postulated that it may play a role in atherosclerosis (by providing growth factors and cytokines to regions of plaque development). In addition, angiogenesis may be involved in plaque destabilisation (by promoting intraplaque haemorrhage from fragile newly-formed vessels) and atherosclerotic plaque rupture (by recruiting inflammatory cells into vulnerable areas of the lesion).

A number of studies have demonstrated a relationship between the presence of intraplaque haemorrhage and patient symptoms (Imparato et al, 1979). Lusby et al (1982) suggested a relationship between the onset of neurological symptoms and the development of plaque haemorrhage, while Persson et al (1983) found that haemorrhage into the plaque appeared more frequently in symptomatic patients than asymptomatic patients. Intraplaque haemorrhage may occur following cap rupture but, conversely, neovascularisation identified in unstable plaques may result in plaque haemorrhage (since new vessels are more fragile and prone to bleeding). The subsequent rapid expansion in core volume may increase the stress upon a weakened cap predisposing it to acute disruption.

A study of coronary atherectomy specimens revealed the presence of neovascularisation in 50% of specimens from patients with unstable angina compared to 10% of specimens from patients with stable angina (Tenaglia et al, 1998), suggesting a possible role in plaque instability. A similar association has been demonstrated in carotid plaques. McCarthy et al
(1999) observed significantly more neovessels in plaques retrieved from symptomatic patients compared to those retrieved from a well-matched asymptomatic group. They also noted that neovessels in symptomatic plaques were significantly larger and more irregular, and concluded that these vessels may contribute to plaque instability and the onset of thromboembolic sequelae.

De Boer et al (1999) have shown a significant increase in microvessel density in lipid-rich compared to fibrous plaques. Importantly, most of these vessels appear to be located in the vulnerable shoulder area of the plaque and immunostaining for inflammatory cells shows a close association between angiogenesis and inflammatory infiltration. In addition, a parallel increase in the expression of leukocyte adhesion molecules in the same vulnerable areas was demonstrated.

Angiogenesis involves interactions between endothelial cells and components of the basement membrane matrix. MMP activity is required for such interactions, especially MMP-2 and MT1-MMP (Haas & Madri, 1999). Up-regulation of MMP activity stimulates angiogenesis, while TIMPs have the opposite effect (Kostoulas et al, 1999).

It is presently unclear as to whether angiogenesis is a cause or an effect of plaque destabilisation. Whilst neovascularisation may promote and sustain inflammatory infiltration, the converse may also be true, whereby changes in the plaque associated with inflammation may themselves promote angiogenesis. Further work in this area is required.

2.5 The role of infectious agents

The role of infectious agents in atherosclerosis and plaque rupture is controversial. Definitive proof of a causal relationship is lacking, although studies have reported associations between plaque development and Chlamydia pneumoniae (Muhlestein et al, 1998; Saikku et al, 1988; Saikku et al, 1992), Helicobacter pylori (Ossei-Gerning et al, 1997) and cytomegalovirus (Nieito et al, 1996; Span et al, 1992).

Certain infectious agents can evoke cellular and molecular changes supportive of a role in atherogenesis (Epstein et al, 2000). Work has shown that Chlamydial interaction with monocytes results in up-regulation of TNF-α and IL-1β (Heinemann et al, 1996; Kaukoranta-Tolvanen et al, 1996), both of which are associated with plaque development.
Chlamydial production of the HSP-60 antigen activates human vascular endothelium, and increases TNF-α and MMP expression in macrophages (Kol et al, 1998; Kol et al, 1999).

There is some doubt about the methods employed for Chlamydia detection (Weiss et al, 1996) and also the role of potential confounding factors in epidemiological studies (Hahn & Golubiatnikov, 1992). A large-scale prospective study of 15,000 healthy men in the United States which was controlled for age, smoking, socio-economic status and other cardiovascular risk factors, failed to show any association between Chlamydia seropositivity and the risk of MI (Ridker et al, 1999a).

The recent STAMINA trial (Stone et al, 2002) demonstrated that eradication therapy (amoxicillin/azithromycin, metronidazole and omeprazole) administered for 1 week after an acute coronary syndrome, significantly reduced cardiac death and acute coronary syndrome readmission rates over the following 12 months. These effects were unrelated to Chlamydia pneumoniae or Helicobacter pylori seropositivity, however, suggesting that the trial therapy prevented lesion progression by a mechanism unrelated to its antibiotic action.

2.6 Risk prediction of plaque instability

2.6.1 Imaging

Angiography can demonstrate ulceration (Eliasziw et al, 1994) but does not appear to be able to adequately distinguish between stable and unstable plaques (Rothwell et al, 1995). In addition, the degree of stenosis detected by angiography does not correlate well with the future risk of events (Ambrose et al, 1985; Giroud et al, 1992; Hackett et al, 1988).

Ultrasound studies have shown an association between carotid plaque morphology and neurological symptoms (Gronholdt, 1999) but have been unable to predict the risk of future events (Golledge et al, 1997). More promisingly, intravascular ultrasound (IVUS) studies have demonstrated an increased incidence of ulcerated and ruptured plaques in patients with acute coronary syndromes (Rioufol et al, 2002).

Increased inflammatory activity occurs prior to plaque rupture, and attempts have been made to detect this increase, using local temperature measurements. Thermography studies have shown that temperature correlates well with macrophage cell density in human carotid plaques (Casscells et al, 1996). The temperature of coronary vessels in patients
with ischaemic heart disease, in particular acute coronary syndromes, is higher than in normal controls (Stefanadis et al, 1999). In addition, increased local plaque temperature has been shown to be an independent predictor of adverse clinical outcome (Stefanadis et al, 2001).

High-resolution MRI appears to characterize the atherosclerotic plaque better than other imaging techniques (Botnar et al, 2000). It is more accurate than angiography in measuring the degree of stenosis and, unlike angiography and IVUS, is non-invasive. Technically, images are limited by small vessel size and movement artifact, and studies have not yet demonstrated the ability to predict risk of future cardiovascular events. However, advances in the technique suggest a potential future role for MRI in detection of the high-risk plaque.

2.6.2 Blood markers

It has long been established that adverse lipid profiles correlate with increased risk of MI and stroke. CRP levels are also associated with increased cardiovascular risk in apparently healthy patients (Ridker et al, 1997; Ridker et al, 1998), and enough evidence now exists to incorporate their use in generating individual risk assessment profiles.

MMP-2 and MMP-9 are raised in the peripheral blood of patients suffering from acute coronary syndromes (Kai et al, 1998), while plasma MMP-9 is raised in patients with unstable carotid plaques (Loftus et al, 2001). A recent study of 1127 patients with coronary artery disease identified baseline plasma MMP-9 levels to be a novel predictor of cardiovascular mortality (Blankenberg et al, 2003). Further work is required to identify the nature and source of these elevated MMPs, but clearly the potential for risk prediction exists.
2.7 Therapy aimed at plaque stabilisation

Pharmacotherapy to induce plaque stabilisation could be targeted at different components of the arterial wall, in particular:

- the endothelium – by increasing endothelial passivation or preventing activation
- the lipid core – by augmenting LDL removal or reducing LDL deposition
- the fibrous cap – by increasing collagen deposition or preventing degradation
- the vessel lumen – by altering the thrombogenicity of the local environment.

Most recent interest has focussed on the role of HMG-CoA reductase inhibitors, which appear capable of influencing plaque stabilisation by multiple actions. The role of pharmacotherapy in the prevention of plaque disruption will be discussed in chapter 4.

2.8 Summary

Acute plaque disruption precedes the onset of clinical ischaemic syndromes. Exposure of the highly thrombogenic core to luminal blood results in platelet adherence and thrombosis. Inflammation is clearly involved in the process of plaque development and acute disruption, though the precise mechanism by which the inflammatory process is initiated remains unclear. The roles of angiogenesis, cellular apoptosis and infectious agents also require further clarification. Unstable plaques have a large lipid core and a thin fibrous cap with reduced collagen content. A major component of plaque destabilisation appears to be increased matrix degradation, and the primary regulators of this process are the MMPs and their inhibitors. There are a number of potential therapeutic options aimed at preventing plaque disruption.
# Chapter 3: The role of collagenases & other MMPs in plaque instability

## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Introduction</td>
<td>33</td>
</tr>
<tr>
<td>3.2 The MMP family</td>
<td>34</td>
</tr>
<tr>
<td>3.3 The control of MMP activity</td>
<td>39</td>
</tr>
<tr>
<td>3.4 The role of MMPs in acute plaque disruption</td>
<td>42</td>
</tr>
<tr>
<td>3.4.1 Gelatinases</td>
<td>42</td>
</tr>
<tr>
<td>3.4.2 Collagenases</td>
<td>46</td>
</tr>
<tr>
<td>3.4.3 Stromelysins</td>
<td>47</td>
</tr>
<tr>
<td>3.4.4 Matrilysins</td>
<td>47</td>
</tr>
<tr>
<td>3.4.5 Membrane-type MMPs</td>
<td>47</td>
</tr>
<tr>
<td>3.5 “Net” proteolytic activity</td>
<td>48</td>
</tr>
<tr>
<td>3.6 Summary</td>
<td>49</td>
</tr>
</tbody>
</table>
3.1 Introduction

Clinical symptoms secondary to atherosclerosis occur as a consequence of 2 pathological processes. Firstly, the stenotic plaque may cause limitation of blood flow resulting in stable conditions, such as exercise-induced angina or intermittent claudication. Secondly, disruption of the cap of the plaque exposes the lipid core to the bloodstream, resulting in thromboembolism and sudden vessel occlusion. As discussed in chapter 1, this latter process is responsible for most of the cardiovascular disease burden. Figure 3.1 shows a carotid plaque retrieved during endarterectomy – the potential for embolisation from this necrotic, friable plaque can be easily appreciated. Atherosclerotic plaque instability is a term used to describe those lesions that have already undergone, or are likely to proceed to, rupture.

Figure 3.1 Plaque retrieved at carotid endarterectomy demonstrating potential for embolisation
The integrity of connective tissue depends upon a balance between degradation of the extracellular matrix (ECM), and its repair. The rate-limiting step of ECM degradation is due to the activity of proteolytic enzymes (Murphy & Docherty, 1992). Matrix metalloproteinases (MMPs) play a major role in the degradation of collagen and other ECM macromolecules, and are the most important group of enzymes in this regard. Their presence in healthy adults is vital to ensure regular turnover of normal tissue. In addition, MMP levels are raised in wound healing (Wysocki et al, 1993), as digestion of injured tissue is as important as deposition of its replacement.

As well as physiological up-regulation of MMPs to repair damaged tissue, increased levels can be found in various disease states. They are thought to play a pathological role in periodontal disease (Page, 1991), rheumatoid arthritis (Harris, 1990) and malignancy (Parsons et al, 1997). These are all conditions in which ECM breakdown is a central characteristic and this led to the hypothesis that MMP inhibition may result in an improved outcome for patients with these diseases. This concept has been tested in clinical trials (Denis & Verweij, 1997; Talbot & Brown, 1996), particularly in the field of cancer.

The evolution of atherosclerosis involves considerable tissue remodelling over many years, and MMPs are intricately involved in each phase of this process (Dollery et al, 1995). This has led to considerable interest in the aetiological role that each MMP plays in various vascular disease states, including aneurysm formation (Saito et al, 2002) and intimal hyperplasia (Loftus et al, 1999).

For the purposes of this chapter we shall focus our attention on their role in atherosclerotic plaque instability. The plaque undergoes a period of destabilisation prior to the onset of symptoms (Falk et al, 1995). The processes involved include infiltration of increased numbers of inflammatory cells (capable of producing MMPs), thinning of the fibrous cap, intraplaque haemorrhage and plaque rupture (Carr et al, 1996; Kolodgie et al, 2001; Moreno et al, 1994).
3.2 The MMP family

The term “metalloproteinase” refers to an enzyme capable of protein degradation, which is dependent on one or more metal atoms at its active centre for catalytic function. Over 200 of these enzymes have been identified and in almost all cases, the metal ion required is zinc (Ye et al, 1998).

The “matrix” metalloproteinases are distinguished from other metalloproteinases by their ability to degrade components of the ECM. These enzymes are also sometimes referred to as “matrixins”. To date, 24 vertebrate MMPs have been identified (see table 3.1) which share many of the following features:

- they are secreted in a latent, propeptide form and are activated by organomercurial compounds
- they possess a “cysteine switch” motif responsible for maintenance of the zymogen form
- they can be blocked by chelators
- they are inhibited by TIMPs (tissue inhibitor of metalloproteinases)
- they have an extracellular site of action
- they share significant sequence homology due to their evolutionary relationship, but are each the product of a separate gene.
<table>
<thead>
<tr>
<th>MMP</th>
<th>Alternative names</th>
<th>Principal substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>Collagenase-1, Interstitial collagenase</td>
<td>Collagens I,II,III, gelatin, proMMP-2 &amp; -9</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase-2, Neutrophil collagenase</td>
<td>Collagens I,II,III, gelatin</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
<td>Collagens I,II,III, gelatin, PAI-2</td>
</tr>
<tr>
<td>MMP-18</td>
<td>Collagenase-4, Xenopus collagenase</td>
<td>Collagen I</td>
</tr>
<tr>
<td>Gelatinases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase-B, 92 kDa gelatinase</td>
<td>Gelatin, collagen types IV, V, VII, X, elastin.</td>
</tr>
<tr>
<td>Stromelysins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin-2</td>
<td>Collagens III, IV, V, gelatin, proMMP-1 &amp; -8</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>Fibronectin, laminin</td>
</tr>
<tr>
<td>Matrilysins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin-1, Pump-1</td>
<td>Collagen IV, elastin, gelatin, proTNF-α</td>
</tr>
<tr>
<td>MMP-26</td>
<td>Matrilysin-2, Endometase</td>
<td></td>
</tr>
<tr>
<td>Membrane types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>Collagens I,II,III, gelatin, proMMP-2 &amp; -13</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>ProMMP-2, gelatin</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>Collagen III, fibronectin, gelatin, proMMP-2</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td></td>
</tr>
<tr>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>ProMMP-2</td>
</tr>
<tr>
<td>MMP-25</td>
<td>MT6-MMP</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-12</td>
<td>Macrophage elastase, metalloelastase</td>
<td>Elastin, fibronectin</td>
</tr>
<tr>
<td>MMP-19</td>
<td>No alternative name</td>
<td>Gelatin</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>Amelogenin</td>
</tr>
<tr>
<td>MMP-21</td>
<td>XMMP (Xenopus)</td>
<td></td>
</tr>
<tr>
<td>MMP-22</td>
<td>CMMP (Chicken)</td>
<td></td>
</tr>
<tr>
<td>MMP-23</td>
<td>Cysteine array MMP (CA-MMP)</td>
<td></td>
</tr>
<tr>
<td>MMP-28</td>
<td>Epilysin</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Vertebrate MMPs and their principal substrates
The majority of MMPs consist of 5 domains (see figure 3.2), although variations do exist.

**Figure 3.2 The domain structure of MMPs**

Starting from the NH$_2$-terminal end, the domains are listed below:

- **Signal domain:** MMPs are synthesised within the cell and this hydrophobic region is required for passage across the cell membrane. The domain is removed during this process and therefore is no longer attached when the enzyme reaches its extracellular site of action.

- **Propeptide domain:** In the middle of this domain exists a highly conserved sequence of 7 amino acids. The cysteine at the centre of this sequence forms a bond with the active zinc ion of the catalytic domain (Springman et al, 1990). Displacement of this cysteine residue, usually by proteolytic cleavage of the propeptide domain causes enzyme activation. Thus, it is apparent the "cysteine switch" mechanism plays a vital role in controlling MMP activity.
• **Catalytic domain:** This domain contains 2 zinc ions and is responsible for the enzymatic activity of the peptide (Woessner, 1994). The catalytic zinc ion is bound by 3 histidine residues and is found towards the COOH-terminal end. Some catalytic domains (notably those of MMP-2 and MMP-9) contain "fibronectin repeat" sequences. These are postulated to provide an extension of the binding cleft making substrate binding and degradation more effective (Banyai et al, 1996). In addition, the bonds formed between these sequences and various matrix components probably act to retain the enzyme in the environment where its action is required (Allan et al, 1995).

• **Hinge domain:** This short polypeptide sequence, also known as a linker sequence, allows for folding of the COOH-terminal hemopexin domain towards the catalytic domain, resulting in firmer binding of substrates.

• **Hemopexin domain:** This region shares homology with serum hemopexin (hence its name) and vitronectin (suggesting that one of its functions may be binding of the enzyme to ECM components). The absence of the hemopexin domain often leads to inability of the catalytic domain to degrade collagen (Clark & Cawston, 1989), implying that the catalytic domain alone is not large enough to bind the collagen molecule securely. Various inhibitors of MMPs (eg, heparin, TIMPs) exert part of their effects via interactions with this region.

The MMPs can be divided into 6 subgroups on the basis of structural characteristics and substrate specificity (also see table 3.1):

• **Collagenases:** MMP-1, MMP-8 and MMP-13 belong to this group, due to their ability to degrade the fibrillar collagens (type I, II and III). They achieve this by cleaving a specific bond (Gly775 - Ile776) three-quarters of the way along the triple helix molecule. The 2 fragments created can then be broken down by non-specific proteases.

• **Gelatinases:** MMP-2 and MMP-9 are the only 2 members of this group. Their main substrates, gelatins, are denatured collagen molecules, although importantly, they also degrade type IV collagen (the major constituent of the basement membrane).
• **Stromelysins**: MMP-3, MMP-10 and MMP-11 are able to digest components of the stroma, such as aggrecan, gelatin and casein. MMP-3 is also sometimes referred to as "collagenase-activating protein" – it is capable of cleaving the propeptide region of MMP-1, MMP-8 and MMP-13.

• **Matrilysins**: These are the smallest MMPs, containing only signal, propeptide and catalytic domains. They degrade a number of ECM components and process cell surface molecules, such as proTNF-α and E-cadherin.

• **Membrane-type MMPs**: MT1-MMP through to MT6-MMP (also known as MMP-14, 15, 16, 17, 24 and 25) possess a transmembrane domain that allows them to exert their actions from a fixed position at the edge of the cell. Their role includes activation of other MMPs (particularly MMP-2).

• **Others**: 7 other vertebrate MMPs have been discovered, that do not fit into any of the other categories listed above. All their functions and substrates are not yet clear, but MMP-12 appears to be particularly important, being essential for macrophage migration.

Human MMP genes are highly conserved modular structures. There is also a high degree of sequence homology preserved between species. A cluster of genes coding for 10 MMPs exists on chromosome 11. The remaining MMP genes are on a variety of the other chromosomes, including MMP-2 on chromosome 16 and MMP-9 on chromosome 20.

### 3.3 The control of MMP activity

The control of MMP activity (see figure 3.3) is obviously essential, if the correct balance between matrix accumulation and degradation is to be achieved. This control is exerted at multiple levels:

- MMP expression and release from cells is performed in a controlled manner in response to molecular signals (Galis et al, 1995). Various cytokines including IL-1β, PDGF and TNF-α all have a stimulatory effect, whereas heparin, corticosteroids and TGF-β are inhibitory.
• MMPs are secreted as latent proenzymes and only become active after enzymatic cleavage of their propeptide region. The major physiological activator of MMPs is plasmin (Lijnen, 2001), the level of which is increased by urokinase plasminogen activator (uPA), and decreased by plasminogen activator inhibitor (PAI). The subsequent stages of activation form a positive feedback loop, whereby plasmin activates MMP-1, MMP-3 and MMP-9 directly, active MMP-3 further activates MMP-1, and active MMP-1 cleaves proMMP-9 (Dollery et al, 1995).

• MMPs often remain bound to matrix components near to their cell of origin, restricting their site of action. The MT-MMPs are important in this regard (fixed as they are to the cell membrane and often being responsible for the activation of other MMPs, especially MMP-2).

• General inhibitors present in the extracellular environment, such as α2-macroglobulin are capable of inactivating excess MMPs by forming complexes with them (Grinnell et al, 1998).

• TIMPs are specific inhibitors that are often secreted by the same cell that produces the MMPs they inhibit – effectively titrating the level of proteolytic activity. Most TIMPs are able to inhibit most MMPs by irreversibly binding to the catalytic site in a 1:1 molar ratio (Murphy & Willenbrock, 1995). There are considerable differences in binding affinities between the TIMPs, and a more complex interaction exists between TIMP-2 & MMP-2 (Hutton et al, 1998), and TIMP-1 & MMP-9 (Overall, 1994), involving folding of the MMP hemopexin domain onto its active centre. Overall proteolytic activity depends on the relative concentrations of the active enzymes and their inhibitors.

• Reactive oxygen species produced by macrophage-derived foam cells modulate both MMP-2 and MMP-9 activity. There is an increase in the steady state levels of reactive oxygen species in a number of disease states including atherosclerosis (Nedeljkovic et al, 2003), and this may directly increase MMP activation in plaques.
Figure 3.3 The control of MMP activity
3.4 The role of MMPs in acute plaque disruption

Atherosclerosis is a chronic inflammatory disease, with continuous inflammation in the arterial wall resulting in smooth muscle cell proliferation, augmented uptake of LDL and atherosclerotic lesion progression (Ross, 1999; Libby, 2001b). Macrophage infiltrates are seen in unstable plaques, and are thought to play a causative role in plaque destabilisation (Buja & Willerson, 1994). These processes are probably mediated by the release of MMPs and cytokines.

The gelatinases are thought to be especially important in plaque instability, as a large number of studies have shown consistently raised levels in unstable plaques. The cap of the plaque is composed of fibrillar collagens that can only be degraded by collagenases, and as such they too are of particular interest.

3.4.1 Gelatinases

MMP-2 and MMP-9 are the 2 largest MMPs due to the triple repeat of fibronectin sequences (and in the case of MMP-9, a collagen type V-like sequence) present in the catalytic domain. These sequences aid in the binding of gelatins (Banyai et al, 1994) and collagens (Banyai & Patthy, 1991).

MMP-2 is the only MMP confirmed to be present in non-diseased human arteries (Galis et al, 1994). In the normal artery, endothelial cells and smooth muscle cells produce this enzyme constitutively along with TIMP-1 and TIMP-2, although MMP-2 activity is undetectable, suggesting tight control of proteolysis (Galis et al, 1994).

High concentrations of MMP-2 can degrade type I, II & III collagen in an in vitro environment devoid of TIMPs (Aimes & Quigley, 1995). However, it is extremely likely that in vivo, only the interstitial collagenases (MMP-1, MMP-8 & MMP-13) are capable of degrading these fibrillar collagens. The gelatinases degrade gelatins (denatured collagens) and by removing the end products of this biochemical reaction are able to influence the rate at which it takes place.

It is difficult to determine the role that each individual MMP plays and to predict what would happen if a particular MMP was absent. Until recently, no pathology secondary to
an MMP gene mutation had been identified. However, the first such disease was described in 2001 – multicentric osteolysis is a rare autosomal recessive disorder caused by the absence of active MMP-2 (Martignetti et al, 2001). It is not clear at present how this mutation results in increased destruction of affected bones.

MMP-2 is capable of degrading the basement membrane (composed of type IV collagen) and therefore, allowing smooth muscle cell migration from the media into the intima, during early plaque development. Animal models demonstrate that smooth muscle cells cultured on a gel of basement membrane proteins secrete MMP-2 and migrate through the basement membrane, a phenomenon that is prevented by the addition of antiserum against MMP-2 (Pauly et al, 1994).

Assumptions and hypotheses regarding the role of MMPs in vivo are generated by the correlation of a high MMP level with a particular situation. For example, Li et al (1996) found an increase in the level and expression of MMP-2 in human atherosclerotic aorta, compared to the non-diseased aortas of trauma victims removed at post-mortem. They also demonstrated that this MMP-2 activity colocalised with areas of macrophage infiltration. Davis et al (1998) found an increasing gradient of MMP-9 levels from normal aorta to occlusive aorta to aneurysmal aorta.

Early work by Vine & Powell (1991) suggested increased MMP-9 activity in aortas affected by occlusive disease compared to controls. Higher MMP-9 mRNA levels were found in atherosclerotic aortic tissue compared to controls by McMillan et al (1995). Thompson et al (1995) quantified MMP-9 using enzyme-linked immunosorbent assay (ELISA), and found concentrations 6 times higher in occlusive aortas compared to controls. Brown et al (1995) found increased immunostaining for MMP-9 in coronary atherectomy specimens removed from patients with unstable angina compared to those with the stable form of the disease. This study was the first to detect increased levels of an MMP in unstable plaques compared to stable ones, as defined by symptomatology.

Loftus et al (2000) investigated the levels and activity of MMP-9 in 75 carotid plaques defined as stable or unstable on the basis of symptomatology, cerebral embolisation and histological findings. Plaques retrieved from patients with carotid territory symptoms in the month prior to surgery, plaques causing pre-operative TCD-detected cerebral embolisation, and plaques demonstrating rupture on histological examination were all defined as
unstable. In each of these 3 categories the MMP-9 levels (as measured by ELISA) and activity (as measured by zymography) were significantly higher in the unstable plaques, suggesting a causative role. Immunohistochemistry confirmed the presence of MMP-9 within the plaque that localised to macrophages in the vulnerable shoulder region, and in situ hybridisation confirmed production within the plaque.

It is clear that genetic factors are likely to play a vital role in determining each individual’s vulnerability to atherosclerotic plaque instability. For example, a polymorphism (cytosine to thymidine transition) has been detected in the MMP-9 gene promoter region (Zhang et al, 1999). In a cohort of 374 post-MI men, a significant increase in this polymorphism was noted in those patients with a >50% stenosis in 3 or more vessels on angiography.

Kai et al (1998) reported elevated peripheral blood levels of MMP-2 and MMP-9 in patients with acute coronary syndromes. Loftus et al (2001) found elevated plasma MMP-9 levels in those patients undergoing spontaneous embolisation from unstable carotid plaques. There are 3 possible explanations for raised MMP-9 in the blood of patients with unstable atherosclerotic plaques. This could be a systemic manifestation of increased local synthesis within one or more plaques throughout the vascular tree (figure 3.4). Alternatively, the excess enzyme may be produced by circulating neutrophils and monocytes and released into the circulation (figure 3.5) - there is mounting evidence to suggest a causal role for systemic inflammation in plaque destabilisation (Libby, 2001b). The final option is that the extra MMP-9 is a result of inflammation secondary to end-organ damage caused by embolisation from the plaque (figure 3.6).
Figures 3.4 to 3.6 Possible sources of elevated plasma MMP-9
In 1962, Gross and Lapiere demonstrated that if the metamorphosing tail of a tadpole was placed on a collagen gel, it was capable of causing degradation of the gel. The constituent responsible for this action was described as “a collagenase” but was, in fact, probably a combination of more than one. MMP-1, MMP-8 and MMP-13 are capable of attacking the triple helix of the collagen molecule at a specific point three-quarters of the way along its length from the N-terminus end (Gross & Nagai, 1965). These 3 collagenases have varying specificities for each of the fibrillar collagens (Welgus et al, 1981; Hasty et al, 1987; Knauper et al, 1996), but the collagen fragments produced are then susceptible to further non-specific degradation by gelatinases.

Galis et al (1994) detected MMP-1 within the fibrous cap and shoulder of the plaque, along with other MMPs and TIMPs, and suggested a possible role in plaque destabilisation. Nikkari et al (1995) then demonstrated MMP-1 expression in carotid plaques that correlated with histological features of instability, such as intraplaque haemorrhage. The expression of MMP-1 is increased in areas of high circumferential stress within the fibrous cap (Lee et al, 1996) and understanding the interaction between physical forces and MMP expression may be crucial (Arroyo & Lee, 1999). Sukhova et al (1999) have shown increased levels of MMP-1 and MMP-13 in “atheromatous” vs “fibrous” plaques in areas that were infiltrated with macrophages and demonstrated collagenase-cleaved collagen.

MMP-8 was originally cloned from the blood of a patient suffering from chronic granulocytic leukaemia and so was historically referred to as “neutrophil collagenase” (Hasty et al, 1990). The action of this enzyme in various disease states including rheumatoid arthritis and periodontal disease has been investigated (Hanemaaijer et al, 1997; Lee et al, 1995), but a role in atherosclerosis had been overlooked until recently, as neutrophils are not a predominant cell type in atheroma. In 2001, evidence was provided that endothelial cells, smooth muscle cells and macrophages are also capable of MMP-8 expression, in addition to neutrophils (Herman et al, 2001a). This discovery led to the hypothesis that the enzyme may be involved in plaque remodelling and rupture.

46
3.4.3 Stromelysins

The substrate specificities of MMP-3 and MMP-10 are similar, but the former enzyme possesses a higher proteolytic efficiency. MMP-11 has different specificities, such that it is sometimes not even grouped with the other stomelysins. One of the most important actions of MMP-3 is in the activation of proMMP-1 – it is known as "collagenase activating protein" (Treadwell et al, 1986).

MMP-3 mRNA (Henney et al, 1991) and protein (Galis et al, 1994) has been identified in the shoulder regions of coronary plaques, colocalising with macrophages. In vitro work has suggested that a common polymorphism of the MMP-3 promoter region results in 2-fold greater enzymatic activity, and therefore, the potential for greater matrix degradation (Ye et al, 1995; Ye et al, 1996). A case-control study then investigated the prevalence of this polymorphism, and found it to be significantly greater in patients with MI, compared to a control population (Terashima et al, 1999).

3.4.4 Matrilysins

MMP-7 and MMP-26 degrade ECM components, and process cell surface molecules (such as proTNF-α) that may influence inflammatory infiltrates in the plaque. Despite this, studies have not yet suggested a clear role in plaque instability.

3.4.5 Membrane-type MMPs

These enzymes are able to reside in the cell membrane due to the presence of a hydrophobic transmembrane domain, which gives them the ability to localise their actions to the leading edge of the cell. With the exception of MT4-MMP, all membrane type MMPs appear capable of activating proMMP-2 bound to the cell surface. MT1-MMP appears capable of degrading various ECM components, including type I, II & III collagen (Ohuchi et al, 1997).

In atheroma, MT1-MMP colocalises with macrophages and smooth muscle cells. In vitro studies have shown that stimulation of these cell types by proinflammatory mediators (IL-1α, TNF-α & oxidised-LDL) results in increased MT1-MMP production (Rajavashisth et al, 1999). Through its activation of proMMP-2, this pathway clearly has the potential to
increase matrix degradation, resulting in plaque instability. Much remains to be learnt about this MMP subgroup, including why MT3-MMP undergoes alternative splicing to produce enzymes of 2 different sizes (Matsumoto et al, 1997).

3.5 “Net” proteolytic activity

There is convincing evidence of increased MMP levels in atherosclerotic plaque instability, as documented above, and many groups now feel that MMPs are highly likely to play a causative role in the disease states with which they are associated (Shah & Galis, 2001). However, the argument as to whether raised MMP levels are “cause” or “effect” of plaque rupture is likely to persist until a large, well-designed, randomised controlled trial of an MMP inhibitor demonstrates atherosclerotic plaque stabilisation in conjunction with decreased proteolysis.

The mere presence of MMPs within plaques does not necessarily confer an increased risk of instability. However, a rise in proteolytic activity, or an imbalance between the levels of enzymes and their inhibitors, may lead to an overall increase in matrix degradation. There is difficulty in investigating this concept since the tissue extraction of MMPs prior to assay may cause activation, or separation from their substrates or inhibitors, all of which may prevent a true assessment of the in vivo state (Vine & Powell, 1991).

In general, most TIMPs are capable of inhibiting most active MMPs, albeit with considerable differences in binding affinities (eg, TIMP-1 & MMP-9; TIMP-2 & MMP-2). In addition, many assays detect TIMPs in the bound and free forms, meaning only a proportion of the TIMP detected is actually available for further inhibition. All these points make assessment of net proteolytic activity a problematic issue.

Despite this, there is evidence of discrepancy between the MMPs and their inhibitors in certain situations. Knox et al (1997) demonstrated an imbalance between MMPs & TIMPs in atherosclerotic aortic tissue compared to normal control aortas. On immunohistochemistry, atherosclerotic aorta demonstrated a marked increase in staining for MMP-1 & MMP-3 plus a smaller increase in TIMP-1. MMP-2 was also increased in the absence of an increase in TIMP-2. Using a novel in situ zymography method, they identified a net increase in proteolytic activity in the diseased aortic tissue. This semi-quantitative technique involved the incubation of frozen tissue sections with fluorescently
labelled substrate (areas of proteolysis appearing as dark lytic zones). Normal tissue failed to exhibit proteolytic activity, but atherosclerotic samples demonstrated gelatinolytic and caseinolytic activity on the luminal aspect.

As previously mentioned, Thompson et al (1995) used ELISA to show a six-fold increase in MMP-9 levels in atherocclusive disease of the aorta, compared to normal control specimens. In that study, there was no difference in the TIMP-1 levels seen between the groups. Also, the study by Loftus et al (2000) that revealed increased MMP-9 levels in unstable carotid plaques, did not show any difference in TIMP-1 levels. Both these studies therefore demonstrate raised levels of proteolytic enzymes in the absence of any corresponding increase in the levels of their inhibitors. This suggests increased proteolytic activity in the diseased tissue.

Sukhova et al (1999) have demonstrated significantly higher levels of MMP-1 & MMP-13 in atheromatous plaques compared to fibrous lesions. Importantly, this study also showed colocalisation of collagenase-cleaved collagen with macrophages expressing MMP-1 and MMP-13, suggesting increased collagenolysis.

Overexpression of TIMPs may well provide protection against plaque instability. Some studies have shown higher levels of TIMPs in atheroma, compared to non-diseased tissue – for example, Fabunmi et al (1998) demonstrated five-fold higher TIMP-3 levels in plaques. As suggested by the authors of this study, this is probably a protective mechanism to compensate for the overexpression of MMPs in the vicinity (the TIMP-3 was found to colocalise with macrophages in the vulnerable areas of the plaque). Zaltsman et al (1999) demonstrated a 2 to 3-fold increase in TIMP-1 & TIMP-2 in animal model atherosclerotic lesions. Again they suggested this rise was to partially counterbalance increased MMP activity – in situ zymography demonstrated excess proteolytic activity, but in the areas of raised TIMP production this was less apparent.

3.6 Summary

Acute plaque disruption precedes the onset of clinical ischaemic syndromes. Exposure of the highly thrombogenic core to luminal blood results in platelet adherence and thrombosis, resulting in acute coronary syndromes and stroke. Plaque rupture is caused by excessive matrix degradation – the primary regulators of this process are the MMPs.
Convincing evidence exists that increased MMP levels and activity are present within unstable plaques, resulting in net proteolysis, although much work remains to be done in identifying the precise MMPs involved.
Chapter 4: The role of COX-2, cytokines & inflammatory markers

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>52</td>
</tr>
<tr>
<td>4.2 COX-2</td>
<td>52</td>
</tr>
<tr>
<td>4.3 Cytokines &amp; inflammatory markers</td>
<td>53</td>
</tr>
<tr>
<td>4.4 Summary</td>
<td>55</td>
</tr>
</tbody>
</table>
4.1 Introduction

Atherosclerotic plaque instability is strongly associated with up-regulation of inflammatory pathways (Ross, 1999). While the precise mechanisms involved are complex, and incompletely understood, it is suggested that the end result is an increase in matrix-degrading potential. Numerous bioactive molecules are hypothesised to play a role, including the inducible enzyme COX-2, the proinflammatory cytokines IL-1β, IL-6 & TNF-α, and the inflammatory marker CRP.

4.2 COX-2

The cyclooxygenase (COX) enzyme has 2 isoforms, each being the product of a distinct gene (Williams & Dubois, 1996). Activity of the 2 COX isoenzymes within cells, as well as the differential expression of downstream tissue-specific synthases, is essential for the generation of prostaglandins. COX-1 is constitutively expressed throughout the body and controls the production of background homeostatic prostaglandins, whereas COX-2 is inducible and plays an important role in the inflammatory response. Expression of COX-2 is low in most tissues, but can be increased by a variety of mediators (Rimarachin et al, 1994).

Atheromatous arteries express COX-2 (Stemme et al, 2000), although it is undetectable in normal vessels (Schonbeck et al, 1999). Activation of PGE$_2$-dependent pathways result in increased production of MMPs by macrophages (Cipollone et al, 2001; Corcoran et al, 1994), and decreased production of collagen by vascular SMCs (Fitzsimmons et al, 1999). COX-2 is capable of activating this pathway, and in addition, has been shown to colocalise with MMP-9 and macrophages in atherosclerotic lesions (Hong et al, 2000). Some studies have suggested increased COX-2 levels in symptomatic carotid plaques and have postulated an aetiological role in plaque rupture (Cipollone et al, 2001; Wijeyaratne et al, 2001).

Since the advent of highly selective COX-2 inhibitors, the role of COX-2 in atherosclerotic plaque instability has been the focus of much attention. There are conflicting reports relating to the effects of COX-2 on the vasculature. The Vioxx Gastrointestinal Outcomes
Research trial (VIGOR) compared the COX-2 inhibitor rofecoxib to naproxen in rheumatoid arthritis patients, and showed a 5-fold increase in cardiovascular events in the rofecoxib group (Bombardier et al, 2000). In such a large trial (8076 patients) these findings raised concerns about the use of this drug in patients with atherosclerotic risk factors, and have been the subject of much debate since (Mukherjee et al, 2001; Pitt et al, 2002). These results could potentially be explained by inhibition of prostacyclin (PGI\(_2\)) formation without a corresponding inhibition of thromboxane A\(_2\) (TXA\(_2\)) by the COX-2 inhibitor. Unopposed TXA\(_2\) increases platelet activation, adhesion, and aggregation with obvious potential for prothrombotic activity.

However, meloxicam (another COX-2 inhibitor) has recently been associated with a significant reduction in adverse outcomes in acute coronary syndrome patients (Altman et al, 2002). In this pilot study, meloxicam was given in addition to the usual combination of aspirin and heparin, and the authors propose that a combination of a COX-2 inhibitor and aspirin (a COX-1 inhibitor) may be cardioprotective. Interestingly, the concomitant use of aspirin was permitted in the Celecoxib Long-term Arthritis Safety Study (CLASS), which did not show an increase in cardiovascular events in the celecoxib group (Silverstein et al, 2000). In contrast, there was a subgroup of patients in VIGOR that were not given aspirin despite having definite indications for cardiovascular event prophylaxis (Bombardier et al, 2000).

A recent double blind, placebo-controlled crossover study has suggested that COX-2 inhibitors may exert a beneficial effect on endothelial function (Chenevard et al, 2003). In this small study, patients with coronary artery disease (on background aspirin therapy) given celecoxib demonstrated improved endothelium-dependent vasodilatation and reduced low-grade chronic inflammation and oxidative stress.

### 4.3 Cytokines & inflammatory markers

Cytokines are mediators of inflammation and immunity. It is not surprising, therefore, that they play a crucial role in atherosclerosis (Young et al, 2002). These low molecular weight glycoproteins usually act via autocrine or paracrine routes. Cytokines can be broadly divided into pro-inflammatory (eg, IL-1\(\beta\), IL-6 & TNF-\(\alpha\)) and anti-inflammatory subtypes (eg, IL-10), and act as part of a complex inter-related network. For this reason, the result of inhibiting individual cytokines is difficult to predict.
Cytokines modulate the production of other molecules that regulate vascular tone. In particular, they control the production of prostaglandins by regulating the activity of the cyclooxygenase enzymes, especially the inducible COX-2 (Cao et al, 1997). Numerous agents and conditions may upregulate the expression of cytokines from the endothelium, such as haemodynamic shear stress, the physical effects of hypertension, oxidised-LDL, and possibly infectious organisms. The resulting increased expression of adhesion molecules and chemokines is responsible for the initiation of atherosclerosis in the early stages, and the propagation of chronic inflammation in the diseased vessel later on.

Cytokines can influence the level of collagen found in atheroma both directly and indirectly. IL-1β has a modest effect on vascular SMC collagen gene expression, causing increased production of type I and type III collagen (Amento et al, 1991). This cytokine, however, has a much more significant ability to upregulate the transcription of collagen-degrading MMPs (Galis et al, 1995). The overall effect, therefore, is one of net collagen loss.

As atherosclerotic plaques are less readily available than blood, most studies have focussed on peripheral cytokine levels in plasma or serum (Lind, 2003). Plasma IL-6 concentrations are elevated in patients with myocardial infarction (Miyao et al, 1993) or unstable angina (Biasucci, 1996), compared to either stable angina or healthy subjects. More interestingly, plasma IL-6 levels can predict the future risk of MI among apparently healthy men (Ridker et al, 2000b).

C-reactive protein, the hepatic by-product of IL-6, activates endothelium and accumulates in the plaque, suggesting an important role in plaque inflammation and destabilisation (Pasceri & Yeh, 2000; Verma et al, 2002). The evidence that CRP levels can accurately predict an adverse cardiovascular event is strong (Ridker et al, 1997; Ridker et al, 2000a), and recent studies suggest it may even be more predictive than LDL (Ridker et al, 2002).
4.4 Summary

Atherosclerosis is an inflammatory disease, and more specifically, there is mounting evidence to suggest a causal role for cytokines in plaque instability. At present, there are numerous inflammatory markers that can be used to generate an individual patient risk assessment profile. In the future, the possibility that cytokine levels could be manipulated to bring about plaque stabilisation is an exciting prospect.
Chapter 5: Pharmacotherapy aimed at plaque stabilisation

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Introduction</td>
<td>57</td>
</tr>
<tr>
<td>5.2 HMG-CoA reductase inhibitors</td>
<td>58</td>
</tr>
<tr>
<td>5.3 TIMPs</td>
<td>61</td>
</tr>
<tr>
<td>5.4 Synthetic MMP inhibitors</td>
<td>62</td>
</tr>
<tr>
<td>5.5 Doxycycline</td>
<td>63</td>
</tr>
<tr>
<td>5.6 ACE inhibitors</td>
<td>64</td>
</tr>
<tr>
<td>5.7 Summary</td>
<td>64</td>
</tr>
</tbody>
</table>
5.1 Introduction

There are several potential mechanisms for inducing plaque stabilisation, such as:

- passivation of the endothelium
- reduction of LDL deposition
- modification of LDL oxidation
- prevention of collagen degradation
- inhibition of blood coagulation.

There are 5 main groups of therapeutic agents to be discussed:

- HMG-CoA reductase inhibitors
- TIMPs
- synthetic MMP inhibitors
- doxycyline
- ACE inhibitors.

These agents have a variety of the effects listed above, but the one action common to all of them is the prevention of collagen degradation (by MMP inhibition). The HMG-CoA reductase inhibitors have justifiably generated the most recent interest and appear to prevent acute coronary syndromes and strokes by stabilising atherosclerotic plaques through multiple actions.
5.2 HMG-CoA reductase inhibitors

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitors, or statins, are well known for their lipid lowering action. They are the most effective group of therapeutic agents for lowering LDL and raising HDL levels. However, recent evidence suggests that they are also capable of decreasing cardiovascular events even in those patients with normal cholesterol levels.

The Oxford Heart Protection Study (Heart Protection Study Collaborative Group, 2002) was a randomised controlled trial of simvastatin vs placebo in 20,536 individuals at high-risk of cardiovascular disease. Coronary death rate and other vascular events were significantly reduced in the simvastatin groups, even in patients with lipid levels below currently recommended targets (<5mmol/l total cholesterol and <3mmol/l LDL-cholesterol).

In the lipid lowering arm of ASCOT, the Anglo-Scandinavian Cardiac Outcomes Trial (Sever et al, 2003), 10,305 individuals with total cholesterol levels <6.5mmol/l were randomised to either atorvastatin or placebo. The trial was stopped for ethical reasons, 1.7 years before the planned 5-year follow-up target was reached, as there were significantly fewer cardiovascular events in the atorvastatin group.

The observed benefits of statins in these clinical trials is probably a combination of lipid lowering below levels previously considered to be normal, and additional lipid-independent plaque-stabilising actions (in particular MMP inhibition). Dietary lipid lowering alone reduces macrophage infiltration and MMP-1, -2, -3 & -9 activity in tandem with increasing collagen content and causing accumulation of mature smooth muscle cells, in rabbit aortas (Aikawa et al, 1998a, Aikawa et al, 1998b).

Statins have "direct" anti-proteolytic actions (independent of their ability to lower cholesterol), which contribute to increased plaque stability (Libby & Aikawa, 2003). In vitro studies have shown that statins reduce the secretion of MMPs. Bellosta et al (1998) demonstrated that the addition of fluvastatin or simvastatin decreased MMP-9 secretion from cultured macrophages, an effect that is also seen when statins are added to SMCs in culture (Porter et al, 2002). Ikeda et al (2000) revealed that fluvastatin decreased MMP-1 expression in cultured human vascular endothelial cells, while Wong et al (2001)
demonstrated that simvastatin inhibited MMP-9 secretion from cultured human monocytes. More recently, cerivastatin, simvastatin and lovastatin were all shown to be capable of decreasing MMP-1, -2, -3 & -9 secretion from vascular smooth muscle cells and macrophages (Luan et al, 2003).

It appears that the ability of statins to reduce MMP secretion is dependent on isoprenoid, and not cholesterol, depletion. When various intermediate metabolites in the cholesterol synthesis pathway were added, it was discovered that mevalonate and geranylgeranylpyrophosphate (GGPP), but not squalene, reversed the effects of the statin (Ikeda et al, 2000; Luan et al, 2003; Wong et al, 2001). Figure 5.1 explains the significance of this finding, using a simplified version of the cholesterol synthesis pathway.

**Figure 5.1** The addition of an HMG-CoA reductase inhibitor blocks the production of mevalonate and its downstream metabolites. In vitro studies suggest this decreases MMP secretion, an effect that can be reversed by the addition of mevalonate or GGPP, but not squalene. It is clear therefore, that it is the reduction of isoprenoids, such as GGPP (and not cholesterol), that is responsible for the corresponding decrease in MMPs.
A small number of in vivo studies have investigated the effect of statin therapy on MMPs. Crisby et al (2001) performed a small, non-randomised, non-blinded study in which 11 patients were given 3 months of pravastatin prior to scheduled carotid endarterectomy. Their plaques were compared to those retrieved from 13 control patients who were not on a statin, and showed increased collagen content and decreased lipid content. Lower MMP-2 levels (measured semi-quantitatively by immunohistochemistry) were also seen in the treated plaques, although no difference was demonstrated with the other MMPs measured (MMP-1 & -9). Cipollone et al (2003) performed a larger (n=128) study in which patients were randomised to simvastatin plus diet, or diet only, for 4 months prior to carotid endarterectomy. Their MMP quantification techniques included immunohistochemistry, Western blotting and zymography. This study demonstrated reduced MMP-2 and MMP-9 levels in the plaques retrieved from simvastatin-treated patients.

Nitric oxide synthase activity is increased by statins (Endres et al, 1998), thereby encouraging endothelial passivation. Nitric oxide causes vasodilatation, inhibition of SMC proliferation and inhibition of platelet aggregation, and has widespread anti-inflammatory and anti-oxidant properties. Therefore, increased bioavailability of this molecule would appear to be beneficial. Numerous studies have suggested that treatment with HMG-CoA reductase inhibitors results in improved endothelial functioning (John et al, 1998; O’Driscoll et al, 1997; Treasure et al, 1995). A recent rat model study demonstrated that pravastatin and cerivastatin increased endothelial nitric oxide synthase activity (Ni et al, 2001). The interesting point about this study was that, in the particular model used, statin treatment did not affect serum lipid levels, and so the actions observed were “lipid-independent”. In addition, HMG-CoA reductase inhibitors appear to inhibit the important plaque-destabilising action of LDL oxidation (Giroma et al, 1999; Giroux et al, 1993; Kleinveld et al, 1993; Palomaki et al, 1999).

Systemic inflammation (most commonly assessed by serum CRP measurement) is suggested as a cause of plaque destabilisation (Libby, 2001b). Statins have been shown to reduce CRP levels in patients with and without ischaemic heart disease (Jialal et al, 2001; Ridker et al, 1998; Ridker et al, 1999b; Ridker et al, 2001). Studies have also demonstrated the ability of statins to lower IL-6 (the precursor of CRP and a key molecule in inflammation) in vitro (Ito et al, 2002; Rezaie-Majd et al, 2002). In addition, the expression of cell adhesion molecules is inhibited by statins (Shovman et al, 2002), preventing adherence of monocytes to the endothelium.
5.3 TIMPs

MMP inhibition aimed at plaque stabilisation aims to redress the imbalance between enzymes and inhibitors, which causes excessive tissue degradation. One possible option is to augment the level of TIMPs, either by the exogenous administration of recombinant TIMPs or by stimulating local production through gene therapy.

Animal and experimental models have suggested that adenovirus-mediated overexpression of TIMPs has a potential therapeutic application in numerous disease processes. The overexpression of TIMP-3 inhibits invasion and promotes apoptosis of melanoma cells (Ahonen et al, 1998), while TIMP-1 overexpression reduces joint damage in a murine rheumatoid arthritis model (Schett et al, 2001).

The majority of TIMP work in vascular disease has focussed on the potential for prevention of neointimal formation and SMC migration, in animal or human saphenous vein models (Cheng et al, 1998; Dollery et al, 1999; George et al, 1998; George et al, 2000; Hu et al, 2001). Promising results have been obtained in these vein graft experiments with overexpression of TIMPs leading to decreased vascular remodelling, a finding that suggests possible benefits to the in vivo arterial system. The lack of a suitable animal model of plaque rupture (Rekhter, 2002; Cullen et al, 2003) has preventing testing the hypothesis that overexpression of TIMPs would result in plaque stabilisation.

Increased TIMP-1 levels in animal models have been shown to reduce plaque development (Rouis et al, 1999). Mice subjected to adenovirus-mediated overexpression of TIMP-1, demonstrate a significant decrease in the size of atherosclerotic lesions. The plaques also appear more stable by virtue of higher collagen, elastin and smooth muscle content, as well as lowered MMP-2, -3 & -13 levels and a significant decrease in macrophage infiltration.

The extrapolation of these data to potential applications in humans is problematic. The major drawback associated with TIMP therapy would be tissue delivery, since exogenous products would be metabolised and denatured with minimal tissue penetration at the intended site of action. Systemic stimulation of TIMPs would almost certainly have significant side effects precluding clinical use. Therefore, treatment would have to take the form of local tissue delivery or gene therapy. Clearly either system would be very
expensive to develop, so more interest has concentrated on the development of synthetic MMP inhibitors.

5.4 Synthetic MMP inhibitors

Synthetic peptides work by binding to the zinc ion at the active site of the MMP, thus preventing cleavage of substrate collagen molecules (Schwartz et al, 1991). Batimastat showed promise in decreasing tumour development and metastasis in animal models (Watson et al, 1996) and limiting aneurysm expansion in experimental models (Bigatet, 1999), but is not available in an oral form.

Marimastat, which is available orally, was shown to limit intimal hyperplasia in vein graft models (Porter et al, 1998) and elastin degradation in aneurysm models (Treharne et al, 1999). Some recent marimastat human cancer trials have shown promise (Groves et al, 2002; Bramhall et al, 2002a), although others have demonstrated no significant survival benefit (Miller et al, 2002; Bramhall et al, 2002b; Shepherd et al, 2002). Significant musculoskeletal side effects are consistently reported in these trials and affect approximately 30% of patients, with a corresponding negative impact on quality of life (Shepherd et al, 2002). However, in a randomised controlled trial of adjuvant marimastat therapy in patients with inoperable hepatic colorectal metastases, a significant survival benefit was seen, but only in those patients who suffered musculoskeletal side effects (King et al, 2003). Interestingly, the authors proposed that these adverse symptoms suggested biological evidence of a tissue effect, with resulting importance for dose optimisation of the drug.

Recent cancer studies of MMI270, a specific inhibitor (of MMP-2, MMP-8 & MMP-9), have shown a similar side effect profile, with 39 patients out of a total of 92 suffering from significant arthralgia and/or myalgia, in addition to 18 patients developing a maculopapular rash (Levitt et al, 2001). These side effects reflect the widespread role played by MMPs in normal physiological function. For the time-being at least, they would appear to exclude their use in a trial of patients at risk of plaque rupture.
5.5 Doxycycline

Doxycycline, a member of the tetracycline antibiotic family, is also a non-selective MMP inhibitor (Greenwald et al, 1998), with a proven safety profile. Clinical trials have shown that doxycycline is capable of decreasing cartilage MMP levels when given to patients prior to hip surgery (Smith et al, 1998b). It has also been shown to limit intimal hyperplasia in vitro (Loftus et al, 1999) and aneurysm expansion in vivo (Petrinec et al, 1996), by reducing MMP-9 activity.

Doxycycline prevents aneurysm formation and lowers MMP-9 levels in rat models (Curci et al, 1998; Petrinec et al, 1996), although concerns were raised that the aortic wall concentration that could be achieved in humans may be insufficient for any effect (Franklin et al, 1999). However, Prall et al (2002) used varying doxycycline doses in rats, and found that a serum concentration equivalent to that achievable in humans, still prevented AAA formation, albeit not as effectively as the higher doses. Doxycycline also reduces aortic wall MMP-9 concentrations when given orally to patients for 7 days prior to AAA repair (Curci et al, 2000).

A randomised clinical trial of doxycycline therapy in small asymptomatic aneurysms was proposed by Thompson & Baxter (1999) and the phase II results addressing issues of compliance, side effects and safety were published recently (Baxter et al, 2002). Completion of the 6-month course was achieved in 92% of patients, with an acceptable side-effect profile, and plasma MMP-9 levels were significantly reduced compared to baseline measurements. The full trial results of the effect on aneurysm expansion are eagerly anticipated.

A randomised clinical trial of doxycycline vs placebo in patients prior to carotid endarterectomy demonstrated decreased MMP-1 levels in the retrieved plaque and acceptable doxycycline penetration (Axisa et al, 2002). Disappointingly, no difference in any of the other MMPs studied was seen – in particular MMP-9. However, a tendency towards reduced levels of pre-operative carotid occlusion was observed in the treated group, suggesting a potential clinical benefit.
5.6 ACE inhibitors

ACE inhibitors decrease cardiovascular events (Fox, 2003; Turnbull, 2003). This is largely achieved by blood pressure control, but evidence exists for other beneficial effects, such as MMP inhibition (Sorbi et al, 1993; Reinhardt et al, 2002), endothelial passivation (Varin et al, 2000), antithrombotic (Vaughan et al, 1997), and anti-inflammatory actions (Di Napoli & Papa, 2003).

Angiotensin II is associated with the progression of coronary artery disease (Ohishi et al, 1997) and promotes endothelial activation (Tummala et al, 1999). It is likely that one of the mechanisms via which ACE inhibitors exert their effects is endothelial passivation (leading to reduced cell adhesion molecule expression and macrophage infiltration). ACE inhibition increases the bioavailability of nitric oxide and performs other regulatory processes in the ECM (Hornig et al, 2001; Varin et al, 2000). Irbesartan (an angiotensin II receptor blocker) can decrease plasma levels of VCAM-1, TNF-α and superoxide resulting in a more stable endothelium (Navalkar et al, 2001).

The ACE inhibitor trandalopril, and the experimental angiotensin II receptor antagonist HR720, decreased the area of atherosclerotic lesions in the thoracic aorta of cholesterol-fed monkeys (Miyazaki et al, 1999). This was achieved without alteration of mean blood pressure or cholesterol levels. The Heart Outcomes Prevention Evaluation (HOPE) study demonstrated a decrease in cardiovascular events in high-risk patients given ramipril as opposed to placebo (Yusuf et al, 2000), an effect that could only be partly explained by the modest decrease in mean blood pressure seen between the 2 groups (3/2mmHg).

5.7 Summary

Statins and ACE inhibitors, which are widely used to lower lipid levels and blood pressure respectively, also have plaque-stabilising properties. Despite the reduction in cardiovascular event risk, people on these medications still undergo disruption of atherosclerotic plaques, so further progress must be made. Of the other therapeutic avenues available, TIMP administration is limited by methods of delivery and the synthetic MMP inhibitors have a side effect profile that presently make them unacceptable for non-cancer studies. Doxycycline shows great promise and if ongoing clinical trials demonstrate reduced
growth of small AAAs, it would seem prudent to then study their use in plaque stabilisation.
Section II: Methods
Chapter 6: Patients & processing of samples

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Aims &amp; scope of thesis</td>
<td>68</td>
</tr>
<tr>
<td>6.2 Ethics</td>
<td>69</td>
</tr>
<tr>
<td>6.3 Patients</td>
<td>69</td>
</tr>
<tr>
<td>6.4 Symptomatology</td>
<td>70</td>
</tr>
<tr>
<td>6.5 Cerebral embolisation</td>
<td>71</td>
</tr>
<tr>
<td>6.6 Collection of samples</td>
<td>72</td>
</tr>
</tbody>
</table>
6.1 Aims & scope of thesis

The burden of cardiovascular disease has been outlined in Chapter 1 of this thesis. It is the main cause of mortality and morbidity in the UK and most of the developed world. In Chapter 2, I identified plaque rupture as the underlying aetiology in most cases of acute coronary syndrome and stroke. There is convincing evidence that an imbalance exists between MMP levels and their inhibitors resulting in increased proteolysis that may be responsible for plaque destabilisation. Present knowledge regarding the role of collagenases and other MMPs, COX-2, cytokines and inflammatory markers in this process was discussed in Chapters 3 and 4.

In this thesis I will quantify the plaque concentrations of the collagenolytic MMPs and their inhibitors in stable and unstable carotid plaques, in an attempt to define the pharmacological profile required for inhibitors with potential to cause plaque stabilisation. The relationship between statin therapy and both clinical and biochemical atherosclerotic plaque instability will be examined. I will also measure COX-2 activity in atherosclerotic plaques and relate these results to features of instability, in order to investigate the potential for plaque stabilisation by COX-2 inhibitors. Plasma MMP-9 will be quantified both pre- and post-operatively, in order to ascertain any change due to instability or the procedure of carotid endarterectomy.

The null hypotheses for this thesis are as follows:

- Collagenase levels are the same in both stable and unstable plaques
- Statin therapy has no effect on clinical or biochemical atherosclerotic plaque instability
- COX-2 activity is not related to plaque instability
- Pre- and post-operative plasma MMP-9 levels are the same.
6.2 Ethics

Prior to study initiation, detailed proposals were submitted to, and approved by, the Leicestershire Research Ethics Committee. The main ethical considerations related to the confidentiality of patient data, the procurement of 2 venous blood samples and the handling of the plaque retrieved at carotid endarterectomy. All patients gave fully informed written consent.

A large amount of patient information was recorded. All paper records were kept in locked offices within the hospital site, and all computerised records were protected by passwords. Once the relevant information was collected, each patient was given a unique identification number for the purpose of future data analysis. Patients were reassured that they would not be identified by any publications arising from the study, including this thesis.

Venous blood samples were obtained from a peripheral vein on the morning of surgery and on the morning of the second post-operative day by a qualified person (usually myself). Two patients withdrew their consent prior to the post-operative blood sample citing a needle phobia, but were happy to allow the analysis of their carotid plaque. No other patients withdrew their consent, and all patients asked to participate in the study accepted the invitation.

6.3 Patients

The total number of patients entered into the study was 159. Consecutive patients were recruited from September 1996 to July 1997 (n=84), and from September 2001 to July 2002 (n=75). All patients entered into the study underwent carotid endarterectomy at a single vascular unit (the Leicester Royal Infirmary). Patients listed for this procedure had at least a 70% stenosis of the internal carotid artery on the operated side, as determined by duplex. This investigation was repeated on the day prior to surgery (to ensure occlusion had not occurred), and this up-to-date reading was recorded for analysis purposes. Patients had ipsilateral carotid territory symptoms or, if asymptomatic, had severe bilateral stenoses or were enrolled in the Asymptomatic Carotid Surgery Trial (ACST).

Some of the data relating to the first 75 of the 84 patients entered between September 1996 & July 1997 has been previously published (Loftus et al, 2000; Loftus et al, 2001). Direct
training was provided to me from the research staff responsible for this group of patients (Mr IM Loftus and Mr S Goodall), to ensure that sample collection and laboratory tests were carried out in an identical manner on patients from both groups.

Small plaque size meant that only a limited amount of tissue was obtained in 22 cases. This resulted in insufficient plaque homogenate for the quantification of cytokines and the measurement of COX-2 activity. The total number of patients in these sections of the study is therefore quoted as 137.

In the first group of 84 patients only pre-operative blood samples were collected, and the results have been published (Loftus et al, 2001). On the basis of the results obtained in this group, it was decided to collect a post-operative sample also. This thesis will present the peripheral blood results from the second group only (n=75).

All patient information was collected pre-operatively and included age, sex, smoking history (past or present), use of medications (in particular aspirin and statins), and presence of IHD (history of MI, or angina requiring medication), HTN (hypertension requiring medication) or DM (diabetes requiring long-term intervention including dietary modification).

6.4 Symptomatology

A detailed clinical history was obtained from each patient with particular reference to carotid territory ischaemic events (amaurosis fugax, transient ischaemic attack, stroke or central retinal artery occlusion). The timing of the most recent symptom in relation to CEA was used for the purpose of dividing patients into groups for comparison (see below). It is now generally accepted that patients with no symptoms in the preceding 6 months should be classified as asymptomatic. In addition, those patients with vague symptoms such as dizziness and headaches were classified as asymptomatic in the absence of any other focal cerebral events.

Classifying plaques as stable or unstable on the basis of timing of symptoms in relation to surgery requires an arbitrary cut-off point, which will always be open to criticism. In the absence of a consensus definition for plaque instability, I divided patients into the following symptom groups for comparison and will present both throughout this thesis:
• Symptoms <6 months pre-operatively vs asymptomatic (or symptoms >6 months pre-operatively)

• Symptoms <1 month pre-operatively vs symptoms >1 month pre-operatively (or asymptomatic)

6.5 Cerebral embolisation

Transcranial Doppler (TCD) insonation of the middle cerebral artery (MCA) was attempted on all patients to detect embolisation from the carotid plaque. In a small number of patients the technique was impossible for anatomical reasons - they lacked a transcranial "window" - and their embolisation status was therefore not determined.

In summary, a probe is secured over the temporal region using a headband (as illustrated in figure 6.1) and focussed on the ipsilateral MCA (distal to the terminal ICA), usually to a depth of approximately 5 cm. The probe is attached to a TC22 transcranial Doppler monitor (SciMed, UK) that allows for visual and audio analysis of detected signals. A more detailed description of the methodology and interpretation of embolic signals has been published previously (Smith et al, 1995).

TCD monitoring was performed for a 30-minute period pre-operatively, as well as during the dissection phase of the operation. Although minimal disturbance of the carotid artery should occur during dissection of the carotid bifurcation, embolisation detected in this phase is not truly spontaneous. However, it has previously been shown to be a reliable marker of plaque instability (Gaunt et al, 1996a), along with truly spontaneous pre-operative embolisation. For this reason, one or more embolus occurring in either phase meant the patient was termed "emboli-positive" and compared to emboli-negative patients.

The numbers of emboli from each patient were recorded. For the purpose of this study, monitoring was discontinued after the application of clamps to the carotid artery, as any emboli occurring after this event are considered to be unrelated to plaque instability.
6.6 Collection of samples

A standard surgical protocol was followed for retrieval of the plaque by carotid endarterectomy. This involved dissection of the common carotid artery proximal to the carotid bulb, and of the internal carotid artery distal to the disease. This took place prior to clamping, but involved minimal dissection of the diseased segment. A qualified person not involved in the operation (usually myself) was available to process the plaque immediately. Therefore, the surgical team did not have to perform any additional procedures for the study.

Without delay, the plaque was divided exactly longitudinally through the point of maximum stenosis. Half was then placed in a cryovial (Sarstedt, Germany), “snap-frozen” in liquid nitrogen and stored at -80°C (see 7.1 for further processing), and half placed in a universal container holding freshly prepared 4% paraformaldehyde solution (see appendix A1) for 24 hours (see 7.7 for further processing).
Blood samples were obtained from a peripheral vein on the morning of surgery using the Monovette blood collection system (Sarstedt, Germany). A 4.7ml serum gel tube was used for CRP measurement and a 4ml EDTA tube for MMP quantification. On the morning of the second post-operative day, a further blood sample was collected into a 4ml EDTA tube for repeat MMP quantification.
## Chapter 7: Laboratory methodology

### Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1 Plaque homogenisation &amp; protein extraction</td>
<td>75</td>
</tr>
<tr>
<td>7.2 Protein standardisation</td>
<td>75</td>
</tr>
<tr>
<td>7.3 MMP &amp; TIMP quantification</td>
<td>76</td>
</tr>
<tr>
<td>7.3.1 MMP-1</td>
<td>77</td>
</tr>
<tr>
<td>7.3.2 MMP-8</td>
<td>79</td>
</tr>
<tr>
<td>7.3.3 MMP-9</td>
<td>80</td>
</tr>
<tr>
<td>7.3.4 MMP-13</td>
<td>81</td>
</tr>
<tr>
<td>7.3.5 TIMP-1</td>
<td>81</td>
</tr>
<tr>
<td>7.3.6 TIMP-2</td>
<td>82</td>
</tr>
<tr>
<td>7.4 Cytokine quantification</td>
<td>82</td>
</tr>
<tr>
<td>7.4.1 IL-1β</td>
<td>82</td>
</tr>
<tr>
<td>7.4.2 IL-6</td>
<td>82</td>
</tr>
<tr>
<td>7.4.3 IL-10</td>
<td>83</td>
</tr>
<tr>
<td>7.4.4 TNF-α</td>
<td>83</td>
</tr>
<tr>
<td>7.5 Tissue factor quantification</td>
<td>84</td>
</tr>
<tr>
<td>7.6 COX activity measurement</td>
<td>84</td>
</tr>
<tr>
<td>7.7 Histology</td>
<td>86</td>
</tr>
<tr>
<td>7.7.1 Routine staining</td>
<td>86</td>
</tr>
<tr>
<td>7.7.2 MMP-8 immunohistochemistry</td>
<td>87</td>
</tr>
<tr>
<td>7.7.3 Colocalisation studies</td>
<td>87</td>
</tr>
<tr>
<td>7.7.4 MMP-8 in situ hybridisation</td>
<td>89</td>
</tr>
</tbody>
</table>
7.1 Plaque homogenisation & protein extraction

Plaque tissue stored in a cryovial at -80°C was thawed on ice and gently rinsed with phosphate-buffered saline (see appendix A2) to remove any traces of luminal blood. The tissue was then weighed prior to being chopped up using a scalpel into pieces of approximately 1mm³ in size. These tiny portions were then added to a Du Pont tube (Du Pont, USA) containing a volume of homogenising buffer (see appendix A3) proportional to the weight of the tissue (1ml of buffer for each 0.1g of tissue). The tissue was then disrupted using an Ultra-turrax T25 homogeniser (IKA, Germany).

The resulting homogenate was centrifuged at 12000 rpm for 60 minutes at 4°C. The supernatant was then aspirated and added to viscing tubing (Fisher, USA), and the cellular debris at the base of the Du Pont tube was discarded. The tubing was then immersed in agitated dialysis buffer (see appendix A4) for 18 hours at 4°C, in order to retain molecules of >14000 kDa weight. This methodology has been demonstrated to extract all forms of enzyme whether bound to proteins in the ECM, dissolved in solution or entrapped in vesicles (Vine and Powell, 1991).

The solution retained in the viscing tubing was then aspirated into vials, which were “snap-frozen” in liquid nitrogen and stored at -80°C. A number of vials were used for each plaque homogenate, so that multiple future freeze-thaw cycles could be avoided. Homogenates were stored in this fashion until enough had been collected to perform batch analysis. Prior to use, each sample was defrosted on ice and sonicated to disrupt protein aggregations using a Soniprep 150 (Sanyo, UK) at 5 amplitude microns for 20 seconds (see figure 7.1).

7.2 Protein standardisation

Throughout the main text of this thesis I will present results in the format “weight of protein of interest (eg, MMP-1) expressed in terms of wet weight of plaque tissue”. However, in appendix B, I will present the results in a protein-standardised format – “weight of protein of interest expressed in terms of total plaque protein".
Figure 7.1 A volume of plaque homogenate undergoing sonication prior to MMP quantification. The purpose of this procedure is to disrupt protein aggregations that may otherwise result in artificially low protein readings.

The total protein concentration in each plaque homogenate was estimated by the dye binding technique. A protein assay dye reagent solution (Bio-Rad Laboratories, Germany) was added to a small volume of each sample (later discarded) and caused a variable alteration in the absorbance of light at 595nm. This change was produced by the binding of reagent solution to protein molecules, and was detected by a UV-visible spectrophotometer (Shimadzu Corporation, Japan). The concentration of protein in each sample is determined by interpolation from a standard curve constructed using known concentrations of bovine serum albumin (Sigma-Aldrich, USA).

7.3 MMP & TIMP quantification

MMP & TIMP concentrations in plaque homogenates were quantified using enzyme-linked immunosorbent assays (Amersham Biosciences, UK). In addition, the MMP-9 &
TIMP-1 ELISAs were used to measure the concentrations of these enzymes in plasma samples. "Biotrak" assay systems provide a specific and precise quantification of enzyme levels. All samples were assayed in duplicate and an average value taken. If there was a significant deviation between these 2 values, the results were discarded and the assay repeated.

7.3.1 MMP-1

This assay is based on a 2-site ELISA "sandwich" format, as shown in figure 7.2.

![MMP-1 ELISA](image)

**Figure 7.2 MMP-1 ELISA**

Standards and samples are incubated in microtitre wells precoated with a monoclonal anti-MMP-1 antibody. Any MMP-1 present will be bound to the wells, other components of the
sample being removed by washing. A primary antibody that recognises a different epitope of the MMP-1 protein is then added. Any excess is removed by washing.

The primary anti-MMP-1 antibody now bound to the wells is detected using a secondary antibody conjugated to horseradish peroxidase (HRP). This HRP enzyme catalyses the conversion of colourless tetramethylbenzidine (TMB) substrate to a coloured product. The amount of HRP (and therefore MMP-1) bound to each well is determined by the intensity of the colour produced when TMB substrate is added. The resultant colour is read at 450nm in a Wellscan automatic microplate reader (Denley Instruments, UK), after the reaction has been stopped by the addition of an acid solution (see figure 7.3). The concentration of MMP-1 in each sample is determined by interpolation from a standard curve.

Figure 7.3 The microtitre plate spectrophotometer detecting absorbances in each individual well of an ELISA, which can be seen in the front tray. A printout of the results is produced.
The assay recognises total human MMP-1 (ie, free or proMMP-1, and that complexed with inhibitors such as TIMP-1). There is no significant cross-reactivity with other MMPs.

7.3.2 MMP-8

On the basis of recent unpublished work within our department we were confident of demonstrating a significant difference in total MMP-8 levels between stable and unstable plaques. We therefore elected to use an activity ELISA that was capable of quantifying both total and active MMP-8 on the same assay system.

This ELISA uses the pro form of a detection enzyme (modified urokinase) that is transformed by captured active MMP-8 into an active detection enzyme, through a single proteolytic event (Verheijen et al, 1997). The natural activation sequence in the pro detection enzyme has been replaced using protein engineering, with an artificial sequence activated by active MMP-8. The activated detection enzyme can then be measured using a specific chromogenic peptide substrate (S-2444). The resultant colour is read at 405nm in a microtiter plate spectrophotometer. The concentration of active MMP-8 is determined by interpolation from a standard curve.

A diagrammatic representation of the kit format is shown in figure 7.4. Primary anti-MMP-8 antibody is immobilised on to microtiter wells precoated with a secondary antibody. Standards and samples are incubated in the plate and any MMP-8 present is bound to the immobilised antibody complex. Washing removes other components of the sample. Either endogenous levels of free active MMP-8 or total MMP-8 (active MMP-8 plus proMMP-8) can be detected. In order to measure the total MMP-8 content, all bound MMP-8 is converted to its active form by the addition of aminophenylmercuric acetate (APMA).

There is minimal cross-reactivity with other MMPs.
7.3.3 MMP-9

This ELISA works in a similar fashion to the 2-site “sandwich” design of the MMP-1 kit. As can be seen in figure 7.5, there is a slight difference in the format, with the primary antibody that detects bound MMP-9 already being conjugated to the HRP, meaning there is no necessity for a secondary antibody. The pro form of MMP-9 (including that bound to
TIMP-1) is detected by this assay and there is no significant cross-reactivity with other MMPs.

![MMP-9 ELISA diagram](image)

**Figure 7.5 MMP-9 ELISA**

### 7.3.4 MMP-13

This ELISA works in the same manner as that for MMP-9. It detects the pro and active form of MMP-13.

### 7.3.5 TIMP-1

This ELISA works in the same manner as that for MMP-9. It recognises total TIMP-1 (ie, free TIMP-1 and that complexed with MMPs). It does not cross-react with TIMP-2.
7.3.6 TIMP-2

This ELISA works in the same manner as that for MMP-9. It recognises free TIMP-2 and that complexed with the active form of MMPs. It does not cross-react with TIMP-1 or TIMP-3.

7.4 Cytokine quantification

Cytokine concentrations in plaque homogenates were also quantified using enzyme-linked immunosorbent assays (Biosource, USA). Again, all samples were assayed in duplicate and an average value taken. If there was a significant deviation between these 2 values, the results were discarded and the assay repeated.

7.4.1 IL-1β

The microtitre wells are coated with a specific antibody for human IL-1β. Standards or samples are added to the wells immediately followed by the addition of a biotinylated primary antibody. During this first incubation, the IL-1β binds simultaneously to the immobilised (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site.

Excess biotinylated antibody is removed by washing prior to the addition of streptavidin-peroxidase. This binds to the biotinylated antibody to complete the “sandwich”. After a further wash to remove unbound enzyme, the TMB substrate solution is added. The peroxidase enzyme then catalyses the conversion of TMB to a coloured solution, the intensity of which is detected at 450nm by the microtiter plate spectrophotometer. The assay format is summarised in figure 7.6.

7.4.2 IL-6

The methodology is identical to that for the IL-1β ELISA.
7.4.3 IL-10

The ultrasensitive version of this assay was used. The methodology is similar to the IL-1β ELISA, with the exception that IL-10 is captured by the immobilised antibody prior to a wash and separate incubation in which it binds to the primary biotinylated antibody. In the IL-1β ELISA these steps occur simultaneously.

7.4.4 TNF-α

The ultrasensitive version of this assay was used and the methodology is identical to that for the IL-1β ELISA.
7.5 Tissue factor quantification

Tissue factor concentrations in plaque homogenates were quantified using the "Imubind" ELISA (American Diagnostica, USA). This assay system uses the same format as that for the IL-1β ELISA (see figure 7.6).

7.6 COX activity measurement

Activity levels of COX and COX-2 in plaque homogenates were measured using a colorimetric assay (Cayman Chemical, USA) that works on the principles shown in figure 7.7. In summary, the colorimetric substrate, tetramethylphenylenediamine (TMPD) possesses reducing potential required for the conversion of arachidonic acid to prostaglandin H₂ by the COX enzyme. Activity is assayed by monitoring the appearance of oxidised-TMPD at 590nm. Potassium hydroxide is required to take the arachidonic acid into aqueous solution, and heme acts as an enzymatic co-factor.

For the purpose of this assay, each plaque homogenate was separated into 3 equal samples. The first sample was boiled at 100°C for 5 minutes (to destroy all COX activity) and this was used in the assay to determine a background value to be subtracted from subsequent readings on the same homogenate. The second sample was used to determine total COX activity. SC-560 (a potent and selective COX-1 inhibitor) was added to the third sample so that COX-2 activity alone could be determined. Each of these 3 samples was assayed in duplicate, and in the case of a significant deviation between the duplicate values, the results were discarded and the assay repeated.
Figure 7.7 The COX activity assay. Arachidonic acid is converted to PGH₂ by the COX enzyme present in the plaque homogenate. COX activity is assayed colorimetrically by monitoring the appearance of oxidised-TMPD at 590nm. A = background values are derived by boiling plaque homogenate for 5 mins (to destroy all COX activity) prior to assay. These are subtracted from the corresponding untreated and inhibitor-treated samples. B = COX-2 activity alone is determined by the addition of SC-560 (a potent and selective COX-1 inhibitor).

The formula used to calculate activity units is shown in figure 7.8. It incorporates a value of 5 minutes (the reaction time) and a value of 0.00826μM⁻¹ (the TMPD extinction coefficient, adjusted for path length of the well). "(0.21/0.01)" is an adjustment factor to account for the 0.21 ml of solution in each well only containing 0.01ml of plaque homogenate (the remainder is assay buffer, heme solution, arachidonic acid, potassium hydroxide, TMPD / SC-560).
COX activity = \[
\frac{\Delta A_{590}}{5\text{min}} \times \frac{0.21\text{ml}}{0.00826\mu\text{M}^{-1}} \times \frac{0.01\text{ml}}{2^*} \times 10^{**}
\]

* Conversion factor as it takes 2 molecules of TMPD to reduce arachidonic acid to PGH₂

** Conversion factor as 1ml of buffer was added for each 0.1g of plaque in the initial homogenisation process

Figure 7.8 Formula for calculation of COX activity units. Background value is subtracted from its corresponding untreated and inhibitor-treated samples to give the “corrected absorbances at 590nm” (ΔA₅₉₀). The reaction rate at 590nm is determined using the TMPD extinction coefficient of 0.00826μM⁻¹ (the actual coefficient for TMPD is adjusted for the pathlength of the solution in the well). ₀.₂₁/₀.₀₁ refers to the fact that only 0.01ml of the 0.21ml of solution is made up of plaque homogenate (the rest consists of assay buffer, heme solution, potassium hydroxide solution, arachidonic acid solution, colorimetric substrate, "/. SC-560).

7.7 Histology

7.7.1 Routine staining

Preservation of the structure and antigens within the plaque is essential prior to routine or immuno staining. After retrieval at endarterectomy, a portion of each sample was immersed in an excess of freshly prepared 4% paraformaldehyde overnight. This fixative fluid prevents autolysis by inactivating lysosomal enzymes and inhibits the growth of bacteria that could give rise to putrefactive changes. It also protects the tissue from the rigors of subsequent processing and staining. The impermeable barriers surrounding viable cells are partly broken down during fixation to allow large molecules (in particular the antibodies used during immunohistochemistry) to enter the cell. Although these actions obviously involve some conformational changes to the structure of proteins, these are limited by the relatively mild nature of the fixative chosen.
Once removed from the paraformaldehyde, samples were paraffin embedded, and 4μm longitudinal sections sliced as required. Sections from all plaques were stained with H&E (Haematoxylin and Eosin) and EVG (Elastic Van Gieson). Assessment of these slides was performed by an experienced histopathologist (Dr Louise Jones, Department of Histopathology, University of Leicester), who was blinded to the patients’ clinical and biochemical findings. In particular, sections were graded for 6 histological features associated with plaque instability (Carr et al, 1996):

- Plaque rupture
- Intraplaque haemorrhage
- Plaque cap thinning
- Intraplaque fibrin
- Plaque necrosis
- Cap foam cells

7.7.2 MMP-8 immunohistochemistry

Immunohistochemistry was performed in a selected group of specimens (4 from highly symptomatic patients, 2 from asymptomatic patients) using a monoclonal antibody (R&D Systems, UK) to identify the presence and location of MMP-8 protein in the carotid plaque. In-house optimisation of monoclonal antibody dilution, antigen retrieval techniques (to allow the exposure of hidden epitopes) and incubation periods resulted in the following protocol being devised:

- Incubate paraffin-embedded section at 60°C for 2mins to melt wax
- Complete dewaxing in xylene
- Rehydrate in graded ethanols to distilled water
- Perform antigen retrieval in trypsin solution (see appendix A5) at 37°C for 10mins
- Wash in PBS for 5mins
- Soak in 6% hydrogen peroxide (to block endogenous peroxidase) for 10mins
- Rinse in distilled water, then wash in running tap water for 2mins, then rinse further in distilled water
- Wash in PBS for 5mins
- Incubate with 100μl of 1:20 normal swine serum (Dako, USA) for 30mins (to prevent non-specific antibody binding)
- Without washing, add 100μl of 1:100 primary anti-MMP-8 antibody and incubate for 60mins
- Wash in PBS for 10mins
- Incubate with 1 drop of link antibody conjugated to HRP from ChemMate Envision bottle A (Dako, USA) for 25mins
- Wash in PBS for 10mins
- React with 200μl of diaminobenzidine (DAB) substrate solution (see appendix A6) for 10mins
- Rinse in distilled water to stop reaction, then wash in running tap water for 5mins
- Counterstain with haematoxylin for 25secs
- Wash in running tap water for 2mins
- Dehydrate in graded ethanol then xylene
- Mount section in xylene medium with a coverslip and observe under the microscope

Negative controls were performed using the same protocol with one alteration – 100μl of 1:20 normal swine serum was added instead of 100μl of 1:100 primary anti-MMP-8 antibody.

7.7.3 Colocalisation studies

Histological studies on serial sections were performed to identify the cell types that colocalised with MMP-8 protein. The particular cells of interest are neutrophils, macrophages, smooth muscle cells & leukocytes. The methodology was as documented in the above protocol, with the exception that primary antibodies directed against antigens on each of the above cell types (see table 7.1) were used rather than antibodies to MMP-8. In addition, the detection of neutrophils and SMCs was not enhanced by any of the antigen retrieval techniques, so in those cases the antigen retrieval stage was omitted.
Table 7.1 The primary antibodies used in my colocalisation studies

<table>
<thead>
<tr>
<th>Cell of interest</th>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Company</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>CD15</td>
<td>1:50</td>
<td>Becton Dickinson, USA</td>
<td>None</td>
</tr>
<tr>
<td>Macrophage</td>
<td>CD68</td>
<td>1:150</td>
<td>Dako, USA</td>
<td>Trypsin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle actin (SMA)</td>
<td>1:600</td>
<td>Dako, USA</td>
<td>None</td>
</tr>
<tr>
<td>Leukocyte</td>
<td>CD45, Leukocyte common antigen (LCA)</td>
<td>1:50</td>
<td>Dako, USA</td>
<td>Trypsin</td>
</tr>
</tbody>
</table>

7.7.4 MMP-8 in situ hybridisation

Immunohistochemistry is capable of confirming the presence of a protein in a histological site, but cannot confirm that the protein is being synthesised at that site. In situ hybridisation is used to identify the sequences of RNA that code for the protein. The technique relies on the re-annealing of complementary sequences of nucleic acid to confirm synthesis and localisation of the hybridising material.

A non-isotopic in situ hybridization technique was performed in a selected group of samples, with a digoxigenin-labelled oligonucleotide probe cocktail based on the published sequences shown below:

\[
\begin{align*}
5' & - tcgacagtctccgactctctttctcgt - 3' \\
5' & - cggaacgacagagggttgatacagaaagc - 3' \\
5' & - ttgtatgaagaaacatttactggttaagac - 3' \\
5' & - tctlgtactaaaccaatcttcctt - 3'
\end{align*}
\]

After optimisation, the following protocol was devised:

- Dewax paraffin-embedded section in xylene
- Rehydrate in graded ethanol to DEPC (diethylpyrocarbonate) water
- Incubate with 0.2ml of proteinase K (10\(\mu\)g/ml) for 60mins at 37\(^\circ\)C
• Wash in DEPC water twice (5mins each wash)
• Dehydrate in graded ethanols
• Add 120μl of hybridisation solution (see appendix A7) containing the labelled
  probe cocktail (see appendix A9)
• Apply coverslip
• Incubate for 15mins at 65°C followed by 2 hours at 37°C
• Remove coverslip
• Wash in 2X SSC/30% formamide at 37°C twice (10mins each wash)
• Wash in DEPC water for 30secs
• Incubate with 0.25ml of blocking solution (see appendix A10) for 5mins
• Incubate with anti-digoxigenin alkaline phosphatase (diluted 1:600 with blocking
  solution) for 60mins
• Wash in 1X TBS twice (5mins each)
• Incubate with 100μl of substrate buffer (see appendix A11) for 5mins
• Add 100μl of substrate solution (see appendix A12)
• Apply coverslip and leave to develop in a moist darkened chamber
• Check with microscope until maximal signal with minimal background develops
• Wash in tap water for 5mins
• Mount in aqueous mountant

The oligonucleotide sequences used in the probe cocktail are “antisense” (ie, they will bind
in a complementary fashion to the single-stranded MMP-8 mRNA). Negative controls
were performed using the same protocol with one alteration – “sense” probes (which will
not bind to MMP-8 mRNA) were used in the cocktail.
## Chapter 8: Statistical analysis

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1 Software</td>
<td>92</td>
</tr>
<tr>
<td>8.2 Groups for comparison</td>
<td>92</td>
</tr>
<tr>
<td>8.3 Discrete variables</td>
<td>92</td>
</tr>
<tr>
<td>8.4 Continuous variables</td>
<td>93</td>
</tr>
<tr>
<td>8.5 Significance</td>
<td>93</td>
</tr>
</tbody>
</table>
8.1 Software

Statistical analysis was performed using GraphPad Prism, Version 3.00 for Windows (GraphPad Software, USA).

8.2 Groups for comparison

In the absence of a consensus definition for plaque instability, patients were divided into the following groups for the purpose of comparison:

- Symptomatic vs asymptomatic. (It is generally accepted that patients with no symptoms in the previous 6 months should be considered as asymptomatic, and therefore this period is used as the cut-off point between the 2 groups.)

- Highly symptomatic vs the rest. (Patients with symptoms in the month prior to surgery are considered as highly symptomatic, and compared to a common group of asymptomatic patients and those with symptoms 1-6 months pre-operatively.)

- Emboli-positive vs emboli-negative. (Some patients lack a TCD window, and therefore the numbers in these 2 groups do not always add up to the total number in the study.)

- Histological feature-positive vs histological feature-negative. (Plaques were grouped for comparison according to the presence or absence of plaque rupture, intraplaque haemorrhage, plaque cap thinning, intraplaque fibrin, plaque necrosis & cap foam cells.)

8.3 Discrete variables

The discrete variables (sex, smoking, medication, ischaemic heart disease, hypertension, diabetes mellitus, symptom group, emboli group, histology group) are presented as actual numbers (and percentages). They are compared using Fisher’s exact test.
8.4 Continuous variables

Deviations from Gaussian (normal) distribution are assessed using the Kolmogorov-Smirnov (KS) test.

The following continuous variables are normally distributed and therefore analysed using parametric statistics:

- Age
- Tissue factor concentration
- COX and COX-2 activity

All are compared using the unpaired t-test. Age is presented as a mean value (and total range) whereas the other variables are presented as means (and standard deviations).

The following continuous variables are not normally distributed and are therefore analysed using non-parametric statistics:

- Degree of carotid stenosis
- Emboli numbers
- MMP, TIMP & cytokine concentrations

All are presented as median values (and total or interquartile ranges) and are compared using the Mann-Whitney U-test. Spearman’s correlation coefficient was used to assess for an association between plaque and plasma MMP-9 levels.

8.5 Significance

Numerous measurements were performed on each carotid plaque homogenate. In addition, each set of results was analysed multiply using 3 slightly different definitions of an unstable plaque (symptoms <6 months, symptoms <1 month and spontaneous embolisation). For these reasons, significance on the homogenate results was assumed at the p<0.01 level.
In contrast, only MMP-9 & TIMP-1 concentrations were measured in plasma samples. The only comparison performed was for levels in patients that were embolising vs levels in patients that were not. Therefore, significance on the plasma results was assumed at the p<0.05 level.

Significant p-values are underlined in the tables and the text.
Section III: Results
Chapter 9: Unstable carotid plaques exhibit raised MMP-8 activity

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1 Patient symptoms</td>
<td>97</td>
</tr>
<tr>
<td>9.2 Cerebral embolisation</td>
<td>97</td>
</tr>
<tr>
<td>9.3 Plaque histology</td>
<td>98</td>
</tr>
<tr>
<td>9.4 Plaque MMP &amp; TIMP concentrations</td>
<td>102</td>
</tr>
<tr>
<td>9.5 Immunohistochemistry &amp; colocalisation studies</td>
<td>108</td>
</tr>
<tr>
<td>9.6 In situ hybridisation</td>
<td>108</td>
</tr>
</tbody>
</table>
9.1 Patient symptoms

Out of 159 patients, 90 were symptomatic in the 6 months prior to surgery, and 30 of these were highly symptomatic (experiencing symptoms in the pre-operative month). There were no significant differences between the symptom groups in terms of age, atherosclerotic risk factors, severity of carotid stenosis or aspirin use (tables 9.1 & 9.2). Table 9.2 shows that highly symptomatic patients were significantly less likely to be on statin therapy than the remainder of the cohort (13% vs 41%; \( p=0.0053 \)). This is an important finding and will be expanded upon in chapter 12.

The types of symptoms experienced by those 90 patients who were symptomatic in the 6 months prior to surgery are listed here - TIAs (n=36), amaurosis fugax (n=21), stroke with recovery (n=13), stroke with residual deficit (n=9), central retinal artery occlusion (n=1), or a combination of the above (n=10).

9.2 Cerebral embolisation

Over 26% of patients had one or more embolus detected in the pre-operative or dissection phases. For emboli-positive patients only, the median number of emboli was 3 (range 1-16) in the symptomatic group and 2 (range 1-8) in the asymptomatic group.

Embolisation status was undefined in 11 patients (4 of these were symptomatic, 2 of whom had symptoms within the pre-operative month) due to the absence of a TCD window. Spontaneous particulate cerebral embolisation was significantly more likely in symptomatic vs asymptomatic patients (41% vs 11%; \( p<0.0001 \)), as shown in table 9.1. Highly symptomatic patients were particularly likely to be emboli-positive compared to the remainder of the group (71% vs 18%; \( p<0.0001 \)), as seen in table 9.2.

There were no significant differences in terms of age, atherosclerotic risk factors, severity of carotid stenosis or aspirin use upon comparison of the emboli groups (table 9.3). Emboli-positive patients were less likely to be on statin therapy (21% vs 42%; \( p=0.0234 \)) although this failed to reach significance at the \( p<0.01 \) level.
9.3 Plaque histology

Tables 9.1 & 9.2 show the presence or absence of plaque histological features related to symptom groups. The finding of rupture was significantly more likely in those plaques from highly symptomatic patients (37% vs 13%; *p*=0.0059) and showed a tendency to occur more frequently in plaques from symptomatic vs asymptomatic patients (23% vs 10%; *p*=0.0361). Intraplaque haemorrhage was also observed significantly more often in those plaques from highly symptomatic patients (47% vs 19%; *p*=0.0034) and tended to be detected more among the symptomatic as opposed to the asymptomatic group (30% vs 16%; *p*=0.0416).

Table 9.3 shows the histological findings in each of the emboli groups. No differences were seen.
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Symptoms &lt;6 months (n=90)</th>
<th>Asymptomatic (n=69)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>70.1 (52-86)</td>
<td>68.7 (49-86)</td>
<td>0.2413</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>64 (71)</td>
<td>43 (62)</td>
<td>0.3063</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>71 (79)</td>
<td>59 (86)</td>
<td>0.3082</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>36 (40)</td>
<td>32 (46)</td>
<td>0.5179</td>
</tr>
<tr>
<td>HTN (%)</td>
<td>54 (60)</td>
<td>50 (72)</td>
<td>0.1301</td>
</tr>
<tr>
<td>DM (%)</td>
<td>16 (18)</td>
<td>12 (17)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>80 (70-88)</td>
<td>80 (75-90)</td>
<td>0.6465</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>30 (33)</td>
<td>27 (39)</td>
<td>0.5059</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>78 (87)</td>
<td>58 (84)</td>
<td>0.6562</td>
</tr>
<tr>
<td>Embolisation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emboli-positive (%)</td>
<td>35 (41)</td>
<td>7 (11)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Emboli no. (range)</td>
<td>3 (1-16)</td>
<td>2 (1-8)</td>
<td>0.6241</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque rupture (%)</td>
<td>21 (23)</td>
<td>7 (10)</td>
<td>0.0361</td>
</tr>
<tr>
<td>IPH (%)</td>
<td>27 (30)</td>
<td>11 (16)</td>
<td>0.0416</td>
</tr>
<tr>
<td>Cap thinning (%)</td>
<td>59 (66)</td>
<td>40 (58)</td>
<td>0.4093</td>
</tr>
<tr>
<td>Intraplaque fibrin (%)</td>
<td>43 (48)</td>
<td>30 (43)</td>
<td>0.6321</td>
</tr>
<tr>
<td>Plaque necrosis (%)</td>
<td>50 (56)</td>
<td>29 (42)</td>
<td>0.1101</td>
</tr>
<tr>
<td>Cap foam cells (%)</td>
<td>48 (53)</td>
<td>29 (42)</td>
<td>0.2003</td>
</tr>
</tbody>
</table>

Table 9.1 Comparison of symptomatic vs asymptomatic groups, in terms of patient characteristics, spontaneous cerebral embolisation and plaque histological features
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Symptoms &lt;1 month (n=30)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=129)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>69.9 (57-84)</td>
<td>69.4 (49-86)</td>
<td>0.7442</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>22 (73)</td>
<td>85 (66)</td>
<td>0.5203</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>22 (73)</td>
<td>108 (84)</td>
<td>0.1953</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>15 (50)</td>
<td>53 (41)</td>
<td>0.4162</td>
</tr>
<tr>
<td>HTN (%)</td>
<td>14 (47)</td>
<td>90 (70)</td>
<td>0.0201</td>
</tr>
<tr>
<td>DM (%)</td>
<td>2 (7)</td>
<td>26 (20)</td>
<td>0.1098</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>80 (73-90)</td>
<td>80 (75-88)</td>
<td>0.5491</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>4 (13)</td>
<td>53 (41)</td>
<td>0.0053</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>21 (70)</td>
<td>115 (89)</td>
<td>0.0172</td>
</tr>
</tbody>
</table>

**Emboliisation**

| Emboli-positive (%)     | 20 (71)                  | 22 (18)                                 | <0.0001 |
| Emboli no. (range)      | 4 (1-16)                 | 2 (1-12)                                | 0.3431  |

**Histology**

| Plaque rupture (%)      | 11 (37)                  | 17 (13)                                 | 0.0059  |
| IPH (%)                 | 14 (47)                  | 24 (19)                                 | 0.0034  |
| Cap thinning (%)        | 20 (67)                  | 79 (61)                                 | 0.6779  |
| Intraplaque fibrin (%)  | 15 (50)                  | 58 (45)                                 | 0.6862  |
| Plaque necrosis (%)     | 21 (70)                  | 58 (45)                                 | 0.0155  |
| Cap foam cells (%)      | 19 (63)                  | 58 (45)                                 | 0.1037  |

Table 9.2 Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups, in terms of patient characteristics, spontaneous cerebral embolisation and plaque histological features
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Emboli-positive (n=42)</th>
<th>Emboli-negative (n=106)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>68.4 (54-80)</td>
<td>70.0 (50-86)</td>
<td>0.2356</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>30 (71)</td>
<td>75 (71)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>35 (83)</td>
<td>88 (83)</td>
<td>1.0000</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>18 (43)</td>
<td>46 (43)</td>
<td>1.0000</td>
</tr>
<tr>
<td>HTN (%)</td>
<td>23 (55)</td>
<td>73 (69)</td>
<td>0.1275</td>
</tr>
<tr>
<td>DM (%)</td>
<td>7 (17)</td>
<td>20 (19)</td>
<td>0.8181</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>80 (80-90)</td>
<td>80 (70-90)</td>
<td>0.0721</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>9 (21)</td>
<td>44 (42)</td>
<td>0.0234</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>36 (86)</td>
<td>90 (85)</td>
<td>1.0000</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque rupture (%)</td>
<td>8 (19)</td>
<td>19 (18)</td>
<td>1.0000</td>
</tr>
<tr>
<td>IPH (%)</td>
<td>13 (31)</td>
<td>23 (22)</td>
<td>0.2885</td>
</tr>
<tr>
<td>Cap thinning (%)</td>
<td>24 (57)</td>
<td>68 (64)</td>
<td>0.4562</td>
</tr>
<tr>
<td>Intraplaque fibrin (%)</td>
<td>18 (43)</td>
<td>50 (47)</td>
<td>0.7156</td>
</tr>
<tr>
<td>Plaque necrosis (%)</td>
<td>21 (50)</td>
<td>53 (50)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Cap foam cells (%)</td>
<td>21 (50)</td>
<td>51 (48)</td>
<td>0.8571</td>
</tr>
</tbody>
</table>

Table 9.3 Comparison of emboli groups in terms of patient characteristics and plaque histological features. NB, 11 patients lacked a TCD window and are therefore not represented in these results.
9.4 Plaque MMP & TIMP concentrations

Tables 9.4, 9.5 & 9.6 show the plaque concentrations of MMPs and TIMPs related to plaque instability (as defined by symptomatology and embolisation). All concentrations are expressed in ng/g wet weight of plaque.

In appendix B, the results are expressed in a protein-standardised format in ng/mg of plaque protein. For ease of comparison, the tables in appendix B are labelled using the same numbering system as in the main text, followed by the symbol: '

There were significantly higher plaque concentrations of both active MMP-8 (20.5 vs 11.4ng/g; \( p=0.0002 \)) and total MMP-8 (275 vs 191ng/g; \( p<0.0001 \)) in symptomatic vs asymptomatic patients (table 9.4).

Significantly higher concentrations of active MMP-8 were also seen in plaques retrieved from highly symptomatic (24.5 vs 13.6ng/g; \( p=0.0018 \)) and emboli-positive patients (22.7 vs 13.5ng/g; \( p=0.0037 \)) as shown in tables 9.5 & 9.6, respectively. No significant differences were seen in the levels of total MMP-8 in these groups, or in the levels of MMP-1 & MMP-13 in any of the groups.

In accordance with previous results (Loftus et al, 2000), the concentrations of MMP-9 were also significantly raised in plaques from symptomatic vs asymptomatic (333 vs 144ng/g; \( p=0.0002 \)), highly symptomatic vs not highly symptomatic (553 vs 185ng/g; \( p<0.0001 \)) and emboli-positive vs emboli-negative (411 vs 193ng/g; \( p=0.0034 \)) patients (see tables 9.4, 9.5 & 9.6).

There was a significantly higher concentration of TIMP-1 in the plaques of symptomatic patients (9738 vs 6439ng/g; \( p=0.0037 \)) as can be seen in table 9.4. However, no difference in TIMP-1 concentration was seen when comparing highly symptomatic patients to those experiencing symptoms >1 month pre-operatively or asymptomatic patients (table 9.5) or when comparing emboli groups (table 9.6).
<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Symptoms &lt;6 months (n=90)</th>
<th>Asymptomatic (n=69)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>39.3 (14.4-111.3)</td>
<td>28.7 (13.9-104.0)</td>
<td>0.2333</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>20.5 (9.5-46.1)</td>
<td>11.4 (5.6-19.3)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>275 (214-358)</td>
<td>191 (106-280)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-9</td>
<td>333 (123-831)</td>
<td>144 (66-381)</td>
<td>0.0002</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.57 (0.33-1.08)</td>
<td>0.63 (0.35-0.87)</td>
<td>0.8294</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>9738 (5294-13,660)</td>
<td>6439 (4641-10,310)</td>
<td>0.0037</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>789 (472-1285)</td>
<td>821 (443-1322)</td>
<td>0.8038</td>
</tr>
</tbody>
</table>

Table 9.4 Comparison of symptomatic vs asymptomatic groups in terms of plaque MMP & TIMP concentrations (see appendix B for protein-standardised results)

There were significantly higher concentrations of active MMP-8 in those plaques with histological evidence of rupture (20.5 vs 14.7ng/g; p=0.0036). No significant differences in active MMP-8 levels were seen when comparing the presence or absence of 5 other histological features (table 9.7).

Levels of MMP-9 were also significantly higher in those plaques with rupture, but no difference was seen with the other histological features. Concentrations of MMP-1, total MMP-8, MMP-13, TIMP-1 & TIMP-2 showed no differences when comparing the presence or absence of all 6 histological features, including plaque rupture (data not shown).
<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Symptoms &lt;1 month (n=30)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=129)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>59.9 (24.8-166.0)</td>
<td>29.5 (12.2-81.2)</td>
<td>0.0118</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>24.5 (11.0-57.4)</td>
<td>13.6 (6.3-24.7)</td>
<td>0.0018</td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>266 (237-336)</td>
<td>239 (142-307)</td>
<td>0.0715</td>
</tr>
<tr>
<td>MMP-9</td>
<td>553 (277-1474)</td>
<td>185 (86-555)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.78 (0.47-1.31)</td>
<td>0.57 (0.32-0.90)</td>
<td>0.0491</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>8911 (5139-14,360)</td>
<td>7705 (5114-11,710)</td>
<td>0.1801</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>821 (506-1295)</td>
<td>684 (370-1290)</td>
<td>0.3417</td>
</tr>
</tbody>
</table>

Table 9.5 Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups in terms of plaque MMP & TIMP concentrations (see appendix B for protein-standardised results)

Active MMP-8 results are represented in graph format in figure 9.1. Total MMP-8 results can be seen in figure 9.2.
Figure 9.1 Comparison of active MMP-8 concentrations in unstable vs stable plaques using the 3 definitions shown. Bars represent median values and whiskers represent interquartile ranges.
<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Emboli-positive (n=42)</th>
<th>Emboli-negative (n=106)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>47.8 (17.4-101.4)</td>
<td>29.1 (12.1-101.6)</td>
<td>0.2491</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>22.7 (9.8-53.2)</td>
<td>13.5 (6.2-25.4)</td>
<td><strong>0.0037</strong></td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>271 (196-330)</td>
<td>238 (140-307)</td>
<td>0.0780</td>
</tr>
<tr>
<td>MMP-9</td>
<td>411 (165-1057)</td>
<td>193 (97-555)</td>
<td><strong>0.0034</strong></td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.77 (0.46-1.16)</td>
<td>0.57 (0.28-0.84)</td>
<td>0.0787</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>7747 (4316-13,540)</td>
<td>7716 (5205-11,470)</td>
<td>0.7465</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>874 (378-1405)</td>
<td>803 (499-1295)</td>
<td>0.6987</td>
</tr>
</tbody>
</table>

Table 9.6 Comparison of emboli groups in terms of plaque MMP & TIMP concentrations (see appendix B for protein-standardised results). NB, 11 patients lacked a TCD window and are therefore not represented in these results.

<table>
<thead>
<tr>
<th>Histological feature</th>
<th>Present</th>
<th>Absent</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque rupture</td>
<td>20.8 (13.2-49.0)</td>
<td>14.7 (6.3-26.4)</td>
<td><strong>0.0036</strong></td>
</tr>
<tr>
<td>IPH</td>
<td>21.4 (10.9-53.5)</td>
<td>13.4 (6.7-25.7)</td>
<td>0.0173</td>
</tr>
<tr>
<td>Cap thinning</td>
<td>13.0 (7.2-25.4)</td>
<td>20.8 (6.6-32.6)</td>
<td>0.1972</td>
</tr>
<tr>
<td>Intraplaque fibrin</td>
<td>15.6 (6.2-33.3)</td>
<td>16.9 (7.2-26.4)</td>
<td>0.8479</td>
</tr>
<tr>
<td>Plaque necrosis</td>
<td>17.3 (8.4-30.1)</td>
<td>14.7 (6.7-27.1)</td>
<td>0.2719</td>
</tr>
<tr>
<td>Cap foam cells</td>
<td>16.6 (6.0-24.3)</td>
<td>16.4 (7.2-32.6)</td>
<td>0.3269</td>
</tr>
</tbody>
</table>

Table 9.7 Comparison of plaque active MMP-8 concentration in presence or absence of 6 histological features associated with plaque instability (see appendix B for protein-standardised results)
Figure 9.2 Comparison of total MMP-8 concentrations in unstable vs stable plaques using the 3 definitions shown. Bars represent median values and whiskers represent interquartile ranges.
9.5 Immunohistochemistry & colocalisation studies

Immunohistochemistry identified the presence of MMP-8 in carotid plaques. Staining was strongest in the shoulder region and corresponded to areas exhibiting inflammatory cell infiltrates. The MMP-8 enzyme colocalised to cells staining for CD45 ("leukocyte common antigen"), also shown to be strongly CD68-positive - this confirmed the identity of these cells as macrophages.

There was no evidence of MMP-8 colocalising to SMA-positive smooth muscle cells or to CD15-positive neutrophils. Figure 9.3, showing histological sections from the shoulder region of a symptomatic carotid plaque, clearly demonstrates the presence of MMP-8 colocalising with macrophages.

Focal, weak staining for MMP-8 was detected within clusters of cells in asymptomatic plaques. Again, these cells were strongly positive for CD68, weakly positive for CD45, and negative for CD15 & SMA – consistent with macrophages. Illustrative sections from an asymptomatic plaque are shown in figure 9.4.

9.6 In situ hybridisation

In situ hybridisation demonstrated the synthesis of MMP-8 protein in a selection of symptomatic carotid plaques. MMP-8 messenger RNA detected was particularly evident within inflammatory cell infiltrates in the shoulder region of plaques. The MMP-8 mRNA localised to the same areas as CD68-positive cells, supporting an origin from the macrophage cell population (fig 9.3).

MMP-8 mRNA was not detected in a selection of asymptomatic plaques, although as described in the immunohistochemistry results, the same plaques did show weak staining for MMP-8 protein colocalising with macrophages. This apparent discrepancy probably relates to the level of MMP-8 synthesis falling below the sensitivity threshold of in situ hybridisation in those asymptomatic plaques (confirmed to have low MMP-8 levels by ELISA). Again, illustrative sections are shown in figure 9.4.
a) H&E

b) H&E

c) MMP-8

d) CD45

e) CD15

f) SMA

g) CD68

h) MMP-8 ISH

i) IHC NEG

j) ISH NEG
Figure 9.3 (page 109). Histological sections taken from the shoulder region of a symptomatic carotid plaque. Some sections show disruption of the friable plaque, in particular due to the harsh pre-treatment required for ISH in slides (h) and (j), but also on the slide showing MMP-8 IHC (c).

(a) Low power H&E section with boxed area delineating high power view shown in (b-j).
(b) High power H&E section demonstrating a cellular infiltrate.
(c) There is strong reactivity for MMP-8 in cells, which display positivity for CD45 (d) and CD68 (g), but are negative for CD15 (e) and SMA (f).
In-situ hybridisation demonstrates signal for MMP-8 mRNA in the same region (h).
(i) and (j) show negative controls for IHC/colocalisation studies and ISH, respectively.
(Magnification x100 for (a), x200 for others).

Figure 9.4 (page 111). Histological sections from an asymptomatic carotid plaque.

(a) Low power H&E section with boxed area delineating high power view shown in (b-j).
(b) High power H&E section.
There is weak reactivity for MMP-8 (c) in cells, which display positivity for CD45 (d) and CD68 (g), but are negative for CD15 (e) and SMA (f).
No signal for MMP-8 mRNA is detected in this region by ISH (h).
(i) and (j) represent negative controls for IHC/colocalisation studies and ISH, respectively.
(Magnification x100 for (a), x200 for others).
a) H&E

b) H&E

c) MMP-8

d) CD45

e) CD15

f) SMA

h) MMP-8 ISH

g) CD68

i) IHC NEG

j) ISH NEG
Chapter 10: COX-2 activity in atherosclerotic plaques

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1 Symptoms</td>
<td>113</td>
</tr>
<tr>
<td>10.2 Cerebral embolisation</td>
<td>113</td>
</tr>
<tr>
<td>10.3 Plaque COX &amp; COX-2 activity levels</td>
<td>115</td>
</tr>
</tbody>
</table>
10.1 Symptoms

The results in this chapter refer to the same cohort of patients (n=159) in whom MMP/TIMP levels were measured, but as previously stated, 22 have been excluded due to insufficient specimen.

Out of 137 patients, 79 were symptomatic in the 6 months prior to surgery, and 24 of these were highly symptomatic (experiencing symptoms in the pre-operative month). There were no significant differences between the symptom groups in terms of age, atherosclerotic risk factors, severity of carotid stenosis, aspirin use or NSAID use (tables 10.1 & 10.2). Table 10.2 shows that highly symptomatic patients were significantly less likely to be on statin therapy (13% vs 43%; p=0.0049).

10.2 Cerebral embolisation

Over 27% of patients had one or more embolus detected in the pre-operative or dissection phases. For emboli-positive patients only, the median number of emboli was 3 (range 1-16) in the symptomatic group and 2 (range 1-6) in the asymptomatic group.

Embolisation status was undefined in 7 patients (one was symptomatic, not within the pre-operative month) due to the absence of a TCD window. Spontaneous particulate cerebral embolisation was significantly more likely in symptomatic vs asymptomatic patients (41% vs 9%; p<0.0001), as shown in table 10.1. Highly symptomatic patients were particularly likely to be emboli-positive compared to the remainder of the group (75% vs 17%; p<0.0001), as seen in table 10.2.

There were no significant differences in terms of age, atherosclerotic risk factors, severity of carotid stenosis, aspirin use or NSAID use, upon comparison of the emboli groups (table 10.3). Emboli-positive patients were less likely to be on statin therapy (24% vs 44%; p=0.0459) although this failed to reach significance at the p<0.01 level.
Table 10.1 Comparison of symptomatic vs asymptomatic groups in terms of patient characteristics, medication use, embolisation and COX plaque activity levels (see appendix B for protein-standardised results)
10.3 Plaque COX & COX-2 activity levels

All COX and COX-2 results are shown in activity units/g wet weight of plaque – see figure 7.8 (in methodology) for definition of activity units. There were no significant differences in the mean activity levels of either total COX (151 vs 146 units/g; p=0.5360) or COX-2 (104 vs 95 units/g; p=0.3247) between the symptomatic and asymptomatic groups (table 10.1). Also, no difference was observed for total COX (162 vs 146 units/g; p=0.1400) or COX-2 (105 vs 99 units/g; p=0.5747) between the highly symptomatic group vs the rest (table 10.2).

In addition, there were no significant differences in the plaque activity levels of total COX (160 vs 146 units/g; p=0.1437) or COX-2 (112 vs 98 units/g; p=0.1326) in the emboli-positive compared to the emboli-negative groups (table 10.3). Despite the lack of statistical significance, it is interesting to note that whichever definition of unstable plaque is used (symptoms <6 months, symptoms <1 month or emboli-positive), the mean values for both COX and COX-2 activity tend to be higher in the unstable group.

Figure 10.1 shows graphs of COX-2 activity in each of the symptom & emboli groups.
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Symptoms &lt;1 month (n=24)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=113)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>69.7 (57-80)</td>
<td>69.9 (50-86)</td>
<td>0.8928</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>20 (83)</td>
<td>77 (68)</td>
<td>0.2153</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>18 (75)</td>
<td>93 (82)</td>
<td>0.4003</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>12 (50)</td>
<td>48 (42)</td>
<td>0.5072</td>
</tr>
<tr>
<td>HTN (%)</td>
<td>11 (46)</td>
<td>79 (70)</td>
<td>0.0328</td>
</tr>
<tr>
<td>DM (%)</td>
<td>1 (4)</td>
<td>25 (22)</td>
<td>0.0457</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>80 (70-90)</td>
<td>80 (75-90)</td>
<td>0.7226</td>
</tr>
</tbody>
</table>

**Medication**

<table>
<thead>
<tr>
<th></th>
<th>Symptoms &lt;1 month (n=24)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=113)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin use (%)</td>
<td>3 (13)</td>
<td>49 (43)</td>
<td>0.0049</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>18 (75)</td>
<td>100 (88)</td>
<td>0.1033</td>
</tr>
<tr>
<td>NSAID use (%)</td>
<td>1 (4)</td>
<td>11 (10)</td>
<td>0.6917</td>
</tr>
</tbody>
</table>

**Embolisation**

<table>
<thead>
<tr>
<th></th>
<th>Symptoms &lt;1 month (n=24)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=113)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emboli-positive (%)</td>
<td>18 (75)</td>
<td>19 (17)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Emboli no. (range)</td>
<td>4 (1-16)</td>
<td>2 (1-10)</td>
<td>0.1517</td>
</tr>
</tbody>
</table>

**Activity levels**

<table>
<thead>
<tr>
<th></th>
<th>Symptoms &lt;1 month (n=24)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=113)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX (s.d.)</td>
<td>162 (51)</td>
<td>146 (47)</td>
<td>0.1400</td>
</tr>
<tr>
<td>COX-2 (s.d.)</td>
<td>105 (48)</td>
<td>99 (50)</td>
<td>0.5747</td>
</tr>
</tbody>
</table>

**Table 10.2** Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups in terms of patient characteristics, medication use, embolisation and COX plaque activity levels (see appendix B for protein-standardised results)
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Emboli-positive (n=37)</th>
<th>Emboli-negative (n=93)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>68.0 (54-80)</td>
<td>70.6 (50-86)</td>
<td>0.0660</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>28 (76)</td>
<td>67 (72)</td>
<td>0.8271</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>30 (81)</td>
<td>77 (83)</td>
<td>0.8034</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>17 (46)</td>
<td>42 (45)</td>
<td>1.0000</td>
</tr>
<tr>
<td>HTN (%)</td>
<td>21 (57)</td>
<td>64 (69)</td>
<td>0.2227</td>
</tr>
<tr>
<td>DM (%)</td>
<td>7 (19)</td>
<td>18 (19)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>80 (80-90)</td>
<td>80 (70-90)</td>
<td>0.0457</td>
</tr>
</tbody>
</table>

**Medication**

<table>
<thead>
<tr>
<th></th>
<th>Emboli-positive (n=37)</th>
<th>Emboli-negative (n=93)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin use (%)</td>
<td>9 (24)</td>
<td>41 (44)</td>
<td>0.0459</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>31 (84)</td>
<td>80 (86)</td>
<td>0.7856</td>
</tr>
<tr>
<td>NSAID use (%)</td>
<td>4 (11)</td>
<td>8 (9)</td>
<td>0.7407</td>
</tr>
</tbody>
</table>

**Activity levels**

<table>
<thead>
<tr>
<th></th>
<th>Emboli-positive (n=37)</th>
<th>Emboli-negative (n=93)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX (s.d.)</td>
<td>160 (47)</td>
<td>146 (48)</td>
<td>0.1437</td>
</tr>
<tr>
<td>COX-2 (s.d.)</td>
<td>112 (46)</td>
<td>98 (50)</td>
<td>0.1326</td>
</tr>
</tbody>
</table>

*Table 10.3 Comparison of emboli groups in terms of patient characteristics, medication use and COX plaque activity levels (see appendix B for protein-standardised results). NB, 7 patients lacked a TCD window and are therefore not represented in these results.*
Figure 10.1 Comparison of COX-2 activity levels in unstable vs stable plaques using the 3 definitions shown. Spots represent individual values and horizontal lines represent mean averages.
Chapter 11: Cytokines, tissue factor & inflammatory markers

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1 Cytokines &amp; tissue factor</td>
<td>120</td>
</tr>
<tr>
<td>11.1.1 Patients, symptoms &amp; cerebral embolisation</td>
<td>120</td>
</tr>
<tr>
<td>11.1.2 Plaque concentrations</td>
<td>120</td>
</tr>
<tr>
<td>11.2 Inflammatory markers</td>
<td>121</td>
</tr>
</tbody>
</table>
11.1 Cytokines & tissue factor

11.1.1 Patients, symptoms & cerebral embolisation

Complete data are available for 137 patients, as discussed previously. These are the same patients on which COX activity levels have already been reported. The results regarding symptomatology and embolisation are therefore identical to those already presented in chapter 10.

11.1.2 Plaque concentrations

An “Ultra sensitive” ELISA (capable of detecting concentrations as low as 0.78pg/ml) was used in an effort to quantify levels of IL-10 in plaques. The first 80 homogenates demonstrated undetectable IL-10 levels and so no further quantification of this cytokine was attempted.

IL-1β, IL-6, TNF-α and tissue factor all had detectable plaque levels. Cytokine concentrations are expressed as median values and compared using the Mann-Whitney U-test. Tissue factor levels are expressed as mean values and compared using the unpaired t-test. Table 11.2 shows plaque IL-6 concentrations were significantly raised in the highly symptomatic group (4075 vs 1929ng/g; p=0.0005). Surprisingly, table 11.3 shows lower tissue factor concentrations in the emboli-positive group (13.54 vs 16.68ng/g; p=0.0138), although this failed to reach significance at the p<0.01 level.

<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Symptoms &lt;6 months (n=79)</th>
<th>Asymptomatic (n=58)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>18.2 (13.1-26.6)</td>
<td>16.7 (11.1-24.9)</td>
<td>0.2859</td>
</tr>
<tr>
<td>IL-6</td>
<td>2471 (1206-4519)</td>
<td>2016 (855-4825)</td>
<td>0.3615</td>
</tr>
<tr>
<td>TNF-α</td>
<td>21.0 (15.4-27.9)</td>
<td>18.8 (13.6-27.1)</td>
<td>0.4241</td>
</tr>
<tr>
<td>Tissue factor (s.d.)</td>
<td>15.95 (6.66)</td>
<td>15.08 (6.44)</td>
<td>0.444</td>
</tr>
</tbody>
</table>

Table 11.1 Comparison of symptomatic vs asymptomatic groups in terms of plaque cytokine & tissue factor concentrations (see appendix B for protein-standardised results)
### Table 11.2

Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups in terms of plaque cytokine & tissue factor concentrations (see appendix B for protein-standardised results)

<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Symptoms &lt;1 month (n=24)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=113)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>19.7 (15.8-26.5)</td>
<td>17.1 (11.1-24.9)</td>
<td>0.1545</td>
</tr>
<tr>
<td>IL-6</td>
<td>4075 (2632-7475)</td>
<td>1929 (939-3731)</td>
<td><strong>0.0005</strong></td>
</tr>
<tr>
<td>TNF-α</td>
<td>23.7 (18.6-29.3)</td>
<td>20.0 (13.8-26.7)</td>
<td>0.0778</td>
</tr>
<tr>
<td>Tissue factor (s.d.)</td>
<td>13.72 (6.00)</td>
<td>15.98 (6.63)</td>
<td>0.1248</td>
</tr>
</tbody>
</table>

### Table 11.3

Comparison of emboli groups in terms of plaque cytokine & tissue factor concentrations (see appendix B for protein-standardised results). NB, 7 patients lacked a TCD window and are therefore not represented in these results.

<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Emboli-positive (n=37)</th>
<th>Emboli-negative (n=93)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>20.5 (14.1-33.7)</td>
<td>17.0 (11.1-23.8)</td>
<td>0.0671</td>
</tr>
<tr>
<td>IL-6</td>
<td>2716 (1648-5005)</td>
<td>1969 (939-4263)</td>
<td>0.0553</td>
</tr>
<tr>
<td>TNF-α</td>
<td>21.5 (14.9-26.8)</td>
<td>20.2 (14.1-27.5)</td>
<td>0.6424</td>
</tr>
<tr>
<td>Tissue factor (s.d.)</td>
<td>13.54 (6.20)</td>
<td>16.68 (6.57)</td>
<td>0.0138</td>
</tr>
</tbody>
</table>

11.2 Inflammatory markers

Data for C-reactive protein and white cell count were obtained on 75 consecutive patients. CRP values (mg/l) were not normally distributed and are therefore shown as median values and compared using the Mann-Whitney U-test. WCC values (x10^9 cells/l) were normally distributed and are therefore shown as mean values and compared using the unpaired t-test.
At its lower range, the CRP assay could only determine whether a value was “<5”. This was the case with the majority (50/75) of pre-operative serum samples. All median CRP values are therefore “<5” which is obviously unsatisfactory for the meaningful analysis of these results. No significant differences in CRP or WCC were demonstrated between the groups, as can be seen in table 11.4.

<table>
<thead>
<tr>
<th>Symptoms &lt;6 months (n=46)</th>
<th>Asymptomatic (n=29)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>&lt;5 (&lt;5-9.0)</td>
<td>&lt;5 (&lt;5-9.5)</td>
</tr>
<tr>
<td>WCC (s.d.)</td>
<td>8.3 (1.7)</td>
<td>7.8 (1.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Symptoms &lt;1 month (n=7)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=68)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>&lt;5 (&lt;5-12.5)</td>
<td>&lt;5 (&lt;5-9.0)</td>
</tr>
<tr>
<td>WCC (s.d.)</td>
<td>7.7 (1.0)</td>
<td>8.1 (1.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Emboli-positive (n=15)</th>
<th>Emboli-negative (n=57)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>&lt;5 (&lt;5-19.5)</td>
<td>&lt;5 (&lt;5-8.5)</td>
</tr>
<tr>
<td>WCC (s.d.)</td>
<td>8.2 (1.8)</td>
<td>8.1 (1.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Statins (n=42)</th>
<th>No statins (n=33)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>&lt;5 (&lt;5-8.5)</td>
<td>&lt;5 (&lt;5-10)</td>
</tr>
<tr>
<td>WCC (s.d.)</td>
<td>8.1 (1.6)</td>
<td>8.0 (1.6)</td>
</tr>
</tbody>
</table>

**Table 11.4 Comparison of pre-operative serum CRP & WCC samples**
Chapter 12: HMG-CoA reductase inhibitors stabilise carotid atheroma by reducing plaque concentrations of MMPs & cytokines

Contents

12.1 Patients & statins 124
12.2 Symptomatology 124
12.3 Cerebral embolisation 124
12.4 MMP & cytokine concentrations 125
12.5 COX & tissue factor concentrations 128
12.1 Patients & statins

Again, insufficient sample from 22 patients means that full data (including cytokine concentrations) are available for 137 patients, which make up this study group. There were 52 patients (38%) taking statin therapy, all for >4 weeks prior to CEA.

A variety of types and dosages were used as listed:

- 25 patients on simvastatin (10mg, n=12; 20mg, n=7; 40mg, n=6)
- 16 patients on atorvastatin (10mg, n=12; 20mg, n=3; 40mg, n=1)
- 6 patients on pravastatin (10mg, n=1; 20mg, n=2; 30mg, n=1; 40mg, n=2)
- 5 patients on fluvastatin (20mg, n=3; 40mg, n=2).

The mean age of patients taking statins tended to be lower than those not on this therapy (68.1 vs 71.0 years, p=0.0269). Patient characteristics in terms of sex, smoking, ischaemic heart disease, hypertension, diabetes mellitus and severity of carotid stenosis were comparable between the 2 groups (table 12.1).

12.2 Symptomatology

Only 3/52 patients (6%) on statin therapy suffered carotid territory symptoms in the 4 weeks prior to CEA (2 with transient ischaemic attacks, 1 with amaurosis fugax) compared to 21/85 patients (25%) not on statins (16 with transient ischaemic attacks, 5 with amaurosis fugax). This difference was highly significant with a p-value of 0.0049 (table 12.1).

12.3 Cerebral embolisation

Embolisation status was unknown in 7 patients lacking TCD windows (2 patients from the statin group and 5 patients from the non-statin group). A lower proportion of patients taking statins (9/50 vs 28/80; p=0.0459) were detected to be undergoing spontaneous cerebral embolisation from their carotid plaque. This failed to reach significance at the p<0.01 level, however. Sub-group analysis of the emboli-positive group only, showed no
difference in the median number of emboli between those patients on statins and those not on therapy (table 12.1).

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Statin therapy for &gt;4 weeks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=52)</td>
<td>No (n=85)</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>68.1 (50-86)</td>
<td>71.0 (54-86)</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>35 (67)</td>
<td>61 (72)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>38 (73)</td>
<td>73 (86)</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>28 (54)</td>
<td>32 (38)</td>
</tr>
<tr>
<td>BP (%)</td>
<td>36 (69)</td>
<td>55 (65)</td>
</tr>
<tr>
<td>DM (%)</td>
<td>13 (25)</td>
<td>13 (15)</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>80 (70-88)</td>
<td>80 (75-90)</td>
</tr>
</tbody>
</table>

**Symptomatology**

| Highly symptomatic (%) | 3 (6) | 21 (25) | 0.0049 |

**Embolisation**

| Emboli-positive (%) | 9 (17) | 28 (33) | 0.0459 |
| Emboli no. (range)  | 2 (1-6) | 3 (1-16) | 0.2946 |

**Table 12.1 Statin groups vs patient characteristics, symptomatology and embolisation**

12.4 MMP and cytokine concentrations

Plaque concentrations, measured in ng/g wet weight of plaque, were significantly lower in the group of patients on statin therapy for MMP-9 (120 vs 308ng/g; p=0.0018) and IL-6 (1372 vs 2732ng/g; p=0.0005). In addition, MMP-1 levels were lower in the statin group (23.3 vs 39.2ng/g; p=0.0176), although this just failed to reach significance at the p<0.01
level. No differences in plaque concentrations were seen for active MMP-8, total MMP-8, MMP-13, TIMP-1, TIMP-2, IL-1β or TNF-α (table 12.2).

<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Statin therapy for &gt;4 weeks</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=52)</td>
<td>No (n=85)</td>
</tr>
<tr>
<td>MMP-1</td>
<td>23.3 (8.5-54.4)</td>
<td>39.2 (18.0-104.2)</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>16.9 (7.1-28.1)</td>
<td>13.4 (7.2-27.9)</td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>282 (152-371)</td>
<td>258 (169-297)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>120 (65-366)</td>
<td>308 (121-722)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.57 (0.38-0.75)</td>
<td>0.57 (0.30-0.91)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>9524 (5147-12240)</td>
<td>7689 (5340-12380)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>949 (739-1466)</td>
<td>739 (438-1259)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>18.2 (12.5-26.1)</td>
<td>17.6 (12.0-24.3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>1372 (842-3000)</td>
<td>2732 (1475-5146)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>22.1 (15.1-28.0)</td>
<td>19.9 (14.5-27.2)</td>
</tr>
</tbody>
</table>

Table 12.2 Statin groups vs MMP, TIMP & cytokine levels (see appendix B for protein-standardised results)

Graphical comparisons of the plaque concentrations of MMP-1, MMP-9 & IL-6 in the statin groups are shown in figure 12.1.
Figure 12.1 Comparison of statin groups in terms of plaque MMP-1, MMP-9 & IL-6 concentrations
12.5 COX & tissue factor concentrations

No differences were seen between the groups of patients on vs not on statin therapy, in terms of total COX or COX-2 activity (table 12.3). Interestingly, patients on statin therapy had *increased* levels of tissue factor in their plaques (17.8 vs 14.2ng/g; p=0.0017). The possible reasons for this unexpected result are discussed in chapter 14.

<table>
<thead>
<tr>
<th>Statin therapy for &gt;4 weeks</th>
<th>Yes (n=52)</th>
<th>No (n=85)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX (s.d.)</td>
<td>151 (46)</td>
<td>148 (50)</td>
<td>0.6805</td>
</tr>
<tr>
<td>COX-2 (s.d.)</td>
<td>101 (49)</td>
<td>99 (50)</td>
<td>0.8481</td>
</tr>
<tr>
<td>Tissue factor (s.d.)</td>
<td>17.8 (6.5)</td>
<td>14.2 (6.2)</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

*Table 12.3* Statin groups vs COX activity and tissue factor concentrations (see appendix B for protein-standardised results). Mean COX activity units/g wet weight of plaque are shown (see fig 7.8 for definition of activity units). Mean plaque concentration of tissue factor shown in ng/g wet weight of plaque.
Chapter 13: Elevation in plasma MMP-9 following carotid endarterectomy is associated with particulate cerebral embolisation

Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.1 Patients</td>
<td>130</td>
</tr>
<tr>
<td>13.2 Cerebral embolisation</td>
<td>130</td>
</tr>
<tr>
<td>13.3 Plasma MMP-9 &amp; TIMP-1</td>
<td>131</td>
</tr>
<tr>
<td>13.3.1 Comparison of emboli groups</td>
<td>131</td>
</tr>
<tr>
<td>13.3.2 Correlation with plaque MMP-9 concentrations</td>
<td>134</td>
</tr>
</tbody>
</table>
13.1 Patients

The initial number of patients recruited for this section of the thesis was 75. For the comparison of pre- & post-operative samples in relation to embolisation, 5 patients were excluded due to lack of a TCD "window" (n=3) or refusal of the post-operative blood sample (n=2).

13.2 Cerebral embolisation

Only 4 emboli (in 3 patients) were recorded during the pre-operative 30-minute period, and all 3 of these patients also demonstrated dissection phase embolisation. These small numbers of emboli (and patients) precluded meaningful separate analysis of this pre-operative embolisation group. Any patient with one or more TCD-detected embolus in the pre-operative or dissection phase was therefore termed "emboli-positive", and compared to emboli-negative patients.

A total of 49 emboli were detected in 15 patients during the dissection phase. Emboli-positive patients were significantly more likely to be symptomatic compared to those who were emboli-negative (100% vs 55%; \(p=0.0006\)). In all other respects the emboli groups were comparable (table 13.1).
<table>
<thead>
<tr>
<th></th>
<th>Emboli-positive (n=15)</th>
<th>Emboli-negative (n=55)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>68.1 (59-80)</td>
<td>71.0 (50-86)</td>
<td>0.1762</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>10 (67)</td>
<td>38 (69)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Ever smoker (%)</td>
<td>11 (73)</td>
<td>42 (76)</td>
<td>1.0000</td>
</tr>
<tr>
<td>IHD (%)</td>
<td>4 (27)</td>
<td>23 (42)</td>
<td>0.3756</td>
</tr>
<tr>
<td>BP (%)</td>
<td>9 (60)</td>
<td>37 (67)</td>
<td>0.7599</td>
</tr>
<tr>
<td>DM (%)</td>
<td>2 (13)</td>
<td>11 (20)</td>
<td>0.7196</td>
</tr>
<tr>
<td>Carotid stenosis (IQR)</td>
<td>85 (80-90)</td>
<td>80 (70-90)</td>
<td>0.0194</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>15 (100)</td>
<td>49 (89)</td>
<td>0.3289</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>6 (40)</td>
<td>34 (62)</td>
<td>0.1515</td>
</tr>
<tr>
<td>Symptoms &lt;6mths (%)</td>
<td>15 (100)</td>
<td>30 (55)</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

Table 13.1 Comparison of patient characteristics, medication use and symptomatology between emboli groups

13.3 Plasma MMP-9 & TIMP-1

13.3.1 Comparison of emboli groups

For the 15 emboli-positive patients, the median post-operative plasma MMP-9 level was significantly higher than the median pre-operative value (14.9ng/ml vs 8.8ng/ml; p=0.0381). However, as can be seen in figure 13.1, no significant change was seen in the plasma MMP-9 level of the 55 emboli-negative patients (7.7ng/ml vs 7.1ng/ml; p=0.3636).
In addition, comparison of pre-operative MMP-9 levels shows no significant difference between emboli-positive and emboli-negative groups (8.8 vs 7.1 ng/ml; \( p=0.5057 \)). However, the median post-operative MMP-9 concentration is significantly higher in the emboli-positive compared to the emboli-negative group (14.9 vs 7.7 ng/ml; \( p=0.0247 \)). These values are shown in table 13.2.
### Table 13.2 Comparison of pre- & post-operative plasma MMP-9 concentrations between emboli groups

<table>
<thead>
<tr>
<th>MMP-9 (ng/ml)</th>
<th>Pre-operative plasma</th>
<th>Post-operative plasma</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emboli-negative (n=55)</td>
<td>7.1 (4.1-13.5)</td>
<td>7.7 (4.2-21.0)</td>
<td>0.3636</td>
</tr>
<tr>
<td>Emboli-positive (n=15)</td>
<td>8.8 (3.4-20.0)</td>
<td>14.9 (9.0-28.6)</td>
<td>0.0381</td>
</tr>
<tr>
<td>p-value</td>
<td>0.5057</td>
<td>0.0247</td>
<td></td>
</tr>
</tbody>
</table>

There were no changes in TIMP-1 concentrations between the pre-operative and post-operative samples in either the emboli-positive or emboli-negative groups, nor when comparing the 2 groups at the pre-operative and post-operative stages (table 13.3).

### Table 13.3 Comparison of pre- & post-operative plasma TIMP-1 concentrations between emboli groups

<table>
<thead>
<tr>
<th>TIMP-1 (ng/ml)</th>
<th>Pre-operative plasma</th>
<th>Post-operative plasma</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emboli-negative (n=55)</td>
<td>967 (875-1084)</td>
<td>1033 (840-1243)</td>
<td>0.3240</td>
</tr>
<tr>
<td>Emboli-positive (n=15)</td>
<td>1011 (884-1105)</td>
<td>1046 (795-1269)</td>
<td>0.9339</td>
</tr>
<tr>
<td>p-value</td>
<td>0.5336</td>
<td>0.9373</td>
<td></td>
</tr>
</tbody>
</table>

On sub-analysis of the emboli-positive group, a significantly greater rise in plasma MMP-9 levels was demonstrated in the 6 patients with >2 emboli detected, compared to the 9 patients with only 1 or 2 emboli (figure 13.2).
Fig 13.2 Graph demonstrating larger rise in plasma MMP-9 levels of patients with more than 2 detected emboli (red) compared to those with 1 or 2 emboli (blue). Spots represent median values and whiskers represent interquartile ranges.

13.3.2 Correlation with plaque MMP-9 concentrations

The non-parametric Spearman’s correlation coefficient (r) was calculated for pre-operative plasma MMP-9 vs plaque MMP-9 levels (see figure 13.3). An r-value of 0.1827 suggested no correlation between these 2 continuous variables. The hypothesis test for r resulted in a non-significant p-value of 0.1167.
Figure 13.3 No significant correlation is seen between plasma & plaque levels of MMP-9
Section IV: Conclusion
Chapter 14: Discussion

Sudden alterations in atherosclerotic plaque structure, such as cap rupture and intraplaque haemorrhage, frequently precede ischaemic cardiovascular events (Falk, 1985; Falk et al, 1995). Studies have shown that there is often an intense inflammatory infiltrate around the rupture site (Moreno et al, 1994) that is probably responsible for destabilisation of the plaque (Buja & Willerson, 1994). There is now considerable evidence that excessive proteolysis leads to plaque rupture, with MMPs being the major group of enzymes involved in this process (Loftus et al, 2002). The gelatinase MMP-9 has been highlighted as one of the most important enzymes in this regard, with increased synthesis observed in atherectomy specimens removed from patients with unstable, as opposed to stable, angina (Brown et al, 1995). More recently, increased levels of MMP-9 have been demonstrated in unstable carotid plaques, defined in terms of symptomatology, cerebral embolisation and histological features (Loftus et al, 2000).

However, type I and type III collagen molecules, which account for the load-bearing strength of the plaque cap, are not substrates for the MMP-9 enzyme (Pourmotabbed et al, 1994; Murphy et al, 1982). There have been reports that high concentrations of other gelatinases (eg, MMP-2) can degrade type I collagen in an in vitro environment, devoid of TIMPs (Aimes & Quigley, 1995). Nonetheless, it is probable that in vivo, only the interstitial collagenases (MMP-1, MMP-8 & MMP-13) are capable of degrading these fibrillar collagens and are therefore likely to play a role in the pathogenesis of cap rupture.

MMP-8 ("neutrophil collagenase") has only very recently been identified in atherosclerotic tissue (Herman et al, 2001a). That study put forward the possibility that MMP-8 may play a role in the pathogenesis of plaque instability, as it colocalised with cleaved collagen. Additionally, an excess of MMP-8 was demonstrated in histologically characterised vulnerable plaques (n=3) compared to stable ones (n=3). The discovery that MMP-8 could be synthesised by atheroma-associated cell types (as opposed to only neutrophils which are not commonly found in plaques) has led to increased interest in this enzyme.
The data presented in this thesis (n=159) suggest active MMP-8 may play an important role in acute plaque rupture. These results are the first to show increased concentrations of the active form of a collagenase, MMP-8, in unstable plaques. Active and total concentrations of MMP-8 were significantly higher in plaques from symptomatic patients (carotid territory symptoms in the 6 months prior to surgery), whilst active MMP-8 was preferentially raised in highly symptomatic patients (symptoms in the pre-operative month) and in those with ongoing cerebral embolisation. It is interesting to note that no differences in the concentrations of the other 2 collagenases (MMP-1 & MMP-13) were detected between stable and unstable plaques. Indeed, the levels of MMP-13 were almost negligible (approximately 400 times lower concentrations than those of MMP-8).

The level of TIMP-1 in plaques from symptomatic patients was significantly higher than the level in asymptomatic patients, but this significant elevation was not seen when analysing plaque TIMP-1 levels from the highly symptomatic or emboli-positive groups. In addition, the raised TIMP-1 level in symptomatic plaques was not of the same magnitude as the increase in MMP-8, suggesting a shift in the equilibrium towards collagenolysis. Calculation of the ratio of an individual MMP to an individual TIMP has been purposely avoided in this thesis. Each TIMP can inhibit numerous MMPs and although they do so preferentially, the total concentration of a particular TIMP will not necessarily be available for inhibition of only that MMP.

The immunohistochemistry results presented in this thesis show that MMP-8 colocalises with macrophages. In addition, in situ hybridisation demonstrated MMP-8 mRNA expression within macrophages, confirming production of the enzyme by this cell type. These findings support the pivotal role of macrophage infiltration in plaque destabilisation. Previous studies have revealed an increase in macrophage-rich areas in unstable coronary (Moreno et al, 1994) and carotid (Cipollone et al, 2001) plaques, and have suggested increased macrophage activation in the plaques of those patients suffering from acute coronary syndromes (Ehara et al, 2001).

Further immunohistochemistry or in situ hybridisation was deemed unnecessary for this thesis, and in particular, no attempt was made to compare enzyme or mRNA intensity using these methods. Computer-assisted image analysis systems are often used to estimate levels from histological slides – these are user-dependent and semi-quantitative at best, and were purposely avoided.
It remains unknown as to whether raised MMP levels cause rupture of the atherosclerotic plaque or are a result of it. Attempts to develop animal models of plaque rupture have been largely unsatisfactory (Rekhter, 2002; Cullen et al, 2003). Proof of a causative role is likely to remain debatable until a randomised controlled trial of an MMP inhibitor versus placebo is completed. Some inhibitors, such as the anti-cancer trial drug, MMI270, are very specific (for MMP-2, MMP-8 & MMP-9), but retain significant musculoskeletal side effects despite this specificity (Levitt et al, 2001).

The results presented in this thesis regarding collagenases in atheroma represent a significant step forward in our understanding of the biological mechanisms likely to be involved in plaque rupture. MMP-8 and MMP-9 are both produced by macrophages within the atherosclerotic plaque and combined, they can degrade all of the major structural components of the plaque extracellular matrix. Pharmacotherapy aimed at plaque stabilisation should therefore target both of these enzymes.

---

In contrast, the data presented on plaque COX-2 activity (in chapter 10) were somewhat disappointing. No significant differences were demonstrated in activity levels between stable and unstable plaques, whichever definition of instability was used. The recent literature gives a mixed view of the effects of COX-2 on the cardiovascular system. As discussed in chapter 4, the VIGOR trial comparing the COX-2 inhibitor rofecoxib to an NSAID (naproxen) in rheumatoid arthritis patients showed a 5-fold increase in cardiovascular events in the rofecoxib group (Bombardier et al, 2000). In comparison, the CLASS trial comparing the COX-2 inhibitor celecoxib to NSAIDs did not show any difference in these events (Silverstein et al, 2000). On the other hand, meloxicam (a preferential COX-2 inhibitor) has been associated with a significant reduction in adverse outcomes in acute coronary syndrome patients (Altman et al, 2002) and indeed, COX-2 inhibitors may exert a beneficial effect on endothelial function (Chenevard et al, 2003).

Of particular relevance to the results presented in this thesis are the findings from 2 studies in 2001 that suggested raised COX-2 protein levels in symptomatic vs asymptomatic carotid plaques (Cipollone et al, 2001; Wijeyaratne et al, 2001). In an attempt to explain the differing results between these studies and those presented in this thesis, it may be of note to point out the combined sizes of the 2 previous study groups (n=50 & n=23,
respectively) were approximately 50% of the study size for the COX-2 section of this thesis (n=137). In addition, the methodology employed was very different – Wijeyaratne et al (2001) used only immunohistochemistry for quantification. Cipollone et al (2001) used immunohistochemistry, reverse transcription-polymerase chain reactions and Western blotting to quantify the levels in their plaques. It is impressive that Cipollone’s methods revealed consistently raised COX-2 in the symptomatic plaques, and also that the same plaques contained raised levels of activated MMP-2 & MMP-9. On the other hand, the division of patients into symptomatic and asymptomatic groups appears unclear, with 16 patients apparently excluded from the study for reasons that are not stated.

To our knowledge, no reliable ELISA kit is available for the accurate quantification of COX-2 protein. If such an assay had been available we would have used it to accurately quantify protein levels alongside measuring activity. However, it is the level of activity and not the absolute amount of protein that is of importance – as highlighted by reports suggesting that NSAIDs may inhibit COX-2 enzyme activity without lowering mRNA or protein expression (Barrios-Rodiles, 1996).

We identified some difficulties in using the COX activity assay that merit discussion at this point. Firstly, the results were time-dependent and were calculated according to a 5-minute reaction time (for the conversion of arachidonic acid to PGH$_2$). With 2 people adding the necessary reagents, we felt confident filling approximately 20 wells with minimal discernible time difference between the first and the last of these wells on the microtitre plate (approximately 20 seconds). In addition, there was inevitably another small time delay between reading the first and the last samples at the end of the reaction time (approximately another 20 seconds) as the microtitre plate reader examines each well sequentially.

Although a standard of known COX activity was used for each run of approximately 20 wells (as quality control), the assay manufacturers did not recommend the construction of a standard curve. This was not suggested as the time taken to construct a standard curve for each run of samples may significantly exceed the 5-minute reaction time. However, interpolation from a standard curve to calculate the activity from the absorbance reading for each sample would have been preferable, as is the case with the ELISAs employed.
COX-2 activity was measured by using a COX-1 inhibitor (SC-560). For each sample, the result obtained in the presence of SC-560 was deducted from the result obtained for total COX activity. This "indirect" method for measuring COX-2 activity could be criticised, but the COX-1 inhibitor employed was highly selective and therefore appropriate for this purpose. SC-560 was used at a well concentration of 0.3\( \mu \text{M} \), which is capable of inhibiting >95% of COX-1 activity (Smith et al, 1998a). The half-maximal inhibition (IC\(_{50}\)) of COX-1 is achieved with a concentration as low as 0.009\( \mu \text{M} \), whereas the IC\(_{50}\) of COX-2 using the same inhibitor is much higher at 6.3\( \mu \text{M} \). This means that at the concentration used there will be negligible COX-2 inhibition with almost complete inhibition of COX-1.

Since the advent of highly selective COX-2 inhibitors, the role of COX-2 in atherosclerotic plaque instability has aroused attention, as the prospect that available pharmacotherapy (with an acceptable side effect profile) may be able to reduce cardiovascular events is exciting. Despite the results in this thesis, this is an area that still holds promise. It is possible that the reason for not detecting a difference is simply that the study does not have enough power, and that this is a type II error. This suggestion is made more likely by the finding that mean average COX & COX-2 activity levels showed a tendency towards higher values in the unstable groups.

Presentation of results that deal with protein concentrations (or activity) can be expressed in terms of "unit weight (or activity) of protein of interest per unit weight of tissue" or "unit weight (or activity) of protein of interest per unit weight of protein". As plaques vary in terms of their lipid composition, some authors suggest that it should always be the latter "protein-standardised" format that is used. Prior experience in our centre has left the impression that in fact, results are not greatly affected whichever format is employed. To avoid this controversy, the results of this thesis have been presented in both formats (the protein-standardised results are in appendix B).

It is interesting to note that despite a 5-fold difference in protein concentrations between plaques, the variation in results between the 2 formats is small. In fact, through all the multiple comparisons performed in this thesis, on only 2 occasions did a significant result become non-significant when protein standardised – the p-value for the comparison of tissue factor between statin groups went from 0.0017 to 0.0805, and for the comparison of
TIMP-1 in symptomatic vs asymptomatic patients it changed from 0.0037 to 0.0903. On one other occasion, a non-significant result became significant – the p-value for the comparison of tissue factor between the emboli groups went from 0.0138 to 0.0026. It must be stressed however, that the p-value chosen to represent a significance difference is merely an arbitrary cut-off point. There will inevitably be discrepancies in p-values using the 2 different methods, but throughout this thesis it can be seen that these variations are minimal.

Atherosclerosis is an inflammatory disease (Ross, 1999) and cytokines play an important role in its initiation and complications (Young, 2002). In this thesis, I have demonstrated that the plaque levels of IL-6 from highly symptomatic patients are significantly raised. I have also shown a tendency for elevated plaque IL-6 levels in emboli-positive patients, although this difference failed to reach significance. No differences were seen for IL-1β or TNF-α, and carotid plaque IL-10 levels were undetectable.

These results are important for a number of reasons. Firstly, they add to the growing body of evidence that suggests peripheral IL-6 levels could be incorporated into an individual patient risk assessment profile, alongside CRP & LDL. This topic is discussed comprehensively in the recent consensus document by Naghavi et al (2003). Additionally, with the unravelling of the human genome, there is now much interest in cytokine polymorphisms, and clearly it is at least a possibility that a polymorphism resulting in increased IL-6 expression may predispose patients to increased cardiovascular risk. Finally, the possibility exists for moderation of cytokine levels by oral medication or gene therapy.

A number of clinical trials have shown that statins markedly reduce the clinical sequelae of atherosclerosis (Heart Protection Study Collaborative Group, 2002; Sever et al, 2003). These and other studies have suggested the beneficial effects reported are at least partly independent of their lipid-lowering properties, and may include anti-proteolytic and anti-inflammatory mechanisms (Libby & Aikawa, 2003). The inhibitory effect of statins on MMP production by macrophages has been already been demonstrated in experimental and animal models (Luan et al, 2003).
In this thesis, I have shown that patients on statin therapy were less likely to have suffered recent symptoms attributable to their carotid disease. The proportion of patients with TCD-detected cerebral embolisation was also lower in this group. The plaque concentrations of MMP-9 and IL-6 were significantly lower in patients treated with statins, and the level of MMP-1 was also reduced, although this fell just short of statistical significance. These data suggest a shift in the balance of proteolysis away from matrix degradation and towards plaque stabilisation.

Levels of MMP-1 (Sukhova et al, 1999) and MMP-9 (Loftus et al, 2000) have been associated with features of carotid plaque instability. It is interesting, therefore, to observe an apparent preferential reduction in the levels of these 2 proteolytic enzymes. Combined, MMP-1 (a collagenase) and MMP-9 (a gelatinase) have the potential to degrade all components of the extracellular matrix, the integrity of which determines the stability of the plaque. IL-6 is a pro-inflammatory cytokine, elevated levels of which have been identified in patients with acute coronary syndromes (Biasucci et al, 1996). Reduced levels within the plaques of patients on statin therapy suggest a lesser degree of localised inflammation.

There are a number of potential criticisms of the statin results presented in chapter 12 of this thesis. Firstly, the proportion of patients on statin therapy appears low (38%). A survey of UK general practices performed in 2001 (DeWilde et al, 2003) showed that in the presence of ischaemic heart disease, 56% of men and 41% of women were prescribed a statin. However, more than half of our patients had no history of ischaemic heart disease, and on this basis, our numbers probably reflect the pattern of statin-prescribing in the UK at the time of our study. This survey by DeWilde et al (2003) also showed that the elderly were less likely to be prescribed a statin than their younger counterparts. Similarly in the results from this thesis, there was a lower mean age in the group of patients taking statins.

The arbitrary choice of 4 weeks to differentiate "highly symptomatic" patients from the rest could also be criticised, in respect of these results. It could be argued that some of the highly symptomatic patients were operated on urgently with insufficient time pre-operatively to commence statin therapy. This could introduce an element of bias. We feel this is unlikely to have significantly affected the results since, on sub-group analysis, only 5/24 of the highly symptomatic patients underwent surgery urgently, of whom 1 patient was already taking a statin. In our unit at the time of this study, non-urgent patients
routinely waited more than 4 weeks for surgery. The discovery that patients on statins tended to experience less pre-operative cerebral embolisation may be viewed as more convincing evidence of plaque stabilisation.

Our findings provide further evidence of a causative role for MMPs in plaque instability and imply that MMP inhibition may play a major role in plaque stabilisation. A true association could only be proven by a randomised controlled trial of statin vs placebo in patients with carotid disease, but in view of the recent large scale statin trials this would be unethical. There is a growing body of opinion suggesting that all patients with evidence of cardiovascular disease should be commenced on statin therapy irrespective of their lipid levels, a view that is reinforced by the data collected for this thesis.

---

Tissue factor is a potent initiator of coagulation and thrombosis. It is located in the subendothelium and in the necrotic core of atherosclerotic plaques (Wilcox et al, 1989), and is exposed to the bloodstream upon cap rupture. The concentrations of tissue factor quantified in carotid plaques for this thesis produced 2 very unexpected findings. Firstly, higher tissue factor concentrations were demonstrated in the plaques of those patients on statin therapy and secondly, a trend towards lower levels was observed in the plaques of emboli-positive patients.

At first glance, these 2 findings are contrary to recent evidence that suggests raised tissue factor expression is a cause of plaque destabilisation (Jander et al, 2001) and that statins decrease tissue factor levels (Aikawa et al, 2001; Libby & Aikawa, 2003). However, our results do not necessarily contradict these earlier findings from other units. One possible explanation for the decreased tissue factor levels in embolising patients is that those plaques that have ruptured and are embolising have used up much of their tissue factor in producing thrombus, and so the levels will be lower when the plaque is eventually retrieved. On the other hand though, a plaque containing low tissue factor levels that ruptures may be less able to satisfactorily heal the ulcer, resulting in continuing embolisation.

Another possibility is that MMPs may be responsible for degrading tissue factor. There is presently no published evidence for this, although interestingly, tissue factor pathway
inhibitor-2 (TFPI-2) is capable of MMP inhibition (Herman et al, 2001b; Baker et al, 2002). If this hypothesis were correct, it could explain how patients on statins have raised tissue factor levels (statins lower MMP levels which could result in less tissue factor degradation) and why embolising patients may have lowered tissue factor (embolising patients less likely to be on statins). The tissue factor results presented in this thesis raise more questions than they answer and, clearly, further work is required in this area.

No association between pre-operative inflammatory markers (CRP & WCC) and carotid plaque instability was demonstrated. This is unsurprising given that this section of the thesis included only 75 patients, which was probably insufficient to show any difference that did exist. Also, the failure of the assay to give a more exact reading than “<5” at the lower range certainly hindered the interpretation of these results, especially as 50 of these patients had such low CRP readings. In addition, although any patient with a serious clinical infection would obviously have their operation postponed, no account was taken of coincidental inflammatory disease, such as arthritis, when analysing these results.

The intraplaque level, expression and activity of MMP-9 are significantly higher in unstable carotid plaques (Loftus et al, 2000). Plasma MMP-9 is also raised in embolising patients awaiting carotid endarterectomy (Loftus et al, 2001), and this may reflect the localised increase in plaque activity, or may perhaps be a sign of a generalised increase in tandem with a systemic inflammatory response promoting plaque degradation. Alternatively, it may be due to cerebral ischaemia caused by particulate embolisation from the plaque (see figures 3.4 to 3.6 for possible sources of elevated MMP-9).

The results presented in chapter 13 have demonstrated a significant rise in plasma MMP-9 after surgery in patients with evidence of embolisation in the dissection phase of their procedure (between the pre-operative and post-operative blood samples). Those with no emboli detected showed no change in plasma MMP-9 levels. The rise in MMP-9 was more marked in those patients with greater than 2 emboli detected. This suggests an association between plasma MMP-9 levels and cerebral embolisation (figure 3.6) rather than a phenomenon related to localised increased plaque production (figure 3.4) or generalised inflammation (figure 3.5). The absence of a correlation between plasma and plaque MMP-9 levels (figure 13.3) supports this finding, as any such positive correlation could have
suggested either raised plaque production (spilling into the circulation) or a generalised increase associated with inflammation.

Human post-mortem studies have shown increased cerebral MMP-9 levels in association with an intense inflammatory reaction following stroke (Anthony et al, 1997). Animal models of cerebral ischaemia have demonstrated a rise in cerebral MMP-9 with ischaemic injury (Rosenberg et al, 1996). Further in vivo studies have shown an increase in plasma MMP-9 levels in a rat model of middle cerebral artery occlusion (Heo et al, 1999).

Kai et al (1998) reported raised peripheral blood levels of MMP-9 in patients with acute coronary syndromes. Early and significant increases in circulating MMP-9 were demonstrated in patients with unstable angina/MI (n=33) compared to stable angina and normal controls. The authors failed to show an association with CK or CK-MB isoforms, and therefore suggested the rise in MMP-9 was unlikely to be secondary to end-organ myocardial ischaemia. However, it should be recognised that CK and CK-MB do not correlate well with the volume of myocardial damage (Madrid et al, 1998).

A more recent study of 1127 patients with coronary artery disease identified baseline plasma MMP-9 levels to be a novel predictor of cardiovascular mortality (Blankenberg et al, 2003). The MMP-9 levels correlated with other acute phase reactants, suggesting a link with a more generalised inflammatory response. Clearly further work is required to fully understand the reasons for, and consequences of, this rise in plasma MMP-9.

Atherosclerotic plaque rupture is a major cause of mortality and morbidity throughout the world. Progress in our ability to recognise and stabilise vulnerable plaques has the potential to both save life and improve quality of life. Great strides forward in this area have been made even during the time it took to perform the research for this thesis, particularly in our understanding of how statins work, and who would benefit from their use.

Much controversy surrounds the topic of statin therapy, as it would appear that very few people would not obtain a benefit, and this leads to important decisions regarding cost, and whether in fact, everyone should have this medication recommended to them – an idea that is to be studied by using a “Polypill” containing statins (Wald & Law, 2003). Detailed
discussion of these issues is obviously beyond my remit here, but some important points are raised by this thesis. Firstly, active MMP-8 is significantly raised in unstable plaques. Secondly, statins do not appear to lower the plaque level of active MMP-8 (although they do reduce MMP-9, IL-6, & to a lesser extent MMP-1). Finally, patients on statins do continue to suffer myocardial infactions and strokes (although at a significantly reduced rate). In conclusion, continuing research into the role and inhibition of the collagenase MMP-8 is warranted.
Chapter 15: Future work

This thesis demonstrates that the active form of MMP-8 is significantly raised in unstable plaques, and may be responsible for their rupture. This important finding needs to be taken into consideration for the development of pharmacotherapy aimed at plaque stabilisation. Specific MMP inhibitors (such as MMI270 which preferentially inhibits MMP-2, MMP-8 & MMP-9) are available, but their use in trials is presently limited by musculoskeletal side effects. These systemic effects of MMP inhibitors could be avoided by local tissue delivery, but regular application to the atherosclerotic plaque is not practical. A one-off delivery of gene therapy to the plaque (to increase local TIMP-1 production, for example) is a possibility for the future, but the present state of knowledge regarding this technique is not yet satisfactory.

A randomised trial of pre-CEA doxycycline therapy vs placebo showed decreased plaque levels of MMP-1 in the treatment group (Axisa et al, 2002). Disappointingly, it did not show any reduction in plaque MMP-9 levels. The gelatinase MMP-9 has been strongly implicated in plaque instability previously (Loftus et al, 2000), a finding that is reinforced by the MMP-9 results presented in this thesis. Large scale trials of doxycycline use for the prevention of small AAA growth are presently underway (Baxter et al, 2002), and it would seem prudent to await these results before considering further studies into the effect of doxycycline on plaque stabilisation, and in particular, whether it is capable of reducing active MMP-8 levels (these were not quantified in the previous study).

The results presented in chapter 12 regarding the effect of statins on plaque concentrations of MMP-1, MMP-9 & IL-6 show large reductions in the statin group. Criticism of this work will point to the non-randomised nature of the study and the lack of serum lipid levels. Many authorities now feel that all patients awaiting carotid endarterectomy (or any other major vascular surgery) should be initiated on statin therapy pre-operatively because of evidence of a reduction in peri-operative mortality (Poldermans et al, 2003). This means that conducting a randomised controlled trial of statin vs placebo in all patients awaiting carotid endarterectomy would now be unethical. A recent RCT of simvastatin vs placebo in
patients awaiting abdominal aortic aneurysm repair was stopped early because of such ethical considerations. Despite its curtailed recruitment, this trial showed a significant reduction in aortic wall MMP-9 content and activity in the treatment arm (Evans et al, 2004).

However, in those units where routine practice is to initiate statin therapy only when cholesterol levels are >5mmol/l, there may be scope for such a trial. Power calculations suggest that to show a difference in carotid plaque MMP-9 levels similar to this thesis, only 10 patients would be required in each study arm (with a power of 90% and a significance level set at 0.01).

Quantifying pre- and post-statin therapy cholesterol levels within such a study may give some indication as to how much of the reduction in MMP levels could be explained by lipid-lowering alone. Investigating the way in which statins are capable of decreasing MMP levels without causing the side effects commonly associated with other MMP inhibitors could prove vital in the development of plaque-stabilising therapy. Measuring pre- and post-statin plasma MMP levels may show whether the MMP-lowering effects of statins are localised to atherosclerotic plaques, or whether they are more generalised. Quantifying tissue factor levels within the plaque may confirm if the unexpected findings of this thesis are correct, and comparison with plaque and plasma MMP concentrations, may provide further information regarding the role of tissue factor in plaque instability.

Another issue for consideration would be the length of pre-operative statin therapy necessary to show a difference. It is now widely accepted that for symptomatic carotid stenoses of >70%, the sooner the surgery the better, and some authorities now recommend CEA is performed within 2 weeks of the patient’s last symptom (Rothwell et al, 2004). This may not leave sufficient time for statin therapy to effect plaque MMP levels. For asymptomatic disease there is not the same urgency, however recruiting only asymptomatic patients (and only those with a cholesterol level <5mmol/l) may prove problematic for any such trial.

The strength of evidence for performing carotid endarterectomy on symptomatic patients with a >70% stenosis (European Carotid Surgery Trialists’ Collaborative Group, 1998; North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1998) is too powerful for the consideration of a randomised controlled trial of MMP inhibition vs
surgery. This would obviously be the gold standard in terms of studying the role of MMPs in plaque rupture and the possible effects of successful inhibition.

However, the evidence for operating on asymptomatic patients is less strong (Asymptomatic Carotid Atherosclerosis Study Executive Committee, 1995; Asymptomatic Carotid Surgery Trial Collaborative Group, 2004). Best medical therapy (BMT) in these 2 trials included statin therapy for hyperlipidaemia. Use of statins was low (albeit appropriate for the years during which the studies were performed), but current knowledge now suggests that BMT should include statin therapy for all patients with carotid stenosis. It seems likely that maximal statin therapy would have reduced the number of strokes in all patients, and reduced the benefits of CEA compared to BMT. As the stroke rate is low in asymptomatic patients, any trial designed to compare a specific MMP inhibitor to CEA and/or BMT would have to be huge to show any significant difference, and is therefore impractical.

Another group of patients with potential for trial recruitment are those with stenoses <70%, as surgery is not indicated in this group. Any such study would therefore be in the form of MMP inhibition vs placebo. Again, the stroke rate is low in such patients, especially as both arms of such a study would need to be on BMT. In addition, the outcome measured is likely to reflect the effects of MMP inhibition on plaque progression and remodelling at least as much as the effects on plaque rupture.

For the reasons outlined, the conception of trials of MMP inhibition with clinical end-points are presently unlikely. However, the identification of the high-risk patient and plaque is important in identifying which patients will benefit most from carotid, or cardiac, intervention. In this regard, the study of various MMP and cytokine polymorphisms may prove useful. Work in our centre is presently underway in an attempt to detect if patients with a particular polymorphism in the MMP-9 promoter region are more prone to plaque instability. If such polymorphisms are found to be associated with plaque levels of MMPs or cytokines, they may prove useful in identifying more vulnerable patients, and altering management accordingly.

This thesis has demonstrated a rise in plasma MMP-9 in those patients who embolised during the dissection phase of their carotid endarterectomy. No such elevation was seen in emboli-negative patients. Clearly further work is required to fully understand the reasons
for, and consequences of, this increase in plasma MMP-9. An increase in systemic MMP-9 has been shown within 12 hours of cardioembolic stroke (Montaner et al, 2001) and it would be interesting to monitor the trend of plasma MMP-9 levels during the CEA post-operative period. This would allow determination of how soon after embolisation the levels rise and would establish the time course of a return to normal plasma concentrations. CT scans were not performed in our study because previous studies have reported a poor association between embolic end-organ damage and CT changes (Gaunt et al, 1996b). Psychometric testing (Gaunt et al, 1996a) or MRI (Guadagno et al, 2003) may be more sensitive markers of embolic damage, and could potentially be employed to confirm the association with MMP-9 levels.

Despite accumulating evidence of a causative role for MMPs in plaque rupture, there remains uncertainty as to whether the raised MMP & cytokine levels found in unstable plaques are a cause or an effect of the rupture. Research in this area must continue in order to discover more about the mechanisms involved in the rupture of atherosclerotic plaques. Simply attributing raised MMP levels to be a consequence of the rupture rather than responsible for it, may represent a missed opportunity for the health of generations to come.
Section V: Appendices & bibliography
Appendix A: Reagents & solutions

A1 Paraformaldehyde solution (4%):
Paraformaldehyde 4g
PBS 100ml
Solution heated to 65°C on a hotplate under the fume hood with continuous stirring.
5M NaOH added in a dropwise fashion until paraformaldehyde dissolved.
Solution cooled to 4°C and stored at this temperature in glass container (light protected by foil wrapping) for no more than 1 week.

A2 Phosphate buffered saline (PBS):
NaCl 8.0g/l
KCl 0.2g/l
Na$_2$HPO$_4$·H$_2$O 1.44g/l
KH$_2$PO$_4$ 0.24g/l
HCl to final pH of 7.4
Sterile distilled water to required volume

A3 Homogenising buffer:
Urea 2mol/l
Tris HCL 50mmol/l
NaCl 1g/l
EDTA 1g/l
BRIJ-35 1ml/l
PMSF 0.1mmol/l
NaOH to final pH of 7.6
Sterile distilled water to required volume
A4 Dialysis buffer:
Tris-base 3.03g/l
CaCl₂·2H₂O 0.97g/l
PMSF 100μl/l
BRIJ-35 1ml/l
HCl to final pH of 8.0
Sterile distilled water total buffer volume of 250ml for each homogenate dialysed

A5 Trypsin solution:
Trypsin 1.0g/l
CaCl₂ 1.2g/l
NaOH to final pH of 7.8

A6 Diaminobenzidine (DAB) solution:
Substrate buffer (ChemMate Envision bottle B, Dako, USA) 20ml
DAB concentrate (ChemMate Envision bottle C, Dako, USA) 400μl

A7 Hybridisation solution
Formamide 300μl/ml
50% dextran sulphate 200μl/ml
2.5M NaCl DEPC 240μl/ml
DEPC water 156μl/ml
Hybridisation mixture (see A8) 100μl/ml
Labelled probe cocktail (see A9) 4μl/ml

A8 Hybridisation mixture
Sodium pyrophosphate 2.5g
Ficoll 4.625g
EDTA 5.0g
Tris 15.14g
DEPC water 250ml
HCl to final pH of 7.5
A9 Digoxigenin-labelled MMP-8 oligonucleotide probe cocktail

500μg/ml custom oligonucleotide cocktail (Sigma-Genosys, USA) 4.0μl
5X TdT buffer 4.0μl
25mM cobalt chloride 4.0μl
1mM digoxigenin-dUTP 1.7μl
5mM dATP 1.7μl
25u/μl TdT 1.8μl
DEPC water 2.8μl

Incubate at 37°C for 15mins. Then add 1μl of EDTA. Store at -20°C until ready for use.

A10 Blocking solution

20X TBS 500μl
Bovine serum albumin 300mg
Triton X-100 10μl
Ultra pure water to final volume of 10ml

A11 Substrate buffer

1M Tris-HCl pH 9.5 1.0ml
1M Magnesium chloride 0.5ml
2.5M NaCl 0.4ml
Ultra pure water to final volume of 10ml

A12 Substrate solution

NBT/BCIP (Nitro-blue-tetrazolium/Bromo-chloro-indolyl phosphate) – dissolve half a tablet in 5ml of double distilled water
Appendix B: Protein-standardised results

Tables are labelled using same numbering system as in the main text followed by the symbol: *.

MMP, TIMP, cytokine & tissue factor values are expressed as ng/mg of plaque protein. COX & COX-2 values are expressed as activity units/mg of plaque protein (see figure 7.8 for definition of activity units).

Statistical analysis and presentation of results are otherwise unchanged from the main text. Values in parentheses are interquartile ranges other than when labelled as s.d. (standard deviation).

Chapter 9 results:

<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Symptoms &lt;6 months (n=90)</th>
<th>Asymptomatic (n=69)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>1.4 (0.5-4.2)</td>
<td>1.1 (0.5-3.4)</td>
<td>0.4925</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>0.67 (0.33-1.61)</td>
<td>0.41 (0.19-0.76)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>10.2 (7.7-12.7)</td>
<td>7.6 (4.3-9.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-9</td>
<td>12.6 (4.4-25.8)</td>
<td>6.3 (2.2-14.9)</td>
<td>0.0021</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.021 (0.011-0.039)</td>
<td>0.022 (0.014-0.041)</td>
<td>0.3946</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>334 (192-440)</td>
<td>276 (180-375)</td>
<td>0.0903</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>25.3 (17.5-44.4)</td>
<td>28.2 (16.5-50.0)</td>
<td>0.7904</td>
</tr>
</tbody>
</table>

Table 9.4' Comparison of symptomatic vs asymptomatic groups in terms of plaque MMP & TIMP concentrations
<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Symptoms &lt;1 month (n=30)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=129)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>2.5 (0.8-5.3)</td>
<td>1.2 (0.5-2.9)</td>
<td>0.0168</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>1.03 (0.44-1.94)</td>
<td>0.48 (0.24-0.91)</td>
<td>0.0012</td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>10.1 (8.1-12.3)</td>
<td>8.6 (5.5-11.7)</td>
<td>0.0575</td>
</tr>
<tr>
<td>MMP-9</td>
<td>20.5 (9.4-58.9)</td>
<td>7.4 (2.9-19.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.027 (0.015-0.048)</td>
<td>0.020 (0.011-0.036)</td>
<td>0.0722</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>355 (169-493)</td>
<td>294 (184-420)</td>
<td>0.2999</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>23.2 (15.9-41.3)</td>
<td>27.4 (18.7-49.2)</td>
<td>0.3103</td>
</tr>
</tbody>
</table>

Table 9.5f Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups in terms of plaque MMP & TIMP concentrations.

<table>
<thead>
<tr>
<th>Plaque concentrations</th>
<th>Emboli-positive (n=42)</th>
<th>Emboli-negative (n=106)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>1.6 (0.6-4.3)</td>
<td>1.2 (0.5-3.3)</td>
<td>0.4452</td>
</tr>
<tr>
<td>Active MMP-8</td>
<td>0.69 (0.34-1.89)</td>
<td>0.48 (0.23-0.92)</td>
<td>0.0075</td>
</tr>
<tr>
<td>Total MMP-8</td>
<td>9.5 (7.4-12.2)</td>
<td>8.5 (5.5-11.8)</td>
<td>0.1242</td>
</tr>
<tr>
<td>MMP-9</td>
<td>13.7 (5.8-43.0)</td>
<td>7.1 (3.3-18.8)</td>
<td>0.0046</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.024 (0.013-0.047)</td>
<td>0.019 (0.010-0.031)</td>
<td>0.1580</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>278 (166-388)</td>
<td>295 (186-432)</td>
<td>0.5876</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>24.5 (13.3-51.8)</td>
<td>27.1 (17.8-46.5)</td>
<td>0.5277</td>
</tr>
</tbody>
</table>

Table 9.6f Comparison of emboli groups in terms of plaque MMP & TIMP concentrations. NB, 11 patients lacked a TCD window and are therefore not represented in these results.
<table>
<thead>
<tr>
<th>Histological feature</th>
<th>Present</th>
<th>Absent</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque rupture</td>
<td>0.74 (0.51-1.80)</td>
<td>0.48 (0.23-1.05)</td>
<td>0.0047</td>
</tr>
<tr>
<td>IPH</td>
<td>0.72 (0.29-1.74)</td>
<td>0.48 (0.24-0.96)</td>
<td>0.0288</td>
</tr>
<tr>
<td>Cap thinning</td>
<td>0.45 (0.26-0.89)</td>
<td>0.67 (0.26-1.18)</td>
<td>0.1595</td>
</tr>
<tr>
<td>Intraplaque fibrin</td>
<td>0.55 (0.26-1.09)</td>
<td>0.57 (0.26-1.10)</td>
<td>0.8222</td>
</tr>
<tr>
<td>Plaque necrosis</td>
<td>0.62 (0.29-1.25)</td>
<td>0.49 (0.24-0.98)</td>
<td>0.1549</td>
</tr>
<tr>
<td>Cap foam cells</td>
<td>0.56 (0.26-0.94)</td>
<td>0.54 (0.25-1.22)</td>
<td>0.5148</td>
</tr>
</tbody>
</table>

Table 9.7' Comparison of plaque active MMP-8 concentration in presence or absence of 6 histological features associated with plaque instability

Chapter 10 results:

<table>
<thead>
<tr>
<th>Activity levels</th>
<th>Symptoms &lt;6 months (n=79)</th>
<th>Asymptomatic (n=58)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX (s.d.)</td>
<td>5.08 (1.31)</td>
<td>5.41 (1.47)</td>
<td>0.1695</td>
</tr>
<tr>
<td>COX-2 (s.d.)</td>
<td>3.40 (1.32)</td>
<td>3.50 (1.75)</td>
<td>0.7053</td>
</tr>
</tbody>
</table>

Table 10.1' Comparison of symptomatic vs asymptomatic groups in terms of COX plaque activity levels

<table>
<thead>
<tr>
<th>Activity levels</th>
<th>Symptoms &lt;1 month (n=24)</th>
<th>Symptoms &gt;1 month or asymptomatic (n=113)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX (s.d.)</td>
<td>5.53 (1.46)</td>
<td>5.15 (1.37)</td>
<td>0.2272</td>
</tr>
<tr>
<td>COX-2 (s.d.)</td>
<td>3.57 (1.47)</td>
<td>3.42 (1.52)</td>
<td>0.6581</td>
</tr>
</tbody>
</table>

Table 10.2' Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups in terms of COX plaque activity levels
Table 10.3' Comparison of emboli groups in terms of COX plaque activity levels. NB, 7 patients lacked a TCD window and are therefore not represented in these results.

Chapter 11 results:

Table 11.1' Comparison of symptomatic vs asymptomatic groups in terms of plaque cytokine & tissue factor concentrations

Table 11.2' Comparison of highly symptomatic vs symptoms >1 month or asymptomatic groups in terms of plaque cytokine & tissue factor concentrations
Table 11.3f Comparison of emboli groups in terms of plaque cytokine & tissue factor concentrations. NB, 7 patients lacked a TCD window and are therefore not represented in these results.

Chapter 12 results:

Table 12.2f Statin groups vs MMP, TIMP & cytokine levels
### Table 12.3

Statin groups vs COX activity and tissue factor concentrations

<table>
<thead>
<tr>
<th></th>
<th>Yes (n=52)</th>
<th>No (n=85)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX (s.d.)</td>
<td>5.04 (1.32)</td>
<td>5.32 (1.42)</td>
<td>0.2545</td>
</tr>
<tr>
<td>COX-2 (s.d.)</td>
<td>3.30 (1.44)</td>
<td>3.53 (1.55)</td>
<td>0.3919</td>
</tr>
<tr>
<td>Tissue factor (s.d.)</td>
<td>0.60 (0.22)</td>
<td>0.53 (0.25)</td>
<td>0.0805</td>
</tr>
</tbody>
</table>
Appendix C: Abbreviations

AAA abdominal aortic aneurysm
ACE angiotensin converting enzyme
ADAMTS A disintegrin and metalloproteinase with thrombospondin motifs
APMA aminophenylmercuric acetate
BMT best medical therapy
CCA common carotid artery
CD cluster of differentiation
CEA carotid endarterectomy
CHD coronary heart disease
CK creatinine kinase
COX cyclooxygenase
CRP C-reactive protein
CT computed tomography
CVD cardiovascular disease
Da Dalton
DAB diaminobenzidine
DEPC diethylpyrocarbonate
DM diabetes mellitus
ECM extracellular matrix
EDTA ethylenediaminetetraacetate
ELISA enzyme-linked immunosorbent assay
EVG elastic Van Gieson
GGPP geranylgeranylpyrophosphate
H&E haematoxylin & eosin
HDL high density lipoprotein
HMG-CoA hydroxymethylglutaryl-coenzyme A
HRP horseradish peroxidase
HSP heat shock protein
HTN hypertension
ICAM intercellular adhesion molecule
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC_{50}</td>
<td>half-maximal inhibition</td>
</tr>
<tr>
<td>ICA</td>
<td>internal carotid artery</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IHD</td>
<td>ischaemic heart disease</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPH</td>
<td>intraplaque haemorrhage</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridisation</td>
</tr>
<tr>
<td>IVUS</td>
<td>intravascular ultrasound</td>
</tr>
<tr>
<td>LCA</td>
<td>leukocyte common antigen</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane-type MMP</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCD</td>
<td>transcranial Doppler</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxytransferase</td>
</tr>
<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIA</td>
<td>transient ischaemic attack</td>
</tr>
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</table>
TIMP  tissue inhibitor of metalloproteinase
TMB  tetramethylbenzidine
TMPD  tetramethylphenylenediamine
TNF  tumour necrosis factor
TX  thromboxane
uPA  urokinase plasminogen activator
uTP  uracil triphosphate
UV  ultraviolet
VCAM  vascular cell adhesion molecule
vs  versus
WCC  white cell count
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